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

EMERGING INFECTIOUS DISEASES

June 2006

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Albrecht Dürer (1471–1528)
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Parchment mounted on canvas (56 cm × 44 cm)
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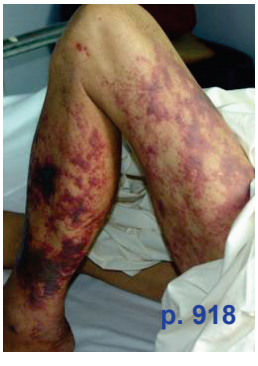

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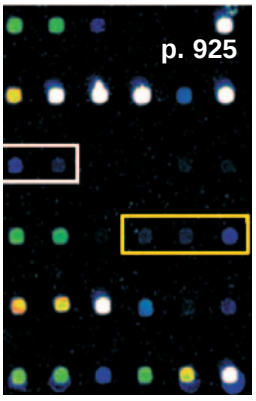

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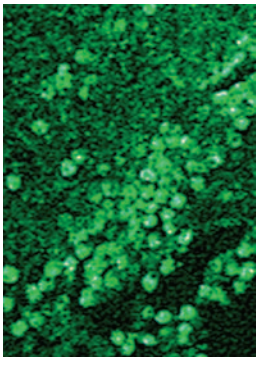

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Host Range Restriction and Pathogenicity in the Context of Influenza Pandemic

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Influenza A viruses cause pandemics at random intervals. Pandemics are caused by viruses that contain a hemagglutinin (HA) surface glycoprotein to which human populations are immunologically naive. Such an HA can be introduced into the human population through reassortment between human and avian virus strains or through the direct transfer of an avian influenza virus to humans. The factors that determine the interspecies transmission and pathogenicity of influenza viruses are still poorly understood; however, the HA protein plays an important role in overcoming the interspecies barrier and in virulence in avian influenza viruses. Recently, the RNA polymerase (PB2) protein has also been recognized as a critical factor in host range restriction, while the nonstructural (NS1) protein affects the initial host immune responses. We summarize current knowledge of viral factors that determine host range restriction and pathogenicity of influenza A viruses.

Of the 3 types of influenza viruses (A, B, and C), only influenza A viruses are established in animals other than humans. Influenza pandemics are caused by viruses that have a hemagglutinin (HA) to which most humans have no immune memory. The strains of the 1957 Asian and 1968 Hong Kong pandemics had HAs derived from an avian virus. Although little information exists about avian influenza viruses at the time of the Spanish influenza pandemic, the HA of the virus responsible for that outbreak is also thought to be of avian origin. Since avian influenza viruses do not replicate efficiently in humans and nonhuman primates, they must overcome host range restriction for the avian virus HA to be introduced into human populations. The molecular basis for host range restriction is not well understood; however, HA plays a key role in the restriction of interspecies transmission.

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The Spanish influenza was among the most devastating infectious diseases in history. At least 20 million people died worldwide. Antimicrobial agents were not available in 1918; however, existing evidence suggests that this high death toll was due to the extreme virulence of the virus. Although all 8 RNA segments of the Spanish influenza virus have been sequenced, these sequences offer no explanation for the high virulence. The Spanish influenza exemplifies how the magnitude of a pandemic can be determined by the pathogenicity of the virus.

In this review, we focus on 2 properties of influenza A viruses as they relate to pandemics, host range restriction and pathogenicity. Viral factors that affect these properties are examined.

Viral Proteins Responsible for Host Range Restriction

Viral Glycoproteins

The HA protein mediates virus binding to sialic acid (SA)–containing host cell surface molecules and promotes the release of viral ribonucleoprotein complexes through membrane fusion. By contrast, the sialidase activity of the neuraminidase (NA) protein removes SA to liberate newly synthesized viruses from infected cells. Thus, efficient virus replication requires the balanced actions of HA receptor-binding specificity and NA sialidase activity.

HA Receptor Specificity

Influenza virus infectivity is influenced by 2 entities: SA species (N-acetylneuraminic acid [NeuAc] and N-glycolylneuramic acid [NeuGc]) and the type of linkage to galactose (sialyloligosaccharides terminated by SA linked to galactose by an α2,6 linkage [Acα2,6Gal] or an α2,3 linkage [Acα2,3Gal]) on the host cell surface. Human influenza viruses preferentially recognize sialyloligosaccharides containing SAα2,6Gal (*1,2*), matched by mainly

NeuAcα2,6Gal linkages on the epithelial cells of the human trachea (*3*). By contrast, avian viruses preferentially recognize SAα2,3Gal sialic acids (*1,2*), in accordance with the predominance of sialyoligosaccharides with SAα2,3Gal linkages on the epithelial cells of duck intestine. The epithelial cells of pig trachea contain both types of SAs and both types of linkages (*4*), which likely explains the high susceptibility of these animals to both human and avian influenza viruses (*5*). Pigs may therefore serve as a "mixing vessel" for reassortment between these 2 viruses and the source of pandemic strains, although no evidence exists that the 1957 or 1968 pandemic viruses originated in pigs.

Despite these differences in receptor specificity, avian viruses can infect humans and have caused lethal infections (*6–8*). This fact may be explained by the recent finding that in differentiated cultures of human tracheobronchial epithelium, α2,3-linked SAs were found on ciliated cells, whereas α2,6-linked SAs were present on nonciliated cells (*9*). The prevalence of cells that possess α2,6 and/or α2,3-linked SAs in the lower respiratory tract remains unknown; however, ciliated cells support avian virus infection. Despite the presence of α2,6-linked SA-bearing cells in differentiated human tracheobronchial epithelium, viruses with avian-type receptor specificity can infect humans, since the index 1997 H5N1 virus isolated from a human preferentially recognized an avian receptor (*10*). Nevertheless, for efficient human-to-human transmission, HA derived from an avian virus must preferentially recognize the human receptor. This notion is supported by the finding that the earliest isolates in the 1918 (*11,12*), 1957, and 1968 pandemics preferentially recognized NeuAcα2,6Gal–containing sialyloligosaccharides (*13*), even though their HAs were derived from avian viruses. Conversion of receptor specificity from SAα2,3Gal to SAα2,6Gal may therefore be critical for generating pandemic influenza viruses.

Sequence comparison, receptor specificity assays, and crystallographic analysis have identified amino acid residues that determine receptor specificity: Gln-226 (found in avian viruses) determines specificity for SAα2,3Gal, whereas Leu-226 correlates with SAα2,6Gal specificity in human H2 and H3, but not H1, viruses (*2,13*). In all human viruses (with the few exceptions of early isolates from the Asian influenza outbreak [*13*]), Leu-226 is associated with Ser-228, while Gln-226 is associated with Gly-228 in avian viruses. For H1 viruses, Asp-190 (found in human and swine virus isolates) or Glu-190 (found in avian virus isolates) determines preferential binding to α2,6 or α2,3 linkages, respectively (*11–14*).

Land-based poultry are thought to play a critical role in the emergence of pandemic influenza viruses. Compared to H5N1 viruses isolated from aquatic birds, those isolated from chickens have significantly lower affinity for NeuAcα2,3Gal (*10*), similar to human virus isolates; however, H5N1 chicken isolates have not acquired preferential specificity for NeuAcα2,6Gal. H5N1 chicken isolates with reduced avian receptor specificity share 2 characteristic features of human viruses, namely, an additional glycosylation site in the globular head region of HA and a deletion in the NA stalk (see below). Similarly, the receptor specificity of H9N2 viruses isolated from land-based poultry, but not of those isolated from aquatic birds, is similar to that of human isolates (*15*). Hence, land-based poultry may serve as an intermediate host that facilitates the conversion of avian to human-type receptors. Avian viruses in land-based poultry may, therefore, pose a greater threat to humans than previously thought.

NA Properties

Since efficient release of virus from infected cells requires the removal of SA by NA, the receptor-binding and receptor-destroying properties of HA and NA, respectively, must be balanced. When an avian virus with an N2 NA was introduced into the human population, its SAα2,6 cleavage activity increased (*16,17*), which suggests it had adapted to the SAα2,6 receptor specificity of human HAs.

The NA stalk, which separates the head region with the enzymatic center from the transmembrane and cytoplasmic domains, varies in sequence and length, depending on the virus (*18*). Typically, shortened stalks result in less efficient virus release since the active site in the head region cannot efficiently access its substrate (*19,20*). However, naturally occurring avian viruses with shortened stalks are virulent in poultry, and the 1997 H5N1 viruses isolated from patients in Hong Kong (which are believed to have been transmitted to humans from poultry) are characterized by a deletion in the NA stalk (*10*). Moreover, most recent highly pathogenic H5N1 viruses isolated from terrestrial poultry possess short NA stalks (*21*).

In avian species, the intestinal tract is the primary site of replication, whereas in humans, influenza virus replication is typically restricted to the respiratory tract. The NA activity of avian H1N1 viruses is more resistant to the low pH environment in the upper digestive tract than is its human or swine-derived counterpart (*22*). In line with this finding, highly pathogenic H5N1 viruses can replicate in the human intestine, causing gastrointestinal symptoms (*23*), and are shed in large amounts in stool.

Internal Proteins

Classical coinfection experiments, or reverse genetics experiments that tested multiple gene combinations of 2 parental viruses, suggest that the genes encoding the "internal proteins"—namely, RNA polymerase (PB2, PB1, PA), nucleoprotein (NP), matrix protein (M1, M2), and

nonstructural protein (NS1, NS2/NEP)—also contribute to host range. The contribution of individual proteins to host range restriction, however, likely varies, depending on the test system and the virus strains under investigation.

PB2

PB2 is a component of the viral polymerase complex and, as such, is essential for viral replication. The 1997 H5N1 human virus isolates in Hong Kong have been divided into 2 groups on the basis of their pathogenicity in mice; this classification also generally corresponds to disease severity in humans (*24*,*25*). Reverse genetics studies have shown that Lys at position 627 of PB2 (found in all human isolates) determines high pathogenicity in mice, while Glu at this position (found in all avian isolates) determines low pathogenicity (*26*). However, the nature of the amino acid at position 627 of PB2 does not affect the cell tropism of the virus but rather its replicative ability in mice and probably in humans.

Several other findings underline the importance of residue 627 of PB2: 1) an H7N7 virus isolated from a patient with fatal pneumonia in the Netherlands in 2003 contained Lys at this position, in contrast to viruses isolated from nonfatal cases and from chickens (*27*); 2) some of the H5N1 viruses isolated from patients in Vietnam are characterized by Lys-627 in PB2 (*28*); 3) a single reassortant virus bearing an avian virus PB2 gene against a human virus background replicated efficiently in avian but not human cells, a feature that could be traced to the nature of the amino acid at position 627 of PB2 (*29*); and 4) ribonucleoprotein complexes reconstituted from human or avian polymerase and NP proteins identified residue 627 of PB2 as the major determinant of replication efficiency in mammalian cells (*30*). Collectively, these findings suggest that a Glu-to-Lys mutation at position 627 of the PB2 protein allows avian viruses to efficiently grow in humans and implicates Lys at this position as an important host range determinant.

Other Components of the Replication Complex

In addition to PB2, the remaining 2 polymerase proteins (PB1 and PA) and the nucleoprotein NP may also contribute to host range. In a minireplicon system, replication in mammalian cells was more efficient with avian than with human virus PB1 proteins (*30*), which suggests that avian PB1 may have greater activity that could provide a replicative advantage in mammalian systems. This scenario is especially appealing in light of the finding that both the 1957 and 1968 pandemic viruses possessed avian PB1 genes, in addition to avian HA, NA, or both genes (*31*,*32*). In one study, however, an avian PB1 gene severely restricted replication of a human virus in mammalian cells and squirrel monkeys (*33*). These findings seem to contradict a role of avian PB1 in replication in mammalian cells. PA and NP proteins have also been implicated in host range restriction; for example, an avian virus NP segment against a background of a human virus resulted in attenuation in squirrel monkeys (*33*). However, because of the limited data available, whether these findings indicate a contribution of these gene products to host range restriction or simply reflect incompatibility among the viral gene segments is unclear.

M Segment

Segment 7 of influenza A viruses encodes the M1 matrix and the M2 ion channel proteins. In coinfection experiments that selected for reassortants containing a human virus M gene and an avian virus HA gene, the M segment of an early human virus (A/PR/8/34, H1N1) cooperated efficiently with avian virus HAs, whereas M segments derived from more recent isolates have gradually lost this ability (*34*). This finding may suggest that currently circulating human viruses are less likely to reassort with avian viruses than their predecessors. If this is the case, the risk for a global pandemic caused by reassortants possessing avian HA, NA, or both segments against a human virus background would be reduced.

Molecular Basis of Pathogenicity

HA Cleavability

The HA protein is synthesized as a precursor protein that is cleaved into 2 subunits (HA1 and HA2) by host cell proteases. HA cleavage is a prerequisite for fusion of the viral and endosomal membranes and, therefore, for viral infectivity (*35*). Low pathogenic avian influenza viruses possess a single Arg residue at the cleavage site, recognized by extracellular, trypsinlike proteases. These proteases are thought to be secreted only by cells of the respiratory and intestinal tract and consequently limit infections to these organs. By contrast, highly pathogenic avian viruses possess multiple basic amino acids at the cleavage that are recognized by ubiquitous, intracellular, subtilisin-like proteases that thus trigger systemic infection. In addition, HA cleavability is affected by the absence or presence of a carbohydrate side chain near the cleavage site that may interfere with the accessibility of host proteases to the cleavage site (*36*). The acquisition of a highly cleavable HA converted an avirulent strain to virulence in Pennsylvania in 1983 (H5N2), Mexico in 1994 (H5N2), Italy in 1997 (H7N1), Chile in 2002 (H7N3), and Canada in 2004 (H7N3) (Table). HA cleavability is, therefore, considered the major determinant of tissue tropism of avian influenza viruses (*41*). This correlation seems to extend to humans, since all avian viruses that have killed humans possess a highly cleavable HA (*6*,*7*,*27*), and an H5N1

Table. Comparison of the hemagglutinin (HA) cleavage sites of highly pathogenic avian influenza viruses and their nonpathogenic predecessors

Isolate	Type	Amino acid sequence*	Reference
A/chicken/Pennsylvania/1/83 (H5N2)	Avirulent	P Q - - - - - - - - - - - - K K K R/ G L F	(*36*)
A/chicken/Pennsylvania/1370/83 (H5N2)	Virulent	P Q - - - - - - - - - - - - K K K R/ G L F†	(*36*)
A/chicken/Mexico/31381-7/94 (H5N2)	Avirulent	P Q - - - - - - - - - - - - R E T R/ G L F	(*37*)
A/chicken/Queretaro/14588-19/95 (H5N2)	Virulent	P Q - - - - - - - - - - R K R K T R/ G L F	(*37*)
A/turkey/Italy/99 (H7N1) consensus	Avirulent	P E I P K G - - - - - - - - - - - - R/ G L F	(*38*)
A/turkey/Italy/99 (H7N1) consensus	Virulent	P E I P K G - - - - - - - S R V R R/ G L F	(*38*)
A/chicken/Chile/176822/02 (H7N3)	Avirulent	P E K P K - - - - - - - - - - - - T R/ G L F	(*39*)
A/chicken/Chile/4957/02 (H7N3)	Virulent	P E K P K T C S P L S R C R K T R/ G L F	(*39*)
A/chicken/Chile/4322/02 (H7N3)	Virulent	P E K P K T C S P L S R C R E T R/ G L F	(*39*)
Isolate CN6/04	Avirulent	P E N P K - - - - - - - - - - - - T R/ G L F	(*40*)
A/chicken/BC/CN12/04(H7N3)	Virulent	P E N P K - - --Q A Y Q K R M T R/ G L F	(*40*)
A/chicken/BC/NS1337-1/04 (H7N3)	Virulent	P E N P K - - - Q A Y K K R M T R/ G L F	(*40*)
A/chicken/BC/NS-1319-2/04(H7N3)	Virulent	P E N P K - - - Q A Y H K R M T R/ G L F	(*40*)
A/chicken/BC/CN7-3/04 (H7N3)	Virulent	P E N P K - - - Q A Y R K R M T R/ G L F	(*40*)
A/chicken/BC/NS-1390-2/04(H7N3)	Virulent	P E N P K - - - Q A H Q K R M T R/ G L F	(*40*)
A/chicken/BC/NS-2035-12/04(H7N3)	Virulent	P E N P K - - - Q A C Q K R M T R/ G L F	(*40*)

*HA cleavage sites are indicated by /. For sequence variants, the amino acids that differ from most sequences found are underlined.
†HA cleavability was enhanced by a single amino acid substitution that abrogated glycosylation near the HA cleavage site.

mutant virus whose HA cleavage site had been changed to an avirulent type was attenuated in mice (*26*).

Role of NS1 in Antagonizing Cellular Immune Responses

Pathogenesis depends partly on the ability of a virus to evade or suppress the host immune response. The NS1 protein, encoded by segment 8, plays a central role in this process by counteracting the cellular interferon (IFN) response in a 2-pronged approach: 1) by binding to double-stranded RNA, thereby suppressing the activation of double-stranded RNA-activated protein kinase, a known stimulator of type I IFN, and 2) by preventing the activation of transcription factors such as ATF-2/c-Jun, NFkB, and IRF-3/5/7, all of which stimulate IFN production (*42*,*43*). The NS gene of the 1918 Spanish flu blocked the expression of IFN-regulated genes in human cells more efficiently than did the NS gene of the A/PR/8/34 (H1N1) virus (*44*), which suggests that the NS genes of highly pathogenic viruses may be more proficient in counteracting the host immune response than those of less pathogenic viruses.

Viruses containing the NS gene of the 1997 H5N1 virus are potent inducers of proinflammatory cytokine genes, particularly tumor necrosis factor-α (TNF-α) and IFN-β in human primary monocyte-derived macrophages (*45*). Similarly, 2003 human H5N1 isolates induce high levels of proinflammatory cytokines in primary human macrophages (*46*). These in vitro findings are substantiated by reports of unusually high serum concentrations of chemokines in patients infected with H5N1 influenza viruses. High levels of macrophage-derived chemokines and cytokines were also induced by a recombinant virus containing a gene segment of the 1918 Spanish flu; in this case, however, the HA segment stimulated the increased levels of chemokines and cytokines (*14*,*47*). This upregulation of cytokine function at later phases of infection may account for the unusual clinical signs and symptoms and the degree of disease severity associated with human infections of highly pathogenic influenza viruses.

Highly pathogenic H5N1 viruses not only trigger the overproduction of proinflammatory cytokines but also are resistant to the antiviral effects of IFN and TNF-α. Pretreatment of porcine lung epithelial cells with IFN-α, IFN-γ, or TNF-α has no effect on the replication of a recombinant human H1N1 virus possessing the NS gene of the 1997 H5N1 virus but abolishes replication of the parental human H1N1 virus (*48*,*49*). Resistance to the antiviral effects of IFN and TNF-α is associated with glutamic acid at position 92 of the NS1 protein, as demonstrated by reverse genetics studies. These in vitro data extend to in vivo findings, since pigs infected with a virus containing Glu-92 in NS1 experience higher virus titers and body temperatures than those infected with a control virus (*48*,*49*). Collectively, these findings indicate that NS1 induces a cytokine imbalance that likely contributes to the extreme pathogenicity of avian influenza viruses in humans.

Conclusions

One might speculate that the next pandemic may be caused by highly pathogenic H5N1 viruses that acquire the ability to be efficiently transmitted among humans, or by H9N2 viruses, which are as prevalent as H5N1 viruses in Asia and in some cases already recognize human receptors. Further investigation of the molecular basis of host range restriction is therefore important. In addition, a better understanding of the mechanisms and consequences of

chemokine/cytokine imbalance caused by highly pathogenic avian viruses is essential, as is a greater appreciation for the contributions of other viral properties, such as replicative ability, to pathogenesis.

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References

1. Rogers GN, Paulson JC. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology. 1983;127: 361–73.
2. Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature. 1983;304:76–8.
3. Couceiro JN, Paulson JC, Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res. 1993;29:155–65.
4. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol. 1998;72:7367–73.
5. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, et al. Potential for transmission of avian influenza viruses to pigs. J Gen Virol. 1994;75:2183–8.
6. Claas EC, Osterhaus AD, Van Beek R, de Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 1998;351:472–7.
7. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science. 1998;279:393–6.
8. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman 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.
9. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A. 2004;101:4620–4.
10. Matrosovich M, Zhou N, Kawaoka Y, Webster R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol. 1999;73:1146–55.
11. Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, et al. The structure and receptor-binding properties of the 1918 influenza hemagglutinin. Science. 2004;303:1838–42.
12. Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA. Structure of the uncleaved human h1 hemagglutinin from the extinct 1918 influenza virus. Science. 2004;303:1866–70.
13. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol. 2000;74:8502–12.
14. Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, et al. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature. 2004;431:703–7.
15. Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology. 2001;281:156–62.
16. Baum LG, Paulson JC. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. Virology. 1991;180:10–5.
17. Kobasa D, Kodihalli S, Luo M, Castrucci MR, Donatelli I, Suzuki Y, et al. Amino acid residues contributing to the substrate specificity of the influenza A virus neuraminidase. J Virol. 1999;73:6743–51.
18. Blok J, Air GM. Variation in the membrane-insertion and "stalk" sequences in eight subtypes of influenza type A virus neuraminidase. Biochemistry. 1982;21:4001–7.
19. Els MC, Air GM, Murti KG, Webster RG, Laver WG. An 18-amino acid deletion in an influenza neuraminidase. Virology. 1985;142:241–7.
20. Luo G, Chung J, Palese P. Alterations of the stalk of the influenza virus neuraminidase: deletions and insertions. Virus Res. 1993;29: 141–53.
21. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature. 2004;430:209–13.
22. Takahashi T, Suzuki Y, Nishinaka D, Kawase N, Kobayashi Y, Hidari KI, et al. Duck and human pandemic influenza A viruses retain sialidase activity under low pH conditions. J Biochem (Tokyo). 2001;130:279–83.
23. de Jong, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med. 2005;352:686–91.
24. Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, et al. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol. 1999;73:3184–9.
25. Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol. 1999;73:5903–11.
26. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science. 2001;293:1840–2.
27. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman 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.
28. Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol. 2005;86:423–33.
29. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol. 1993;67:1761–4.
30. Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S. Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol. 2000;81 (Part 5):1283–91.
31. Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology. 1978;87:13–20.

32. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol. 1989;63:4603–8.
33. Snyder MH, Buckler-White AJ, London WT, Tierney EL, Murphy BR. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. J Virol. 1987;61:2857–63.
34. Scholtissek C, Stech J, Krauss S, Webster RG. Cooperation between the hemagglutinin of avian viruses and the matrix protein of human influenza A viruses. J Virol. 2002;76:1781–6.
35. Garten W, Klenk HD. Understanding influenza virus pathogenicity. Trends Microbiol. 1999;7:99–100.
36. Kawaoka Y, Naeve CW, Webster RG. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology. 1984;139:303–16.
37. Garcia M, Crawford JM, Latimer JW, Rivera-Cruz E, Perdue ML. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J Gen Virol. 1996;77:1493–504.
38. Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, et al. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol. 2001;146:963–73.
39. Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, Lee CW, et al. Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis. 2004;10:693–9.
40. Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, et al. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol. 2005;86:727–31.
41. Horimoto T, Kawaoka Y. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol. 1994;68:3120–8.
42. Garcia-Sastre A. Identification and characterization of viral antagonists of type I interferon in negative-strand RNA viruses. Curr Top Microbiol Immunol. 2004;283:249–80.
43. Krug RM, Yuan W, Noah DL, Latham AG. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology. 2003;309:181–9.
44. Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, et al. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci U S A. 2002;99:10736–41.
45. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet. 2002;360:1831–7.
46. Guan Y, Poon LL, Cheung CY, Ellis TM, Lim W, Lipatov AS, et al. H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci U S A. 2004;101:8156–61.
47. Kash JC, Basler CF, Garcia-Sastre A, Carter V, Billharz R, Swayne DE, et al. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. J Virol. 2004;78:9499–511.
48. Seo SH, Hoffmann E, Webster RG. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med. 2002;8:950–4.
49. Seo SH, Hoffmann E, Webster RG. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res. 2004;103:107–13.

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Dengue Prevention and 35 Years of Vector Control in Singapore

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After a 15-year period of low incidence, dengue has reemerged in Singapore in the past decade. We identify potential causes of this resurgence. A combination of lowered herd immunity, virus transmission outside the home, an increase in the age of infection, and the adoption of a case-reactive approach to vector control contribute to the increased dengue incidence. Singapore's experience with dengue indicates that prevention efforts may not be sustainable. For renewed success, Singapore needs to return to a vector control program that is based on carefully collected entomologic and epidemiologic data. Singapore's taking on a leadership role in strengthening disease surveillance and control in Southeast Asia may also be useful in reducing virus importation.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are reemerging diseases that are endemic in the tropical world. Disease is caused by 4 closely related dengue viruses that belong to the genus *Flavivirus* and are transmitted principally by the *Aedes aegypti* mosquito. Other mosquito species, such as *A. albopictus* and *A. polynesiensis*, can transmit epidemic dengue but do so less efficiently (*1*). The virus has 4 antigenically similar but immunologically distinct serotypes. Infection confers lifelong immunity to the infecting serotype but not to the remaining 3; therefore, a person can be infected with dengue virus up to 4 times during his or her lifetime. Furthermore, epidemiologic observations suggest that previous infection increases risk for DHF and dengue shock syndrome (DSS) in subsequent infections (*2*). These conditions are characterized by plasma leakage as a result of alteration in microvascular permeability (*3*). While DF may cause substantial morbidity, the death ratio of DHF and DSS can be as high as 30% if the disease is not properly managed (*4*). As yet, no specific treatment for DF or DHF is available, although efforts to develop an antidengue drug are in progress.

While vaccines for other flaviviruses such as yellow fever and Japanese encephalitis have been developed, dengue vaccine development is complicated by the need to incorporate all 4 virus serotypes into a single preparation. An approved vaccine is not likely to be available for 5 to 7 years; the only way to prevent dengue transmission, therefore, is to reduce the population of its principal vector, *A. aegypti*.

Dengue has been successfully prevented through vector control in 3 instances. The first of these was the highly successful, vertically structured paramilitary hemispheric eradication campaign directed by the Pan American Sanitary Board from 1946 to 1970 (*5*). The second was also a rigorous, top-down, military-like vector control operation in Cuba that was based on intensive insecticidal treatment followed by reduction of available larval habitats (source reduction) in 1981 (*6*). Neither of these programs, however, was sustainable. The third successful program was in Singapore.

Vector Control in Singapore

DHF appeared in Singapore in the 1960s and quickly became a major cause of childhood death. Public health response to dengue began in 1966, when the Vector Control Unit was set up within the Quarantine and Epidemiology Branch, initially in the Ministry of Health but transferred to the Ministry of the Environment in 1972, when DHF was made a notifiable disease (*7*); DF was made notifiable in 1977. From 1966 to 1968, following a series of entomologic surveys (*8–12*) and a pilot project to control the *Aedes* vectors in an area with high incidence of DHF (*13*), a vector control system based on entomologic surveillance and larval source reduction (i.e., reducing the availability of *Aedes* larval habitats) was developed; the system was implemented in 1968 (*7*). The thrust of this program was that mosquito breeding precedes disease

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transmission and controlling the vector population before disease is detected would reduce transmission. In a pilot project, this approach reduced the *A. aegypti* population in a 3-month period from 16% to 2%, as measured by the premises index, which is the percentage of inspected premises found to have containers with *A. aegypti* larvae or pupae (*13*). To maintain this low vector population density, however, the pilot study concluded that public involvement was necessary because the vector repopulates the area soon after vector control operations move to another site (*13*). The vector control program thus has 2 elements in addition to source reduction: public education and law enforcement. The Destruction of Disease Bearing Insects Act of 1968 was enacted to discourage persons from intentionally or unintentionally propagating mosquitoes.

The implementation of this vector control program was completed in 1973. The premises index since then has been ≈2%; achieving an index of zero has been difficult since natural breeding habitats are created as quickly as they are eliminated (*14*). With the reduced *A. aegypti* population, Singapore experienced a 15-year period of low dengue incidence. However, since the 1990s, the incidence of dengue has surged despite the low premises index (Figure 1).

Singapore's experience with dengue bodes ill for the sustainability of preventive efforts. Vector control may have worsened the dengue situation in Singapore because overt dengue attack rates in the 1990s and early 2000s were severalfold higher than those in the 1960s. We have identified several factors that may have contributed to this resurgence: lowered herd immunity, increasing virus transmission outside the home, more clinically overt infection as a consequence of adult infection, and a shift in the surveillance emphasis of the vector control program. We discuss each of these factors and suggest possible solutions.

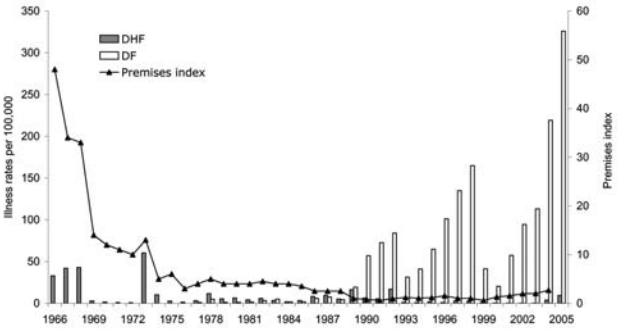

Figure 1. Annual incidence dengue fever (DF) and dengue hemorrhagic fever (DHF) and the premises index, Singapore, 1966–2005. DHF was made a notifiable disease in 1966, while DF became a notifiable disease in 1977. The annual incidences of DF and DHF reported in this figure were calculated from the number of reported cases each year from 1966 to 2004. The annual premises index is expressed as a percentage of the premises in which *Aedes aegypti* or *A. albopictus* larvae were found divided by the number of premises visited by environmental health officers.

Lowered Herd Immunity

Several explanations have been put forth to account for the resurgence of dengue in Singapore despite the vector control program (Figure 1). Reduced dengue transmission in the 1970s and 1980s resulted in a concomitant reduction in herd immunity to dengue virus (*15*). Low levels of population immunity provide an ideal condition for dengue transmission despite low *Aedes* mosquito density (*16*). This hypothesis is supported by observations made from a series of serologic surveys conducted in 1982–1984, 1990–1991, and 1993, in which a declining trend of seroprevalence among children was observed (*17*).

Low herd immunity in the Singapore population could also be deduced by comparing seroprevalence ratios with those of other dengue-endemic countries. The seropositive ratios of 6.7% in primary school children and 42% in adults (*18*) are in contrast to ratios reported in other dengue-endemic countries such as Thailand, where primary school children in Ratchaburi Province had a seropositive rate of 71% (*19*).

Transmission Outside the Home

Lowered herd immunity is, however, insufficient to account for the resurgence of dengue in Singapore. Dengue is predominantly a childhood disease in most parts of Southeast Asia, and more women than men are infected as adults. This disease pattern fits the behavior of *A. aegypti*. This species of mosquito is highly domesticated, lives and breeds indoors, has a limited flight range, and feeds almost exclusively on humans. Consequently, persons who spend more time at home during the daytime, i.e., mothers and children, are more likely to be infected than those who leave the home for work. In Singapore, however, the incidence of DF/DHF is lower in children than in adults (*14*). This finding could be due to a high proportion of subclinical infection in children or a lack of infection in the domestic environment.

To investigate this observation, a serologic survey of 1,068 children ≤15 years of age was conducted during an 18-month period in 1996 and 1997 (*17*). All children who were born at or who visited outpatient clinics of the National University Hospital, which serves the entire country, for routine check ups and vaccinations were included in this study, with parental consent. This population would have grown up during dengue resurgence. The results of this survey showed that preschool children, 10 months to 5 years of age, had a seroprevalence ratio of 0.77%, children 6–10 years of age and 11–15 years of age has prevalence ratios of 6.7% and 6.5%, respectively (*17*).

School-age children were therefore 9× more likely to have antibodies to dengue than were preschool children (*17*).

Preschool children spend most of their time either at home or at a nursery or kindergarten. Most of these facilities are run out of residences or shophouses in government-owned, high-rise accommodations. Formal half-day schooling starts at the age of 6 years, often with after-school extracurricular activities. The significant difference in seropositivity between preschool and school-age children suggests that the risk of acquiring dengue in Singapore is greater when a person spends more time away from home (*17*).

This hypothesis is supported by the lower premises index in residences than nonresidences in 1997. Residential properties in 1997 had low premises indexes; 2.1% in landed premises and 0.6% in apartments compared to indexes in schools (27.0%), construction sites (8.3%), factories (7.8%), and vacant properties (14.6%) (*20*). In contrast, the premises index in 1966 was highest in residences: slum housing (27.2%), shophouses (16.4%), and apartments (5.0%) (*9*). Furthermore, women, who are more likely than men to care for children at home, have a lower incidence of dengue, as indicated by the male-to-female disease ratio of 1.6:1 (*21*). Collectively, these findings suggest that substantial virus transmission occurs away from the home.

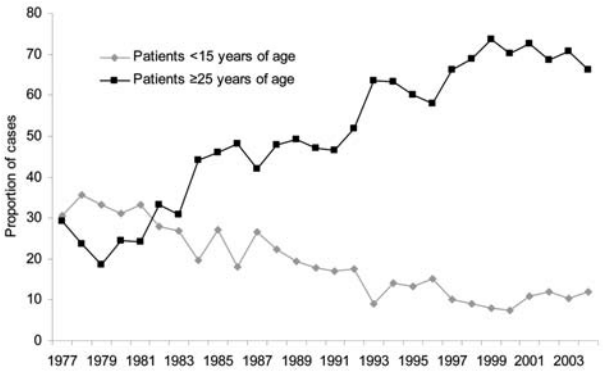

Figure 2. Proportion of indigenous dengue fever cases in patients <15 or ≥25 years of age, Singapore, 1977–2004. Indigenous cases are those that were acquired locally, among permanent and temporary residents of Singapore. Data were obtained from Communicable Disease Surveillance in Singapore, an annual publication of the Ministry of the Environment until 2002 and the Ministry of Health since 2003.

Dengue in Adults

As a consequence of lowered herd immunity and transmission outside the home, cases in adults predominate in Singapore. This fact is reflected in the steady decline in the proportion of patients <15 years of age, while the proportion of patients ≥25 years of age has increased over the years (Figure 2). This predominance of cases in adults may also contribute to the resurgence in dengue incidence. While most dengue infections, particularly primary infections in young children, are mild or silent (*22*,*23*), infections in adults are more likely to be clinically overt. In a recent dengue fever outbreak at a construction site in Singapore, patients had serologic and virologic evidence of primary dengue infection with serotype 2 virus (*24*). A serologic survey was conducted in the affected construction site; 274 of 360 workers volunteered for the study. With anti-dengue immunoglobulin M used as a marker, the survey identified 27 workers with recent infection. The illness was sufficiently debilitating for 24 (88.9%) of them to seek medical attention (*24*). Results support the commonly held perception that dengue infection in adults is more likely to be clinically overt than in children, contributing to the increase in the overall incidence of dengue.

A second consequence of the increase in patient age may be in the outcome of dengue infection. With the increase in patient age, most dengue cases in Singapore manifest as DF instead of DHF (Figure 1), even though a substantial proportion of adults have neutralizing antibodies to >2 serotypes of the dengue virus (*25*). The observed epidemiologic trend in Singapore therefore suggests that adults, while still susceptible, are at lower risk for DHF than are children. In the 1981 dengue outbreak in Cuba, hospitalization and death rates for severe and very severe dengue, postulated to be equivalent to DHF and DSS, were highest in those <15 years of age and those >60 years of age (*26*). Hospitalization and death rates were lower for those whose ages fell between these age groups, despite the same secondary infection with dengue serotype 2 virus (*26*). Results from Cuba support the hypothesis that adults are at lower risk for DHF and DSS than are children.

These age-dependent differences in the outcome of dengue infection may be due to differences in vascular permeability; children have a greater propensity for vascular leakage, under normal physiologic conditions, than do adults (*27*). This higher baseline of microvascular permeability in children could result in less ability to accommodate extraneous factors, such as dengue infection, that increase vascular permeability (*27*).

While the risk for DHF in adults is low compared to that in children, it is not absent. Besides host factors and secondary infection, certain strains of dengue viruses have been associated with severe disease (*28*,*29*). Although more work is needed to elucidate the role that age and other host and viral factors play in the pathogenesis of DHF, the current low DHF incidence cannot be taken as invulnerability to DHF outbreaks.

Shift in Surveillance Emphasis

Without a vaccine or antiviral drug, an effective vector control program is the only means to reduce dengue transmission. While most components of the vector control program remain similar to those of the 1970s, differences exist. Over time, the program evolved and its strategy changed. In particular, emphasis is now placed on early detection of cases and identifying whether they cluster in time and space, which is taken to indicate active virus transmission in the area. Detecting such clusters triggers emergency vector control operations, as was observed during a recent review of dengue in Singapore (*30*).

This shift of emphasis away from vector surveillance toward case detection cannot be linked with certainty to specific factors or events. Previous reviews of the dengue control program in Singapore in 1993 (*31*), 1994 (*32*), and 1997 (*33*) made the same observation. The shift probably took place in the late 1980s or early 1990s since Chan's report on the program in 1985 continued to emphasize vector surveillance (*7*). The shift in emphasis coincides with the latter stages of the 15-year period of low dengue incidence. With vector control, dengue transmission may have become sporadic and isolated, making perifocal mosquito control in response to reported cases more widely practiced as an efficient means of using public resources. Entomologic surveillance-based vector control still exists but only in limited, dengue-sensitive areas (*31–33*).

Responding to dengue cases and clusters, however, has limited effectiveness in preventing virus transmission, since such an approach ignores virus transmission from persons with subclinical infection or mild undifferentiated fever to uninfected mosquitoes. Furthermore, only ≈30% of cases can be mapped to a cluster. Most reported dengue cases occur outside known clusters. No evidence shows that emergency control measures, particularly the use of chemical insecticides, are effective after cases have already been detected (*31*).

Solutions

Observations made on epidemiologic features of dengue in Singapore indicate that further studies on the exact sites of dengue transmission need to be conducted. While the serologic study in children and the increasing proportion of adult infections, particularly among men, suggest that transmission may occur outside the home, further epidemiologic and virologic studies are needed. An ongoing case-control study, combining virus isolation, serotype identification, and genetic characterization of the virus by genome sequence analysis, may prove useful. Shedding more light on virus transmission dynamics would guide use of public health resources.

In addition to epidemiologic studies, more detailed entomologic research is needed. Larval source reduction and control are the most effective methods to deal with the *Aedes* vector. With lowered herd immunity and the possibility of virus transmission in nondomestic places, the vector control program in Singapore must return to an approach that emphasizes vector surveillance instead of early case detection. A repeat of some of the entomologic studies that were performed from 1966 to 1968 (*8–12*) may be fruitful. Results from such studies could help in revising the current vector control strategy and devising effective systems for surveillance of *A. aegypti*. Research on vector bionomics and evaluation of the cost-effectiveness of various control strategies may also be rewarding. Alternative approaches that complement larval source reduction should also be considered. We suggest 2 such approaches.

Ovitraps

Public involvement in Singapore is crucial to the sustainability of a vector control program (*7,13*). Public education is therefore essential for Singapore's vector control effort. National campaigns, such as the month-long "Keep Singapore Clean and Mosquito Free" campaign in 1969, have been conducted, and schoolchildren have been educated to carry out source reduction in their homes. However, 2 community-based surveys in 1992 and 1995 showed that while the population's awareness of the need for dengue control is high, many respondents did not believe that mosquitoes were in their homes and did not carry out necessary preventive measures (*34*). The survey population also reported that they checked their homes for mosquito breeding after having been fined under the Destruction of Disease-Bearing Insects Act, which has been superseded by the 1998 Control of Vectors and Pesticide Act. The problem with threatening the public with legal repercussions is that in the absence of checks by vector control officers, the public is not motivated to prevent mosquito breeding. What may be necessary would be a method of engaging the public through tools that provide regular positive feedback to the users. The recent positive experience in Vietnam is a case in point (*35*). Members of the public were closely engaged in the vector control effort by cleaning public areas and using copepods in water storage tanks. While the water supply system in Singapore is vastly different from that in Vietnam, the principle of engaging the public with an effective larvicidal tool could be adopted.

A larvicidal ovitrap was introduced by Lok et al. (*36*) that consists of a black, water-filled cylindrical container with a flotation device made up of a wire mesh and 2 wooden paddles. Eggs laid by mosquitoes on the wooden paddle hatch, and larvae develop in the water under the wire mesh. Resultant adult mosquitoes are trapped under the wire mesh and drown (Figure 3).

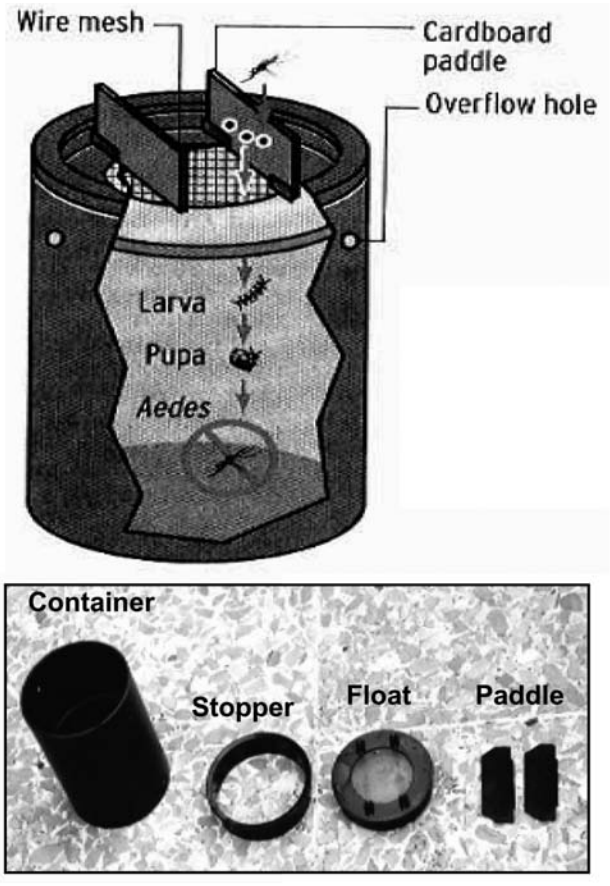

Figure 3. Diagrammatic representation of an autocidal ovitrap made up of a black cylinder, a wire mesh on a flotation device, and 2 pieces of cardboard. The gravid female *Aedes* mosquito lays its eggs on the cardboard. The larvae that hatch from the eggs go through the immature stages of the mosquito's lifecycle, but the resultant adult mosquito will be trapped underneath the wire mesh and drown. Picture inset shows the different components of the ovitrap.

The use of such an ovitrap has, in 2 previous instances, significantly reduced the *A. aegypti* population (*37*,*38*). The drawback in both these instances was that a large number of vector control officers were required to inspect and maintain the ovitraps. We suggest that such a trap could be used and maintained by members of the public instead of limiting its use to public employees. While training in the use and maintenance of ovitraps may be needed, seeing trapped mosquitoes may provide both positive feedback and a regular reminder of the need to be vigilant in efforts to curb the growth of the mosquito population.

An alternative to this ovitrap is a gravid female mosquito trap recently developed by Liew and Giger (patent pending). This device makes use of nondrying glue to trap gravid female mosquitoes that are attracted to water in the cylindrical device. In cage studies, this device maintained its effectiveness for up to 9 months (C. Liew, pers. comm.). Coupled with public education, using ovitraps or gravid female traps may sustain vector control more effectively than law enforcement.

Strengthening Regional Vector Control

A bolder, but possibly more rewarding, approach would be for Singapore to take the lead in strengthening disease surveillance and vector control in the Southeast Asian region, where dengue remains hyperendemic. The constant importation of dengue virus by travelers to Singapore may contribute to the observed dengue resurgence (*39*,*40*). Each year, 8 million visitors arrive in Singapore, not including residents who travel abroad or the thousands who commute across the causeway from the southern peninsula of Malaysia. The Singapore Changi Airport alone handles >20 million passengers per year, a rate that might better illustrate the amount of human traffic through Singapore. In the past 5 years, 5%–10% of dengue cases in Singapore have been imported. Most of these cases are from Indonesia, Thailand, and Malaysia. Collectively, these data suggest that symptomatic and asymptomatic persons can easily enter Singapore and infect vector mosquitoes. This problem will continue to expand, since travel and trade in the region are likely to increase. Expanding resources and effort toward achieving vector control in Southeast Asian countries may reduce importation of dengue and overall dengue incidence in Singapore.

Indeed, the mechanism to facilitate regional cooperation for disease surveillance and control is already being established. The Regional Emerging Disease Intervention Centre officially opened in Singapore on May 24, 2004. A joint United States–Singapore collaboration, its mission involves extending the perimeter of defense for emerging infectious diseases, widening the international network for research, and translating research findings into improved public health. While its immediate focus is on avian influenza and the threat of a pandemic, dengue could become a key item on its agenda, and a regional, surveillance-based vector control effort could be initiated.

Conclusions

In the absence of a safe and effective tetravalent vaccine for dengue viruses, vector control is the only method to prevent viral disease. The main lesson learned from Singapore's experience is that for a vector control program to be effective, it must be based on carefully collected and analyzed epidemiologic and entomologic surveillance data, with particular emphasis on ecologic factors that determine where, how, and when to initiate vector control, which Chan termed "vector epidemiology" (*7*). Reacting to cases, despite early and rapid diagnosis, is unlikely to reduce the incidence of dengue. An effective vector control

program will require an increase in expenditures, new strategies to lower and limit the *A. aegypti* population, and limiting importation of dengue virus into Singapore.

The Singapore experience also underscores the fact that dengue control must be a regional effort. Barring eradication of the mosquito vector, countries that control dengue transmission are doomed to failure if neighboring countries do nothing to prevent continued epidemic transmission. Thus, the combination of decreasing herd immunity and increasing imported dengue infection make preventing dengue transmission difficult, even with *A. aegypti* indexes as low as 2%, as exists in Singapore.

A final justification for regional *A. aegypti* control in Southeast Asia is the potential for epidemic urban yellow fever in the American tropics, risk for which is at its highest level in 60 years. With modern transportation, urban yellow fever could move quickly from the American tropics to the Asia-Pacific region, where ≈2 billion people are at risk. While a safe, effective vaccine for yellow fever is available, it is not manufactured in large enough quantities to prevent or control epidemics in Asia. Thus, regional *A. aegypti* control would be an effective preventive measure for epidemic dengue and yellow fever in Asia.

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References

1. Gubler DJ. Current research on dengue. In: Harris KF, editor. Current topics in vector research. New York: Springer Verlag Inc.; 1987. p. 37–56.
2. Halstead SB, Nimmannitya S, Cohen SN. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med. 1970;42:311–28.
3. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science. 1988;239:476–81.
4. Nimmannitya S. Dengue hemorrhagic fever: diagnosis and management. In: Gubler DJ, Kuno G, editors. Dengue and dengue hemorrhagic fever. Oxford: CABI Publishing; 1997. p. 133–45.
5. Schliessmann DJ, Calheiros LB. A review of the status of yellow fever and *Aedes aegypti* eradication programs in the Americas. Mosq News. 1974;34:1–9.
6. Kouri GP, Gusman MG, Bravo JR, Triana C. Dengue hemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. Bull World Health Organ. 1989;67:375–80.
7. Chan KL. Singapore's dengue haemorrhagic fever control program: a case study on the successful control of *Aedes aegypti* and *Aedes albopictus* using mainly environmental measures as a part of integrated vector control. Tokyo: Southeast Asian Medical Information Center; 1985.
8. Chan YC, Chan KL, Ho BC. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. 1. Distribution and density. Bull World Health Organ. 1971;44:617–27.
9. Chan KL, Ho BC, Chan YC. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. 2. Larval habitats. Bull World Health Organ. 1971;44:629–33.
10. Ho BC, Chan KL, Chan YC. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. 3. Population fluctuations. Bull World Health Organ. 1971;44:635–41.
11. Chan KL, Chan YC, Ho BC. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. 4. Competition between species. Bull World Health Organ. 1971;44:643–9.
12. Chan YC, Ho BC, Chan KL. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City. 5. Observations in relation to dengue haemorrhagic fever. Bull World Health Organ. 1971;44:651–8.
13. Chan KL. A report on the pilot control scheme in mosquito eradication at the Geylang–Tanjong Rhu area in Singapore. Singapore Public Health Bulletin. 1967;1:52–72.
14. Goh KT, Ng SK, Chan YC, Lim SJ, Chua EC. Epidemiological aspects of an outbreak of dengue fever/dengue hemorrhagic fever in Singapore. Southeast Asian J Trop Med Public Health. 1987;18:295–302.
15. Goh KT. Changing epidemiology of dengue in Singapore. Lancet. 1995;346:1098.
16. Newton EA, Reiter P. A model of the transmission of dengue fever with an evaluation of the impact of ultra-low volume (ULV) insecticide applications on dengue epidemics. Am J Trop Med Hyg. 1992;47:709–20.
17. Goh KT. Seroepidemiology of dengue in Singapore. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 50–72.
18. Ooi EE, Hart TJ, Tan HC, Chan SH. Dengue seroepidemiology in Singapore. Lancet. 2001;357:685–6.
19. Tuntaprasart W, Barbazan P, Nitatpattana N, Rongsriyam Y, Yoksan S, Gonzalez JP. Seroepidemiological survey among schoolchildren during the 2000–2001 dengue outbreak of Ratchaburi Province, Thailand. Southeast Asian J Trop Med Public Health. 2003;34:564–8.
20. Tan BT. Control of dengue fever/dengue hemorrhagic fever in Singapore. Dengue Bulletin. 1997;21:30–4.
21. Goh KT. Dengue-a remerging infectious disease in Singapore. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 33–49.
22. Burke DS, Nisalak A, Johnson DE, Scott RM. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg. 1988;38:172–80.
23. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, et al. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol. 2002;156:40–51.
24. Seet RC, Ooi EE, Wong HB, Paton NI. An outbreak of primary dengue infection among migrant Chinese workers in Singapore characterized by prominent gastrointestinal symptoms and a high proportion of symptomatic cases. J Clin Virol. 2005;33:336–40.
25. Wilder-Smith A, Yoksan S, Earnest A, Subramaniam R, Paton NI. Serological evidence for the co-circulation of multiple dengue virus serotypes in Singapore. Epidemiol Infect. 2005;133:667–71.
26. Guzman MG, Kouri G, Bravo J, Valdes L, Vazquez S, Halstead SB. Effect of age on outcome of secondary dengue 2 infections. Int J Infect Dis. 2002;6:118–24.
27. Gamble J, Bethell D, Day NP, Loc PP, Phu NH, Gartside IB, et al. Age-related changes in microvascular permeability: a significant factor in the susceptibility of children to shock? Clin Sci (Lond). 2000;98:211–6.

28. Gubler DJ, Reed D, Rosen L, Hitchcock JR Jr. Epidemiologic, clinical, and virology observations on dengue in the Kingdom of Tonga. Am J Trop Med Hyg. 1978;27:581–9.
29. Messer WB, Vitarana UT, Sivananthan K, Elvtigaala J, Preethimala LD, Ramesh R, et al. Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. Am J Trop Med Hyg. 2002;66:765–73.
30. Singapore Ministry of Health. Report of the expert panel on dengue [monograph on the Internet]. 2005 Oct 7 [cited 2006 Apr 6]. Available from http://www.moh.gov.sg/cmaweb/attachments/topic/3625c5ae51QU/Final_Report-dengue_7_Oct_05.pdf
31. Reiter P. Dengue control in Singapore. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 213–38.
32. Halstead SB. The dengue situation in Singapore. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 242–9.
33. Leake C. Singapore's dengue hemorrhagic fever control program. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 250–62.
34. Heng BH, Goh KT, How ST, Chua LT. Knowledge, attitude, belief and practice on dengue and *Aedes* mosquito. In: Goh KT, editor. Dengue in Singapore. Singapore: Institute of Environmental Epidemiology, Ministry of the Environment; 1998. p. 167–83.
35. Kay B, Vu SN. New strategy against *Aedes aegypti* in Vietnam. Lancet. 2005;365:613–7.
36. Lok CK, Kiat NS, Koh TK. An autocidal ovitrap for the control and possible eradication of *Aedes aegypti.* Southeast Asian J Trop Med Public Health. 1977;8:56–62.
37. Chan KL. The eradication of *Aedes aegypti* at the Singapore Paya Lebar International Airport. In: Vector Control in Southeast Asia. Proceedings of the 1st SEAMEO-TROPMED Workshop; Singapore; 1972. p. 85–8.
38. Cheng ML, Ho BC, Bartnett RE, Goodwin N. Role of a modified ovitrap in the control of *Aedes aegypti* in Houston, Texas, USA. Bull World Health Organ. 1982;60:291–6.
39. Cummings DA, Irizarry RA, Huang NE, Endy TP, Nisalak A, Ungchusak K, et al. Travelling waves in the occurrence of dengue haemorrhagic fever in Thailand. Nature. 2004;427:344–7.
40. Gubler DJ. Cities spawn epidemic dengue virus. Nat Med. 2004;10:129–30.

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etymologia

dengue

[den′gē]

An acute, self-limited disease characterized by fever, headache, myalgia, and rash caused by any of 4 related but distinct viruses of the genus *Flavivirus* and spread by *Aedes* mosquitos. Dengue (a Spanish homonym for the Swahili *ki denga pepo*, which describes a sudden, cramplike seizure caused by an evil spirit) is believed to have been first recorded in a Chinese medical encyclopedia from the Chin Dynasty (265–420 AD). The Chinese called dengue "water poison" and knew that it was somehow associated with flying insects.

Sources: Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11:480–96; and Halstead SB. Dengue hemorrhagic fever—a public health problem and a field for research. Bull World Health Organ. 1980;58:1–21.

Severe Community-acquired Pneumonia Due to *Staphylococcus aureus*, 2003–04 Influenza Season

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During the 2003–04 influenza season, 17 cases of *Staphylococcus aureus* community-acquired pneumonia (CAP) were reported from 9 states; 15 (88%) were associated with methicillin-resistant *S. aureus* (MRSA). The median age of patients was 21 years; 5 (29%) had underlying diseases, and 4 (24%) had risk factors for MRSA. Twelve (71%) had laboratory evidence of influenza virus infection. All but 1 patient, who died on arrival, were hospitalized. Death occurred in 5 (4 with MRSA). *S. aureus* isolates were available from 13 (76%) patients (11 MRSA). Toxin genes were detected in all isolates; 11 (85%) had only genes for Panton-Valentine leukocidin. All isolates had community-associated pulsed-field gel electrophoresis patterns; all MRSA isolates had the staphylococcal cassette chromosome *mec* type IVa. In communities with a high prevalence of MRSA, empiric therapy of severe CAP during periods of high influenza activity should include consideration for MRSA.

Staphylococcus aureus is an infrequent cause of community-acquired pneumonia (CAP), accounting for ≈3% of cases in which a bacterial cause is identified, but it is a recognized cause of influenza-associated CAP (*1–4*). Methicillin-resistant *S. aureus* (MRSA) commonly causes nosocomial pneumonia, but relatively few cases of MRSA CAP have been reported (*5,6*).

Recent reports have shown that MRSA is an emerging cause of skin and soft tissue disease among otherwise healthy persons who have little or no contact with healthcare settings (*7,8*). These community-associated strains of MRSA differ from healthcare-associated strains by having a characteristic methicillin-resistant gene cassette (staphylococcal cassette chromosome *mec* [SCC*mec*] type IV) that elicits certain toxins, notably Panton-Valentine leukocidin (PVL), resistance generally limited to the β-lactams and macrolides, and specific molecular typing patterns (*8–10*).

During the 2003–04 influenza season, the Centers for Disease Control and Prevention (CDC) received reports of severe complications after influenza virus infection, including pneumonia caused by *S. aureus* and MRSA, among previously healthy children and adults. We report the demographic and clinical features of 17 patients with *S. aureus* and MRSA CAP associated with influenza or influenzalike illness (ILI) and describe the microbiologic characteristics of the *S. aureus* isolates.

Methods

Case Definition and Case Finding

A case of *S. aureus* CAP associated with ILI (*S. aureus* CAP-ILI) was defined as pneumonia occurring during the 2003–04 influenza season in a person with either laboratory-confirmed influenza virus infection, clinician-determined ILI (e.g., fever plus sore throat or cough), or both during the 2003–04 influenza season from whom a speci-

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men (i.e., blood, sputum, or pleural fluid) collected <48 hours after hospitalization yielded *S. aureus*. Cases were identified by following up on reports of influenza-associated staphylococcal complications on 2 influenza assessment surveys conducted in December 2003 by the Infectious Diseases Society of America Emerging Infections Network, which consists of 859 infectious disease consultants (*11*). These surveys collected information on the 2003–04 influenza outbreak, including influenza-related complications, such as secondary bacterial infections, among pediatric and adult populations. Reports were also received through state and local health departments. Detailed clinical information on 4 cases was presented previously (*12*). We contacted clinicians and collected information by using a standardized data collection form on patient demographics, past medical history, signs and symptoms at the time the patient sought medical care, hospitalization, laboratory data including influenza testing, and risk factors for acquisition of MRSA (i.e., hospitalization, dialysis, surgery, or residence in a long-term care facility in the previous year; ever having an MRSA infection; and presence of percutaneous device or catheter at time of positive *S. aureus* culture). In addition, data on empiric (i.e., before *S. aureus* culture results were known) and targeted (i.e., after *S. aureus* culture results known) antimicrobial therapy and clinical outcomes were collected. Discordant empiric or targeted therapy was defined as a drug regimen that did not include an antimicrobial agent to which *S. aureus* was susceptible.

Laboratory Procedures

S. aureus isolates from patients were collected and sent to CDC for characterization. All available isolates were tested for susceptibility to chloramphenicol, clindamycin, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, penicillin, rifampin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin by using broth microdilution, as recommended by the Clinical Laboratory Standards Institute (*13*). Inducible clindamycin resistance was determined for isolates with the erythromycin-resistant/clindamycin-susceptible phenotype by using the double-disk diffusion test (D-zone test) (*13*). All isolates were tested for genes encoding selected toxins (staphylococcal enterotoxin [SE] A–E, H; PVL; and toxic shock syndrome toxin 1) by multiplex real-time polymerase chain reaction (PCR) using primers prepared at CDC. All MRSA isolates underwent typing of their SCC*mec* gene cassette with PCR (*14*). Genotyping of all isolates was performed by pulsed-field gel electrophoresis (PFGE) with *Sma*I-digested DNA, and gels were analyzed as previously described (*9*).

Results

Case Characteristics

From November 10, 2003, to January 4, 2004, 17 cases of *S. aureus* CAP-ILI were reported from 9 states (Alabama, Arkansas, Illinois, Maryland, Michigan, Missouri, Oklahoma, Texas, and Washington); 15 (88%) were due to MRSA. The median age of the 17 case-patients was 21 years; 5 (29%) patients had underlying diseases, and 4 (24%) had risk factors for MRSA (Table 1)

Table 1. Demographic and clinical characteristics of cases of *Staphylococcus aureus* community-acquired pneumonia associated with influenzalike illness, influenza season 2003–04*

Characteristic	No. (%), N = 17
Median age, y (range)	21 (3 mo–62 y)
Sex, male	8 (47)
Race	
White	10 (59)
Black	7 (41)
Underlying disease†	5 (29)
MRSA risk factors‡	4 (24)
Documented influenza vaccination	1 (6)
Evidence of influenza infection	
Laboratory-confirmed	12 (71)§
Rapid antigen test	10 (59)
Paired serology	2 (12)
Fluorescent antibody staining	2 (12)
Clinical symptoms	
Cough	14 (82)
Myalgias	9 (53)
Sore throat	6 (35)
Headache	6 (35)
Shortness of breath	5 (29)
Rigors	4 (24)
Clinical signs	
Temperature >38°C	12/13 (92)
Hypotension (systolic blood pressure <90 mm Hg)	12/13 (93)
Normal or elevated leukocyte count† (≥3,500/mm^3)	12/16 (75)
Median leukocyte count (range)	16,500 mm^3 (6,000–295,000)
Leukopenia (<3,500/mm^3)	4/16 (25)
Thrombocytopenia (<150,000/mm^3)	6/16 (38)
Radiologic documentation of pneumonia¶#	
Lobar	3/16 (19)
Multiple lobe involvement	4/16 (25)
Diffuse/patchy infiltrates	6/16 (38)
Effusions/empyema	5/16 (31)
Cavitation/necrosis	4/16 (25)

*MRSA, methicillin-resistant *S. aureus*.
†One each of diabetes, multiple sclerosis, prune belly syndrome, cystic fibrosis, eczema.
‡Hospitalization, dialysis, surgery, or residence in a long-term care facility in the previous year; ever having an MRSA infection; and presence of percutaneous device or catheter at time of culture.
§One patient had influenza infection confirmed by all methods.
¶One patient died on arrival at the hospital.
#Not mutually exclusive.

Although 5 (29%) patients were in the primary target groups (i.e., underlying illness [n = 2], age 50–64 years [n = 3]) recommended for annual influenza vaccination under current guidelines, only 1 (20%) had documented influenza vaccination during 2003–04. All case-patients had clinician-determined ILI. Twelve (71%) of the 17 patients had laboratory-confirmed influenza virus infection; 10 of these were confirmed by rapid antigen testing. *S. aureus* was recovered from multiple sources including sputum (14 [82%]), blood (8 [47%]), and pleural fluid (4 [24%].

Respiratory symptoms began a median of 4 days (range 1–17 days) before *S. aureus* specimen collection. All patients had ≥1of the following at the time they sought medical care: cough, myalgias, sore throat, headache, or shortness of breath. Most had fever, hypotension, and normal or elevated leukocyte counts. Four (25%) had leukopenia, and 6 (38%) had thrombocytopenia. Radiologic information was available for review for 16 patients, and all had documentation of an infiltrate. Information on empiric antimicrobial therapy was available for 15 patients; most received a third-generation cephalosporin (9 [60%]), respiratory fluoroquinolone (i.e., levofloxacin, gatifloxacin, or moxifloxacin) (7 [47%]), or vancomycin (10 [67%]); most patients (13 [87%]) received multiple antimicrobial agents. Discordant empiric antimicrobial therapy was documented in 3 (20%) patients, all of whom received a third-generation cephalosporin with or without a macrolide. Information on targeted antimicrobial therapy was provided for 13 patients (2 died before targeted treatment could be initiated) and consisted of vancomycin (10 [77%]), linezolid (2 [15%]), clindamycin (5 [38%]), and fluoroquinolones (4 [31%]); many patients (9 [69%]) received multiple antimicrobial drugs.

One patient was pronounced dead on arrival at the emergency department. Most patients were admitted to the intensive care unit and required intubation, and placement of chest tubes (Table 2). The median number of hospital days for patients was 13 (range 1–108 days). Five patients (4 with MRSA) died; their median age was 28 years (range 2 months–52 years), and only 1 had underlying illness (diabetes). Most died within 1 week of symptom onset.

Table 2. Outcomes of cases of *Staphylococcus aureus* community-acquired pneumonia associated with influenzalike illness, influenza season 2003–04

Outcome	No. (%), N = 17
Hospitalization	16 (94)*
Admitted to ICU†	13 (81)
Required intubation	8 (62)
Chest tube placement	6 (46)
Median length of stay (range)	13 days (1–108)
Death	5 (29)
Median age, y	28 (2–53)
Symptom onset to death, median days (range)	7 (3–73)
Underlying disease	1/5 (20)‡

*One patient died on arrival at the hospital.
†ICU, Intensive care unit.
‡Diabetes.

Laboratory Findings

S. aureus isolates were available from 13 (76%) patients (11 MRSA, 2 methicillin-susceptible *S. aureus*) from 9 states. Toxin genes were detected in all isolates; 11 (85%) had only the PVL genes, whereas 2 (15%) had genes for SEA, SEB, and SEH (Figure) All MRSA isolates had the SCC*mec* type IVa resistance gene cassette. Antimicrobial drug–susceptibility testing results for the MRSA isolates showed that all were resistant to oxacillin and erythromycin but susceptible to linezolid, rifampin, trimethoprim-sulfamethoxazole, and vancomycin; 10 (91%) and 6 (55%) isolates, respectively, were susceptible to clindamycin and levofloxacin. The 1 MRSA isolate that was not susceptible to clindamycin demonstrated inducible resistance by the D-zone test. In 4 cases, isolates were not available for testing at CDC. Antimicrobial drug susceptibility test results performed at the treating facility indicated that these 4 isolates were MRSA and nonsusceptible to macrolides (n = 4), clindamycin (n = 1), and levofloxacin (n = 1).

Analysis of PFGE results showed that 11 (85%) were community-associated pulsed-field types USA300, and 2 (15%) were USA400, according to CDC criteria (Figure). Of the 10 MRSA isolates that were classified as pulsed-field type USA300, 8 (80%) from 6 different states had indistinguishable banding patterns and were further classified as USA300 subtype 0114. These MRSA isolates differed from pulsed-field types associated with healthcare-related strains (USA100 and 200) (*9*).

Discussion

We report the emergence of *S. aureus* and MRSA as a cause of CAP-ILI resulting in severe illness and death in otherwise healthy persons in the United States during the 2003–04 influenza season. Most infections were caused by MRSA strains that contained PVL genes and were uniformly resistant to macrolides; half were nonsusceptible to fluoroquinolones. However, the isolates were susceptible to other antimicrobial agents, including vancomycin and linezolid. Although some phenotypic differences were noted, most cases of pneumonia appeared to be attributable to a single strain of MRSA found in diverse geographic areas. This strain, USA300 subtype 0114, is a predominant strain responsible for community outbreaks of MRSA skin disease in the United States (*8*,*9*,*15*).

Postinfluenza staphylococcal pneumonia has been reported in healthy adults during influenza pandemics and epidemics for the last century; it has been reported in the literature less frequently during the past 30 years (*1*–*3*). The recognition of MRSA as a cause of CAP-ILI has

occurred concomitant with reports of MRSA as an increasingly common cause of skin and soft tissue infection in the community. Molecular typing of isolates in our series demonstrates that the CAP-ILI isolates are indistinguishable from MRSA associated with numerous outbreaks of skin and soft tissue infections (*8*). Given this association, MRSA might become a more common cause of *S. aureus* CAP following or coincident to influenza infection in regions where the MRSA strain is prevalent as a cause of skin and soft tissue infection. Antecedent *S. aureus* skin infection or colonization may be associated with postinfluenza *S. aureus* CAP, as was reported during the 1957 influenza pandemic (*16*). Although we did not systematically collect information on antecedent skin infections in our study, skin infections occurring among families of case-patients were noted. Given the apparent wide national distribution of MRSA as a cause of skin disease, physicians should be aware that MRSA can cause not only skin and soft tissue infections but also CAP.

Although most of the reported patients had laboratory confirmation of influenza virus as a cause of preceding illness, those diagnoses based solely on clinical symptoms may have been caused by other viral respiratory pathogens. However, growing evidence of mechanisms by which influenza may interact specifically with *S. aureus* to increase the risk for influenza–*S. aureus* co-infections suggests that these *S. aureus* CAP infections were likely associated with influenza (*17*). These include an influenza-induced increase in *S. aureus*–specific adhesion throughout the respiratory tract and *S. aureus*–specific proteases, which may increase influenza viral replication (*18–20*). This latter mechanism actually points to a synergistic relationship in which *S. aureus* increases influenza disease severity while influenza increases *S. aureus* infection and severity. Strains of influenza A virus also decrease phagocytic killing of *S. aureus*, leading to increased host susceptibility to bacterial superinfection (*21*). No other respiratory virus appears to share with influenza such a prominent role in predisposing to and increasing the severity of *S. aureus* pneumonia.

Risk factors for postinfluenza *S. aureus* CAP are undefined, but annual influenza vaccination is not recommended for half of the patients reported in our series under current guidelines (*22*). However, influenza vaccination is a major preventive strategy for influenza-associated pneumonia in older adults and in children 6–23 months of age (*22*,*23*). Moreover, studies have demonstrated that influenza vaccination can decrease the incidence of upper respiratory infections and lessen the need for antimicrobial drug use in healthy adults (*24*,*25*). Although these studies do not focus on specific bacterial complications, many studies have shown that influenza vaccination reduces overall pneumonia risk; thus one can reasonably assume that influenza vaccination would prevent secondary bacterial infections, including MRSA, in immunocompetent adults (*24*,*26*). Because information on antiviral treatment was not collected and most patients in this series sought medical care >2 days after illness onset, we could not assess the effects of early antiviral treatment. Although 1 study reported that early antiviral treatment of influenza with oseltamivir can decrease the incidence of lower respiratory tract complications, further studies are needed to determine whether early antiviral treatment of influenza can help reduce the risk for *S. aureus* pneumonia associated with influenza (*22*,*27*).

The incidence of MRSA CAP is unknown. In 2004, to monitor the incidence of MRSA, CDC initiated active population-based surveillance for invasive MRSA disease in 9 locations in the United States through the Emerging Infections Programs, Active Bacterial Core surveillance.

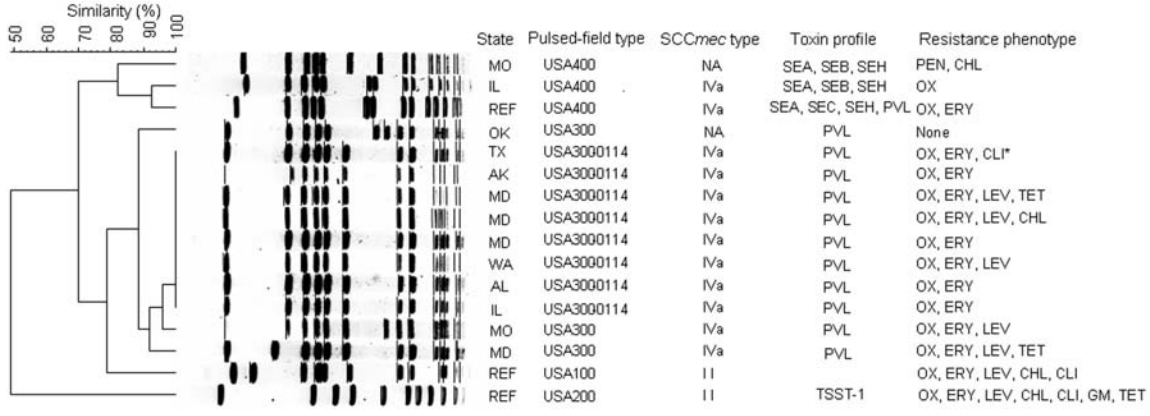

Figure. Dendrogram of *Staphylococcus aureus* isolates determined by using *SmaI*–digested DNA recovered from patients with community-acquired pneumonia associated with influenzalike illness, influenza season, 2003-04. NA, not applicable (methicillin-susceptible); SE, staphylococcal enterotoxin A, B, C, H; REF, reference strain; PVL, Panton-Valentine leukocidin; TSST, toxin shock syndrome toxin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; GM, gentamicin; LEV, levofloxacin; OX, oxacillin; PEN, penicillin; TET, tetracycline. *Inducible clindamycin resistance.

These data will help characterize the further emergence of MRSA as a cause of CAP, guide public health interventions to prevent these infections, and provide information to guide empiric therapy recommendations. Currently recommended empiric therapy of CAP in immunocompetent adults with bacterial superinfection following influenza consists of a β-lactam or respiratory fluoroquinolone and may not adequately provide activity against community strains of MRSA (*28*). Whenever possible, physicians should obtain specimens (e.g., sputum or blood cultures) for diagnostic and antimicrobial drug–susceptibility testing to target therapy (*28*,*29*). Most patients in our series had severe disease and received broad-spectrum antimicrobial drugs, including coverage for resistant gram-positive bacteria. Whether initial inadequate empiric therapy plays a role in patient outcomes is therefore unknown.

Our cases suggest that empiric therapy of severe CAP during periods of high influenza activity should include coverage for MRSA, including among those without recognized risk factors for MRSA. In this regard, our concerns echo those of Martin et al. in 1959. Following these researchers' experience with the emergence of penicillin-resistant staphylococci during the 1957–58 Asian influenza pandemic, they commented "…during epidemics of influenza in localities in which staphylococci are known to be prevalent, all patients with signs of severe, potentially fatal influenza should—until proven otherwise—be diagnosed and treated promptly as cases of staphylococcal pneumonia caused by relatively antibiotic-resistant staphylococci" (*1*).

Acknowledgments

We thank Benjamin Estrada, Lisa Carollo, Jean Kirk, Melissa Tucker, Bette J. Jensen, David Lonsway, and Susan Webb for assisting in data collection and acquisition of isolates.

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References

1. Martin CM, Kunin CM, Gottlieb LS, Finland M. Asian influenza A in Boston, 1957–1958. II. Severe staphylococcal pneumonia complicating influenza. Arch Intern Med. 1959;103:532–42.
2. Schwarzmann SW, Adler JL, Sullivan RJ Jr, Marine WM. Bacterial pneumonia during the Hong Kong influenza epidemic of 1968–1969. Arch Intern Med. 1971;127:1037–41.
3. Chickering HT, Park JH. *Staphylococcus aureus* pneumonia. JAMA. 1919;72:617–26.
4. Fine MJ, Smith MA, Carson CA, Mutha SS, Sankey SS, Weissfeld LA, et al. Prognosis and outcomes of patients with community-acquired pneumonia. A meta-analysis. JAMA. 1996;275:134–41.
5. Johnston BL. Methicillin-resistant *Staphylococcus aureus* as a cause of community-acquired pneumonia—a critical review. Semin Respir Infect. 1994;9:199–206.
6. Centers for Disease Control and Prevention. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999. MMWR Morb Mortal Wkly Rep. 1999;48:707–10.
7. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, et al. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA. 2003;290:2976–84.
8. Kazakova SV, Hageman JC, Matava M, Srinivasan A, Phelan L, Garfinkel B, et al. A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. N Engl J Med. 2005;352:28–35.
9. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC, et al. 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.
10. 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.
11. Podewils LJ, Liedtke LA, McDonald LC, Hageman JC, Strausaugh LJ, Fischer TK, et al. A national survey of severe influenza-associated complications among children and adults, 2003–04. Clin Infect Dis. 2005;40:1693–6.
12. Francis JS, Doherty MC, Lopatin U, Johnston CP, Sinha G, Ross T, et al. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. Clin Infect Dis. 2005;40:100–7.
13. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility tests. Supplement M100-S14. Wayne (PA): The Committee; 2004.
14. Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, Boyle-Vavra S, et al. Novel type of staphylococcal cassette chromosome mec identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother. 2002;46:1147–52.
15. Centers for Disease Control and Prevention. Methicillin-resistant *Staphylococcus aureus* infections among competitive sports participants—Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000–2003. MMWR Morb Mortal Wkly Rep. 2003;52:793–5.
16. Goslings WR, Mulder J, Djajadiningrat J, Masurel N. Staphylococcal pneumonia in influenza in relation to antecedent staphylococcal skin infection. Lancet. 1959;2:428–30.
17. Nickerson CL, Jakab GJ. Pulmonary antibacterial defenses during mild and severe influenza virus infection. Infect Immun. 1990;58: 2809–14.
18. Sanford BA, Ramsay MA. Bacterial adherence to the upper respiratory tract of ferrets infected with influenza A virus. Proc Soc Exp Biol Med. 1987;185:120–8.
19. Tashiro M, Ciborowski P, Reinacher M, Pulverer G, Klenk HD, Rott R.. Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. Virology. 1987;157:421–30.
20. Davison VE, Sanford BA. Adherence of *Staphylococcus aureus* to influenza A virus–infected Madin-Darby canine kidney cell cultures. Infect Immun. 1981;32:118–26.
21. Abramson JS, Lewis JC, Lyles DS, Heller KA, Mills EL, Bass DA. Inhibition of neutrophil lysosome-phagosome fusion associated with influenza virus infection in vitro. Role in depressed bactericidal activity. J Clin Invest. 1982;69:1393–7.
22. Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB, Advisory Committee on Immunization Practices, et al. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2005;54(RR-8):1–40.

23. Gross PA, Hermogenes AW, Sacks HS, Lau J, Levandowski RA. The efficacy of influenza vaccine in elderly persons. A meta-analysis and review of the literature. Ann Intern Med. 1995;123:518–27.
24. Nichol KL, Lind A, Margolis KL, Murdoch M, McFadden R, Hauge M, et al. The effectiveness of vaccination against influenza in healthy, working adults. N Engl J Med. 1995;333:889–93.
25. Bridges CB, Thompson WW, Meltzer MI, Reeve GR, Talamonti WJ, Cox NJ, et al. Effectiveness and cost-benefit of influenza vaccination of healthy working adults: a randomized controlled trial. JAMA. 2000;284:1655–63.
26. Hak E, Hoes AW, Grobbee DE, Lammers JW, van Essen GA, van Loon AM, et al. Conventional influenza vaccination is not associated with complications in working-age patients with asthma or chronic obstructive pulmonary disease. Am J Epidemiol. 2003;157:692–700.
27. Kaiser L, Wat C, Mills T, Mahoney P, Ward P, Hayden F. Impact of oseltamivir treatment on influenza-related lower respiratory tract complications and hospitalizations. Arch Intern Med. 2003;163: 1667–72.
28. Mandell LA, Bartlett JG, Dowell SF, File TM Jr, Musher DM, Whitney C, et al. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis. 2003;37:1405–33.
29. Bartlett JG. Diagnostic test for etiologic agents of community-acquired pneumonia. Infect Dis Clin North Am. 2004;18:809–27.

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Temple Monkeys and Health Implications of Commensalism, Kathmandu, Nepal

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The threat of zoonotic transmission of infectious agents at monkey temples highlights the necessity of investigating the prevalence of enzootic infectious agents in these primate populations. Biological samples were collected from 39 rhesus macaques at the Swoyambhu Temple and tested by enzyme-linked immunosorbent assay, Western blot, polymerase chain reaction, or combination of these tests for evidence of infection with rhesus cytomegalovirus (RhCMV), *Cercopithecine herpesvirus* 1 (CHV-1), simian virus 40 (SV40), simian retrovirus (SRV), simian T-cell lymphotropic virus (STLV), simian immunodeficiency virus (SIV), and simian foamy virus (SFV). Antibody seroprevalence was 94.9% to RhCMV (37/39), 89.7% to SV40 (35/39), 64.1% to CHV-1 (25/39), and 97.4% to SFV (38/39). Humans who come into contact with macaques at Swoyambhu risk exposure to enzootic primateborne viruses. We discuss implications for public health and primate management strategies that would reduce contact between humans and primates.

Most pathogens that affect humans are thought to have originated in animals and subsequently evolved to successfully parasitize human populations (*1*). Proximity and physical contact between animals and humans provide the opportunity for infectious agents to pass between the groups. Whether a particular infectious agent can successfully make the cross-species jump depends in part on the new host environment (*1*). By virtue of their genetic, physiologic, and behavioral similarity to humans, nonhuman primates (hereafter referred to as primates) are particularly likely sources of emerging infectious agents with the capacity to infect humans, and primate-to-human cross-species transmission of infectious agents has become a focus of scientific inquiry. Because human-primate contact is common in Asia, this continent is a rich area in which to pursue this research. We examine the prevalence of selected enzootic primateborne viruses in a population of rhesus macaques (*Macaca mulatta*) that lives in close proximity to humans.

Monkey Temple Context for Cross-species Transmission

Monkey temples can be found throughout South and Southeast Asia, where primates play a role in Hindu and Buddhist culture (*2*). Macaque species, because they can thrive in human-altered environments, are the primates most often associated with temples. Extensive, unregulated, and often close contact between humans and primates occurs at these sites (*3*). Persons who live or work in or around monkey temples are among those who frequently come into contact with temple monkeys (*4*). Other persons may come into contact with temple macaques when they visit for purposes of worship, recreation, or tourism. Worldwide, monkey temples may account for more human-primate contact than any other context (*5*).

Swoyambhu Temple is 1 of 2 temple sites in the densely populated Kathmandu valley with a large population of free-ranging rhesus monkeys (*6*) (Figure 1). As 1 of the region's oldest and most important Buddhist holy places, Swoyambhu has been designated a world heritage site and continues to play a vibrant role in Kathmandu's cultural life. In addition to the Tibetan monks, Brahmin priests, and

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Figure 1. Swoyambhu Temple in Kathmandu, Nepal, is home to 400 free-ranging rhesus macaques (*Macaca mulatta*). (Photo by R. Kyes.)

Newar nuns who live on the site, a brisk flow of local worshipers and visitors from around the world passes through Swoyambhu. Persons who live and work in and around Swoyambhu share common water sources with the macaques and report that the macaques frequently invade their homes and gardens in search of food. The macaques at Swoyambhu have become a tourist attraction in their own right, and many visitors interact with the monkeys, often by feeding or teasing them (Figure 2). A growing literature documents human-macaque interactions at monkey temples in Asia (*2*–*5*). Macaques climb on the heads and shoulders of visitors, which may bring macaque body fluids into contact with visitors' eyes and nasal and oral mucosa, potential portals of entry for infectious agents. Visitors may also be bitten or scratched by macaques during aggressive encounters, resulting in transcutaneous exposure to infectious agents present in macaque body fluids.

Enzootic Simian Viruses

Cercopithecine herpesvirus 1

Cercopithecine herpesvirus 1 (CHV-1), also known as herpes B virus, is a member of the taxonomic subfamily *Alphaherpesviridae*. Serologic evidence of infection with CHV-1 has been documented in several species of macaques (*7*). Seroprevalence of anti-herpesvirus antibodies is 10%–80% among wild populations and can reach 100% among captive populations, though the percentage of infected monkeys who shed virus at a given time is only 1%–2% (*8*).

While CHV-1 infection in primates is almost always benign, CHV-1 infection in humans causes severe meningoencephalitis with a death rate approaching 70% (*9*). Several routes of primate-to-human transmission have been implicated, most involving direct exposure to tissue or fluid from an infected macaque. One case of human-to-human transmission of CHV-1 has been documented (*10*). No cases of CHV-1 infection have been documented in persons exposed to free-ranging macaques, in spite of a long history of human-macaque commensalism in Asia.

SV40

Simian virus 40 (SV40) is a polyomavirus enzootic among some species of Asian macaques, including rhesus macaques of northern India and Nepal. SV40 is present in the genitourinary tract of infected macaques and is thought to be transmitted through ingestion of urine containing the virus (*11*). SV40 first became an object of public health interest in the 1960s when millions of doses of polio vaccine, produced in tissue cultures of monkey kidney cells, were contaminated with SV40. Shah (*12*) examined the seroprevalence of antibodies to polyomavirus among workers at 2 monkey export firms in India who had abundant, long-term contact with rhesus macaques; he found a seroprevalence of 27% among these workers and noted that SV40 seroprevalence increased with duration of work in the export firms. Recent technological advances have improved the specificity of immunoassays that detect antibodies to SV40 (*13*). Using these new methods, Engels and colleagues reported evidence of human SV40 infection among zoo employees who worked with primates (*14*).

Rhesus Cytomegalovirus

Rhesus cytomegalovirus (RhCMV), *Cercopithecine herpesvirus* 8, is a β-herpesvirus enzootic among *M. mulatta*, infecting up to 100% of rhesus macaques >1 year of age in breeding populations of captive animals (*15*). In

Figure 2. Rhesus macaques at Swoyambhu Temple routinely get food handouts from local inhabitants and visitors. (Photo by L. Jones-Engel.)

immunologically intact animals, RhCMV infections are asymptomatic. RhCMV can cause illness and death in rhesus macaques co-infected with immunosuppressive retroviruses (simian type D retrovirus and simian immunodeficiency virus) (*16*) or in experimentally infected rhesus macaque fetuses (*17*). Infection is lifelong, with continued viral shedding from mucosal surfaces (*18*). Though growth of RhCMV in human cells has been demonstrated in vitro, human infection with RhCMV has yet to be reported (*19*).

Enzootic Simian Retroviruses

Macaques harbor several enzootic retroviruses, including simian foamy virus (SFV), simian type D retrovirus (SRV), and simian T-cell lymphotropic virus (STLV). SRV and SFV are present in saliva and other body fluids of infected macaques, which suggests that bites, scratches, and mucosal splashes with macaque body fluids can transmit infection (*20*,*21*). Previous studies examining laboratory and zoo workers as well as bushmeat hunters in Africa and monkey temple workers in Indonesia have shown that humans can be infected with SFV and SRV (*5*,*22*–*24*).

STLV is closely related to human T-cell lymphotropic virus (HTLV-1). Asymptomatic infection with STLV-1 is common among primate hosts. STLV is hypothesized to be the progenitor of HTLV through multiple cross-species transmissions (*25*).

Simian immunodeficiency virus (SIV) is widely distributed among African primates and has been shown to infect humans who come into contact with them (*26*). Though SIV has not, to date, been detected among Asian primates, several species of Asian macaques have been experimentally infected with the virus (*27*). And though international trade in primates is regulated by the Convention on International Trade in Endangered Species, illicit import and export of primates continues, potentially exposing Asian primates to infectious agents, such as SIV, that are enzootic among African primate species.

Materials and Methods

Macaque Population

The rhesus macaques at Swoyambhu number ≈400, distributed among 5 to 7 groups with overlapping home ranges (*6*). Physical contact among macaque groups is common. Natural forage is extremely limited at Swoyambhu (Figure 3). Almost all of the macaques' daily food comes from handouts given by persons who frequent the temple site.

Field Methods

During a 4-day period in May 2003, a total of 39 macaques from 3 different groups (12 from group 1, 11 from group 2, and 16 from group 3) were trapped, sampled, and released. Samples were obtained as part of a comprehensive health screening effort conducted at the request of the Federation of Swoyambhu Management and Conservation Committee. Macaques were trapped in a portable cage measuring $2.5 \times 2.5 \times 1.5$ m and sedated with 3 mg/kg intramuscular tiletamine HCl/zolazepam HCl. To avoid stressing young animals, infants were not anaesthetized or sampled as part of this protocol. All anesthetized macaques were given a complete physical examination, and using universal precautions and sterile technique, we collected 10 mL blood by venipuncture of the femoral vein; 8 mL blood was centrifuged to extract serum. The remaining blood was aliquotted into Vacutainer vials containing EDTA. Unique study identification numbers were assigned to all specimens collected from each animal. Serum and whole blood were frozen in the field, then stored at −70°C. Animals were tattooed on their inner right thigh for identification and future follow-up. Each macaque's weight and dental formula were collected and recorded for age assessment. Age was estimated on the basis of observed dental eruption sequence. After sample collection, animals were placed in a cage and allowed to recover fully from anesthesia before being released as a group back into their home range. This data collection protocol was reviewed and approved by the University of Washington Institutional Animal Care and Use Committee (#3143-03).

Laboratory and Data Analysis Methods

After necessary national and international permits were obtained, the samples were shipped to the United States, where they were analyzed at several institutions. Enzyme immunoassays for anticapsid antibodies to SV40 were performed as described previously (*13*). Serologic status to RhCMV was determined by enzyme-linked immunosorbent assay (ELISA) with an infected cell extract to detect

Figure 3. Natural forage is extremely limited at Swoyambhu. Rhesus macaques routinely raid garbage bins and people's homes in search of food. (Photo by R. Kyes.)

RhCMV-specific immunoglobulin G (IgG) (*28*). ELISAs were used to detect antibodies to SRV, STLV, SIV, and CHV-1 as previously described (*29*,*30*). Because of endogenous seroreactivity to retroviral proteins in macaques, immunoblot assays for STLV and SRV serotypes 1, 2, 4, and 5 and were performed on all samples to confirm antibody status. Reactions were deemed positive if core and envelope bands were present, indeterminate if only core or only envelope were present, and negative if bands did not appear or were lighter than those for negative control plasma. Real-time polymerase chain reaction (PCR) for SRV was performed as previously described (*30*). To produce large volumes of SRV-1, 2, 4, and 5 antigens for enzyme immunoassay, infected cell supernatants were collected, concentrated, and purified on sucrose gradients as previously described (*29*). Nested PCR to detect STLV in these samples was performed as previously described (*30*). Western blot immunoassays for SFV were performed as previously described, with a few modifications (*5*,*31*).

Demographic and serologic data were entered into a spreadsheet, and univariate analysis was performed with the JUMP-IN 4 statistical software package (SAS Institute, Inc., Cary, NC, USA). Statistical associations between macaque viral seropositivity, sex, age class, and group number were determined by χ^2 test.

Results

Table 1 presents the demographic distribution of the macaques sampled. The animals sampled may not reflect the demographic breakdown of the Swoyambhu population as a whole because animals were trapped opportunistically, and infants were excluded from the study. Approximately 9.75% of Swoyambhu's estimated macaque population was sampled. Table 2 presents seroprevalence data for the 39 macaques sampled. Seven samples reacted to SRV on ELISA; 4 of these 7 were indeterminate on immunoblot, and 3 were negative. Repeated attempts to amplify SRV from all samples, including those indeterminate by immunoblot, by using PCR primers in 2 different regions of the genome were not successful. STLV testing showed 9 samples to be reactive on ELISA, but none were confirmed positive by immunoblot. Nested PCR did not detect STLV DNA in peripheral blood mononuclear cells. Additional tests for SRV and STLV by PCR were performed to rule out latent virus in genomic DNA, since some apparent false reactivity was seen on ELISA and Western blot. None of the 39 serum samples was reactive on SIV ELISA.

The results of the serologic and PCR assays were analyzed by using χ^2 to test associations by sex, group number, and age category. The results from these tests show no significant association between seropositivity for antibodies to RhCMV, SV40, or SFV and age, sex, or group number of the macaque. However, a significant (χ^2 $p<0.0001$) age-related effect was seen for CHV-1. Seroprevalence of antibodies to CHV-1 increased from 23.1% (3/13) among juveniles to 100% (19/19) among adult macaques.

Table 1. Demographic distribution of rhesus macaques sampled at Swoyambhu

Age class	n	Males	Females
Juvenile	13	6	7
Subadult	7	1	6
Adult	19	10	9
Total	39	17	22

Discussion

Relatively little is known about enzootic primate viruses in free-ranging populations of macaques. CHV-1 antibody prevalence has been measured among temple monkeys in Bali (*4*), rhesus monkeys (*M. mulatta)* from India (*32*), and free-ranging rhesus monkeys transplanted to the Caribbean Island of Cayo Santiago (*11*). The CHV-1 seroprevalence among these populations is similar to that of the Swoyambhu macaques, with a similar positive association between age and seroprevalence. SFV prevalence among the Bali macaques was also similar to that measured in the Swoyambhu macaques (*5*). The high seroprevalence of RhCMV in the Swoyambhu macaque population mirrors that measured in other studies that assessed seroprevalence in both captive and free-ranging populations of macaques and other primates (*15*,*33*).

Evidence of STLV-1 infection was not found among the Swoyambhu macaques with either serologic or PCR detection methods. In comparison, a survey measuring STLV-1 prevalence among wild-caught *M. fascicularis* in Indonesia by serologic methods and PCR suggested an STLV prevalence between 3.3% and 10% (*34*), and a sample of wild-caught *M. fascicularis* from 9 localities in Thailand were all antibody-negative for STLV-1 (*35*).

No conclusive serologic or PCR evidence of SRV infection was found among the Swoyambhu macaques. SRV infection is commonly seen among laboratory primates, but far less so among other free-ranging primate populations examined to date (L. Jones-Engel, unpub. data). Increased population densities characteristic of captive settings may facilitate viral transmission, providing a possible explanation for this observation.

The absence of SIV in the Swoyambhu macaque population is unsurprising, given that SIV has yet to be detected in natural populations of Asian primates. This finding, however, does not eliminate the possibility that the situation could change in the future. While SIV is typically found only among African primates, this virus could be introduced into Asian primate populations through the global market trade in animals, in which pet primates can

Table 2. Seroprevalence of select enzootic simian viruses among Swoyambhu rhesus macaques*†

Characteristic	n	RhCMV (% ELISA-reactive)	SV40 (% EIA-reactive)	CHV-1 (% ELISA-reactive)	SFV (% WB-reactive)
Male	17	94.1	94.1	64.7	94.1
Female	22	95.5	86.4	63.6	100.0
Juvenile	13	84.6	76.9	23.1	92.3
Subadult	7	100.0	100.0	42.9	100.0
Adult	19	100.0	94.7	100.0	100.0
Total	39	94.9	89.7	64.1	97.4

*RhCMV, rhesus cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; SV40, simian virus 40; EIA, enzyme immunoassay; CHV-1, cercopithecine herpesvirus 1; SFV, simian foamy virus; WB, Western blot.
†Seven samples were ELISA-positive for simian retrovirus (SRV); 4 of these were indeterminate on WB, and 3 were negative. Polymerase chain reaction (PCR) failed to amplify SRV from any sample. Nine samples were ELISA-positive for simian T-cell lymphotropic virus (STLV), but none were positive on immunoblot, and nested PCR detected no STLV DNA. None of the samples was reactive to simian immunodeficiency virus.

be purchased (*36*). Abandoned pet primates are commonly seen in monkey forests in Asia, and future surveys of Asian primates should continue to test for SIV.

Public Health Implications

A growing literature suggests that cross-species transmission of infectious agents occurs between humans and several primate species in a variety of contexts and in diverse areas (*4*,*5*,*22*–*24*,*37*,*38*). Indeed, wherever humans and primates come into contact, the potential for cross-species transmission exists. Whether cross-species transmission occurs depends on several factors, including the prevalence of infectious agents in primate reservoirs, the context of interspecies contact, and the frequency with which contact occurs (*39*). To date, cross-species transmission has been most thoroughly studied in primate laboratories and zoos because of the ready availability of biological samples from both primates and exposed humans (*14*,*22*,*23*,*40*). Research on humans exposed to primates in these contexts has documented an SFV seroconversion rate of 1% to 5.3%, an SV40 seroconversion rate of 3% to 10%, and an SRV rate of 0.9%. Human CHV-1 infection is rare and has only been documented among persons directly or indirectly exposed to laboratory primates (*9*).

The dynamics of human-macaque contact at Asian monkey temples differ substantially from those in laboratories and zoos, which may make cross-species transmission more likely at monkey temples. Because primate laboratories have been promoting specific pathogen-free colonies, the risk for primate-to-human transmission of enzootic agents in these settings is likely to diminish over time. Additionally, primate laboratories require the routine use of eye protection, gloves, and protective garments. Injury protocols call for thorough irrigation of wounds and close follow-up of exposed persons. In contrast, research examining wound care practices among exposed workers at the Sangeh monkey forest in Bali (*4*) found no use of protective eyewear, gloves, or protective clothing. Bleeding wounds from macaque scratches and bites were often not cleansed, and only 6 of 51 persons bitten or scratched by a macaque sought medical care (*4*). As a result, exposure to bites, scratches, and mucosal splashes at monkey temples may carry a higher risk for primate-to-human viral transmission than does exposure in primate laboratories and zoos.

From a global infection control standpoint, learning about primate-to-human transmission at monkey temples like Swoyambhu is particularly relevant. Because the number of humans who come into contact with primates at monkey temples around the world is probably several million per year (*3*), monkey temples are an important interface between humans and primates. Additionally, many of the visitors to Swoyambhu are foreign tourists, which makes Swoyambhu a potential point source for the global dispersal of infectious agents, as world travelers can return to their homes carrying novel infectious agents transmitted from macaques. Monkey temples of South and Southeast Asia are also near large human population centers. The combination creates the potential for rapid global dispersal of primateborne infectious agents to human populations around the world.

In spite of centuries of human-primate commensalism in Asia, human disease has yet to be causally linked to enzootic primateborne viruses. However, disease may go undetected because of low incidence, inadequate surveillance, and lack of awareness. Latency between infection and disease manifestation could mask the association between primate exposure and disease. Though long-term commensalism may lead to increased immunity to primateborne pathogens among exposed human populations, non-Asian visitors might be vulnerable, and increased travel to Asia would expose more nonimmune persons to primateborne pathogens. Finally, as HIV continues to spread in Asia, increasing numbers of immunosuppressed persons will likely be exposed to primateborne infectious agents. Immunocompromised hosts could provide pathogens a "permissive" environment in which to evolve into more pathogenic forms.

Management Strategies

In taking steps to reduce the risk for cross-species transmission between humans and primates, we should first

understand how transmission occurs, i.e., the situations, conditions, and behavior that lead to contact and transmission. Because feeding macaques is thought to account for most interactions between humans and macaques, adopting protocols to restrict feeding to persons specifically trained for that purpose could reduce the number of visitors who come into direct contact with macaque body fluids. Educating visitors as well as persons who live near monkey temples to avoid behavior that leads to bites and scratches could reduce risk. Finally, availability and awareness of proper protocol for effective wound irrigation has the potential to reduce transmission of infection. No data on the efficacy of postexposure prophylaxis with antiviral agents are available, but pharmacologic therapy is another tool that could reduce the likelihood of cross-species viral transmission. In addition, monitoring human populations for infection with primate viruses at the human-primate interface is a prudent strategy to facilitate the early detection of primateborne zoonoses.

This information must be put into context. The recent culling of macaques at a wildlife park in England and at monkey temples in Hong Kong and Taiwan in response to the perceived threat of zoonotic transmission of CHV-1 is an example of an exaggerated response to an inadequately understood risk. Improving awareness of zoonotic transmission and effective management strategies among the public as well as among persons who manage primate parks and temples will be instrumental in allowing humans and primates to continue to coexist.

Acknowledgments

We thank Mahendra R. Budhdajracharya and the members of the Federation of Swoyambhu Management and Conservation Committee, the Swoyambhu Temple staff, Radha K. Gharti, Dipesh R. Shakya, and Hanna and Leah Engel for their outstanding logistic support and expert assistance with the health assessment of the rhesus macaques at Swoyambhu Temple; Robin Watanabe and LaRene Kuller and the WaNPRC Diagnostic Laboratory for their excellent work; the Department of National Parks and Wildlife Conservation in Nepal for their assistance with permit acquisition; and N. Lerche for providing SRV-4 and 5 antigens.

This study was supported in part by a Defense Advanced Research Projects Agency contract N660001-02-C-8702 and grants from the National Institutes of Health RR00166 and RR013986-06.

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References

1. Cleaveland S, Laurenson MK, Taylor LH. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond B Biol Sci. 2001;356:991–9.
2. Fuentes A, Gamerl S. Disproportionate participation by age/sex classes in aggressive interactions between long-tailed macaques (*Macaca fascicularis*) and human tourists at Padangtegal Monkey Forest in Bali, Indonesia. Am J Primatol. 2005;66:197–204.
3. Fuentes A, Southern M, Suaryana KG. Monkey forests and human landscapes: is extensive sympatry sustainable for *Homo sapiens* and *Macaca fascicularis* on Bali? In: Patterson J, Wallis J, editors. Commensalism and conflict: the human-primate interface. Norman (OK): ASP Press; 2005. p. 165–95.
4. Engel GA, Jones-Engel L, Schillaci MA, Suaryana KG, Putra A, Fuentes A, et al. Human exposure to herpesvirus B-seropositive macaques, Bali, Indonesia. Emerg Infect Dis. 2002;8:789–95.
5. Jones-Engel L, Engel G, Schillaci M, Rompis A, Putra A, Suaryana KG, et al. Primate-to-human retroviral transmission in Asia. Emerg Infect Dis. 2005;11:1028–35.
6. Chalise MK, Ghimire M. Non-human primate census in different parts of Nepal. Bulletin of the Natural History Society of Nepal. 1998;8:11–5.
7. Kalter SS, Heberling RL, Cooke AW, Barry JD, Tian PY, Northam WJ. Viral infections of nonhuman primates. Lab Anim Sci. 1997;47:461–7.
8. Kessler MJ, Hilliard JK. Seroprevalence of B virus (*Herpesvirus simiae*) antibodies in a naturally formed group of rhesus macaques. J Med Primatol. 1990;19:155–60.
9. Huff JL, Barry PA. B-virus (cercopithecine herpesvirus 1) infection in humans and macaques: potential for zoonotic disease. Emerg Infect Dis. 2003;9:246–50.
10. Centers for Disease Control and Prevention. B-virus infection in humans—Pensacola, Florida. MMWR Morb Mortal Wkly Rep. 1987;36:289–90.
11. Shah KV, Morrison JA. Comparison of three rhesus groups for antibody patterns to some viruses: absence of active simian virus 40 transmission in the free-ranging rhesus of Cayo Santiago. Am J Epidemiol. 1969;89:308–15.
12. Shah KV. Neutralizing antibodies to simian virus 40 (SV40) in human sera from India. Proc Soc Exp Biol Med. 1966;121:303–7.
13. Viscidi RP, Rollison DEM, Viscidi E, Clayman B, Rubalcaba E, Daniel R, et al. Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays. Clin Diagn Lab Immunol. 2003;10:278–85.
14. Engels EA, Switzer WM, Heneine W, Viscidi RP. Serologic evidence for exposure to simian virus 40 in North American zoo workers. J Infect Dis. 2004;190:2065–9.
15. Vogel P, Weigler BJ, Kerr H, Hendrickx A, Barry PA. Seroepidemiologic studies of cytomegalovirus infection in a breeding population of rhesus macaques. Lab Anim Sci. 1994;44:25–30.
16. Kaur A, Kassis N, Hale CL, Simon M, Elliott M, Gomez-Yafal A, et al. Direct relationship between suppression of virus-specific immunity and emergence of cytomegalovirus disease in simian AIDS. J Virol. 2003;77:5749–58.
17. Chang WL, Tarantal AF, Zhou SS, Borowsky AD, Barry PA. A recombinant rhesus cytomegalovirus expressing enhanced green fluorescent protein retains the wild-type phenotype and pathogenicity in fetal macaques. J Virol. 2002;76:9493–504.
18. Huff JL, Eberle R, Capitanio J, Zhou SS, Barry PA. Differential detection of B virus and rhesus cytomegalovirus in rhesus macaques. J Gen Virol. 2003;84:83–92.

19. Alcendor DJ, Barry PA, Pratt-Lowe E, Luciw PA. Analysis of the rhesus cytomegalovirus immediate-early gene promoter. Virology. 1993;194:815–21.
20. Lerche NW, Osborn KG, Marx PA, Prahalada S, Maul DH, Lowenstine LJ, et al. Inapparent carriers of simian acquired immune deficiency syndrome type D retrovirus and disease transmission with saliva. J Natl Cancer Inst. 1986;77:489–96.
21. Murray SM, Picker LJ, Axthelm MK, Linial ML. Expanded tissue targets for foamy virus replication with simian immunodeficiency virus–induced immunosuppression. J Virol. 2006;80:663–70.
22. Lerche NW, Switzer WM, Yee JL, Shanmugam V, Rosenthal AN, Chapman LE, et al. Evidence of infection with simian type D retrovirus in persons occupationally exposed to nonhuman primates. J Virol. 2001;75:1783–9.
23. Switzer WM, Bhullar V, Shanmugam V, Cong ME, Parekh B, Lerche NW, et al. Frequent simian foamy virus infection in persons occupationally exposed to nonhuman primates. J Virol. 2004;78:2780–9.
24. Wolfe ND, Switzer WM, Carr JK, Bhullar VB, Shanmugam V, Tamoufe U, et al. Naturally acquired simian retrovirus infections in central African hunters. Lancet. 2004;363:932–7.
25. Vandamme AM, Salemi M, Desmyter J. The simian origins of the pathogenic human T-cell lymphotropic virus type I. Trends Microbiol. 1998;6:477–83.
26. Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. Science. 2000;287:607–14.
27. Allan JS, Short M, Taylor ME, Su S, Hirsch VM, Johnson PR, et al. Species-specific diversity among simian immunodeficiency viruses from African green monkeys. J Virol. 1991;65:2816–28.
28. Yue Y, Zhou SS, Barry PA. Antibody responses to rhesus cytomegalovirus glycoprotein B in naturally infected rhesus macaques. J Gen Virol. 2003;84:3371–9.
29. Kuller L, Watanabe R, Anderson D, Grant R. Development of a whole virus multiplex flow cytometric assay for rapid screening of specific pathogen-free primate colony. Diagn Microbiol Infect Dis. 2005;53:185–93.
30. Schillaci MA, Jones-Engel L, Engel GA, Paramastri Y, Iskandar E, Wilson B, et al. Prevalence of enzootic simian viruses among urban performance monkeys in Indonesia. Trop Med Int Health. 2005;10:1305–14.
31. Khan AS, Sears JF, Muller J, Galvin TA, Shahabuddin M. Sensitive assays for isolation and detection of simian foamy retroviruses. J Clin Microbiol. 1999;37:2678–86.
32. Orcutt RP, Pucak GJ, Foster HL, Kilcourse JY. Multiple testing for the detection of B virus antibody in specially handled rhesus monkeys after capture from virgin trapping grounds. Lab Anim Sci. 1976;26:70–4.
33. Blewett EL, Lewis J, Gadsby EL, Neubauer SR, Eberle R. Isolation of cytomegalovirus and foamy virus from the drill monkey (*Mandrillus leucophaeus*) and prevalence of antibodies to these viruses amongst wild-born and captive-bred individuals. Arch Virol. 2003;148:423–33.
34. Richards AL, Giri A, Iskandriati D, Pamungkas J, Sie A, Rosen L, et al. Simian T-lymphotropic virus type I infection among wild-caught Indonesian pig-tailed macaques (*Macaca nemestrina*). J Acquir Immune Defic Syndr Hum Retrovirol. 1998;19:542–5.
35. Ishida T, Varavudhi P. Wild long-tailed macaques (*Macaca fascicularis*) in Thailand are highly infected with gamma herpes virus but not with simian T-lymphotropic retrovirus of type 1. Folia Primatol (Basel). 1992;59:163–8.
36. Karesh WB, Cook RA, Bennett EL, Newcomb J. Wildlife trade and global disease emergence. Emerg Infect Dis. 2005;11:1000–2.
37. Jones-Engel L, Engel G, Schillaci M, Lee B, Heidrich J, Chalise M, et al. Considering human to primate transmission of measles virus through the prism of risk analysis. Am J Primatol. In press 2006.
38. Jones-Engel L, Engel GA, Schillaci MA, Babo R, Froehlich J. Detection of antibodies to selected human pathogens among wild and pet macaques (*Macaca tonkeana*) in Sulawesi, Indonesia. Am J Primatol. 2001;54:171–8.
39. Engel G, Hungerford L, Jones-Engel L, Travis D, Fuentes A, Grant R, et al. Risk assessment: a model for predicting cross-species transmission of SFV from macaques (*M. fascicularis*) to humans at a monkey temple in Bali, Indonesia. Am J Primatol. In press 2006.
40. Sandstrom PA, Phan KO, Switzer WM, Fredeking T, Chapman L, Heneine W, et al. Simian foamy virus infection among zoo keepers. Lancet. 2000;355:551–2.

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Multidrug-resistant Commensal *Escherichia coli* in Children, Peru and Bolivia

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Using a rapid screening method, we investigated the prevalence in fecal carriage of antimicrobial drug–resistant *Escherichia coli* in 3,174 healthy children from 4 urban settings in Peru and Bolivia. High resistance rates were observed for ampicillin (95%), trimethoprim-sulfamethoxazole (94%), tetracycline (93%), streptomycin (82%), and chloramphenicol (70%). Lower resistance rates were observed for nalidixic acid (35%), kanamycin (28%), gentamicin (21%), and ciprofloxacin (18%); resistance to ceftriaxone and amikacin was uncommon (<0.5%). In a random sample of 1,080 resistant *E. coli* isolates, 90% exhibited a multidrug-resistance (MDR) phenotype. The 2 most common MDR phenotypes (ampicillin/tetracycline/trimethoprim-sulfamethoxazole and ampicillin/tetracycline/ trimethoprim-sulfamethoxazole/chloramphenicol) could be transferred en bloc in conjugation experiments. The most common acquired resistance genes were bla_{TEM}, *tet*(A), *tet*(B), *drfA8*, *sul1*, *sul2*, and *catI*. These findings underscore the magnitude of the problem of antimicrobial drug resistance in low-resource settings and the urgent need for surveillance and control of this phenomenon.

The spread of microbial drug resistance is a global public health challenge, which impairs the efficacy of antimicrobial agents and results in substantial increased illness and death rates and healthcare-associated costs (*1–3*). In low-resource countries, the extent and the impact of the phenomenon tend to be even larger than in industrialized countries. In fact, high resistance rates have often been reported in surveillance studies dealing with clinical isolates (*1,4,5*) and in prevalence studies of commensal bacteria taken as indicators to estimate spread of acquired resistance (*6–15*). Moreover, in low-resource countries the impact of antimicrobial drug resistance on illness and death rates tends to be greater because of the high prevalence of bacterial infections and the major role of antimicrobial agents in combating infectious diseases (*1,3,4,16,17*). The high antimicrobial drug resistance rates observed in low-resource countries are likely due to a combination of several factors, among which irrational antimicrobial drug usage and conditions of poor sanitation are thought to play a major role, even if the relative importance of additional factors remains unclear (*1,4,8,9*).

ANTRES (Towards Controlling Antimicrobial Use and Resistance in Low-Income Countries: An intervention Study in Latin America) is a research project that aims to investigate this phenomenon on a large scale. The project is carried out in 2 Latin American countries, Bolivia and Peru, where 4 urban areas have been selected for studying antimicrobial drug use and bacterial resistance in healthy children by a prospective approach. An information-education-communication strategy will be developed based on the collected information and involving local health services. The impact on antimicrobial drug use and bacterial resistance trends will be evaluated (http://www.unifi.it/infdis/antres/default.htm).

We report the results of the baseline study, carried out at the beginning of the project, to assess the antimicrobial drug resistance rates in the studied areas. Very high

*Università di Firenze, Florence, Italy; †Università di Siena, Siena, Italy; ‡Hospital Apoyo Yurimaguas, Yurimaguas–Loreto, Peru; §Servicio Departamental de Salud Santa Cruz, Camiri, Bolivia; ¶Hospital Moyobamba, Moyobamba–San Martin, Peru; #Karolinska Institute, Stockholm, Sweden; and **Universidad Peruana Cayetano Heredia, Lima, Peru

resistance rates to several antimicrobial agents were observed in commensal bacteria from the population of each area. Strains showing multidrug-resistance (MDR) phenotypes were widely disseminated.

Materials and Methods

Study Design and Population

The study population was represented by healthy children 6–72 months of age from 4 urban areas, 2 in Bolivia (Camiri, Santa Cruz Department; Villa Montes, Tarija Department) and 2 in Peru (Yurimaguas, Loreto Department; Moyobamba, San Martin Department). The urban areas were communities of ≈25,000 to 30,000 inhabitants who had comparable demographic and socioeconomic characteristics. Eight hundred children from each area were enrolled in the study to cover at least 25% of all households with children in the target age cohort. Only children who had not had diarrhea (as defined by the World Health Organization [*18*]) during the previous 24 hours were eligible for inclusion in the study. In each household, the youngest recruitable child in the target age cohort was selected. The studied households were selected with a modified cluster sampling: detailed maps outlining the distribution of households were obtained from each study area, and each city was divided into 80 small clusters. In each cluster a number of households selected at random were visited until 10 children were reached. A rectal swab was obtained from each child enrolled in the study, after informed consent was obtained from parents or other legal guardians. Before the sample was obtained, adult members of the household were interviewed to collect information on the family's socioeconomic and cultural setting, the household antimicrobial drug use, and the health status of the selected child. In each country, full ethical clearance was obtained from the qualified authorities who had revised and approved the study design.

The overall acceptance rate was >95% in all studied areas. Rectal swabs were obtained from a total of 3,174 children (Figure 1): 794 from Camiri and 790 from Villa Montes, Bolivia; 797 from Yurimaguas, and 793 from Moyobamba, Peru. The study participants were 6–72 months of age (mean 34.7 ± 18.2 months; median age 33.5 months).The female:male ratio was 0.95. No significant differences in age or sex were observed among children enrolled from the different areas. The study was carried out for 4 months (August–November 2002). In each area, the sampling period was not longer than 7 weeks.

Screening for Resistant *Escherichia coli* in Commensal Microbiota

Rectal swabs, stored in Amies transport medium (Oxoid, Milan, Italy), were transferred in a cold box to the laboratory of the corresponding district hospital within 3 hours of collection. The 4 laboratories participate in national quality control programs. A training phase and a pilot study preceded the survey. Three European investigators (AB, MB, LP) participated in the sample analysis in the 4 laboratories. This approach was chosen to limit variability in the microbiologic procedures during the field studies.

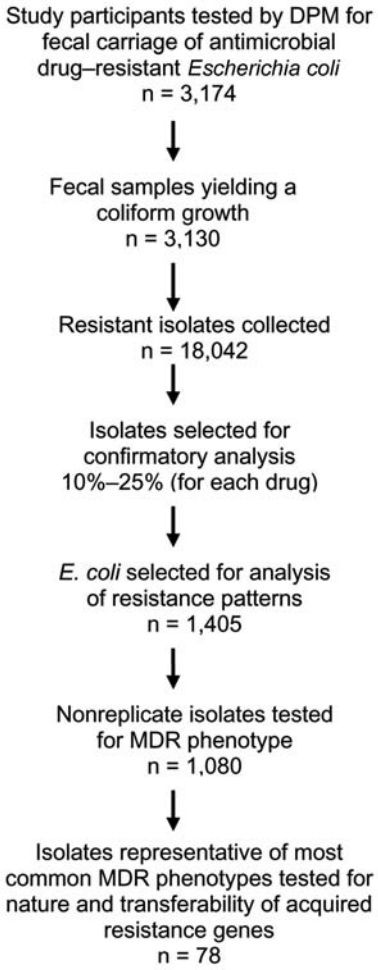

Figure 1. Flow chart of microbiologic analysis of fecal samples. DPM, direct plating method.

Screening for antimicrobial-resistant *E. coli* in the fecal microbiota was carried out by a direct plating method, as described previously (*7*,*19*). This method is preferred because it correlates well with methods based on testing of randomly collected colonies from primary stool culture, but it is more sensitive (*11*,*19*).

Briefly, each swab was spread on a McConkey agar no. 3 plate (Oxoid) to yield uniform growth, and antimicrobial drug–containing disks were directly placed onto the seeded plate. Antimicrobial agents tested included ampicillin, ceftriaxone, tetracycline, trimethoprim-sulfamethoxazole,

chloramphenicol, streptomycin, kanamycin, gentamicin, amikacin, nalidixic acid, and ciprofloxacin (Oxoid). After incubation at 37°C for 12 to 14 hours, plates were inspected for growth, and inhibition zone diameters were measured. The presence of a growth inhibition zone larger than the established breakpoint diameter was considered to indicate susceptibility to that agent. The presence of a growth inhibition zone smaller than the breakpoint diameter, the absence of any inhibition zone, or the presence of isolated colonies growing inside an inhibition zone of any size was considered indicative of resistance. In the latter case, however, resistance was considered not to be represented in the dominant flora. Breakpoints were as previously described for ampicillin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, amikacin, nalidixic acid, and ciprofloxacin (*19*), and 14 mm, 12 mm, 12 mm, and 14 mm for kanamycin, streptomycin, gentamicin, and ceftriaxone, respectively (A. Bartoloni, unpub. data). Only bacterial growth exhibiting a shape typical of *E. coli* was considered valid for the analysis. Specially prepared forms were used to record microbiologic results, including growth features and susceptibility patterns. Confluent bacterial growth was obtained from 3,130 (99%) of the 3,174 collected rectal swabs (Figure 1). In a few cases insufficient growth (11 swabs) or noncoliform growth (33 swabs) was observed. From each plate and for each drug, the putatively resistant coliform growth (i.e., a pool of the colonies grown inside the zone of inhibition or a loopful of the microbial lawn grown in proximity of the disk) were collected (n = 18,042) (Figure 1). Of these, a random sample (10%–25% for each drug, depending on the prevalence of the resistance phenotype) were subjected to confirmatory analysis for resistant *E. coli* (Figure 1) by using the API20E identification system (bioMérieux, Marcy l'Étoile, France) and the standard agar disk diffusion method (*20*,*21*). Confirmation was obtained in ≥96% of the tested samples, without significant differences among the 4 laboratories.

Analysis of Resistance Patterns of Commensal *E. coli*

Susceptibility to several antimicrobial agents representative of various classes (ampicillin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, kanamycin, gentamicin, nalidixic acid, ciprofloxacin) was tested in 1,405 (8%) *E. coli* isolates randomly selected from the 18,042 collected samples. Each isolate was spread onto Mueller-Hinton (MH) agar plates containing each antimicrobial agent at a concentration 20% higher than the breakpoints for resistance of each antimicrobial agent, as per National Committee of Laboratory Standards guidelines (*20*,*21*). The inoculum size was $\approx 2 \times 10^5$ CFU per spot. A plate of antimicrobial drug–free medium was always included as a growth control. *E. coli* ATCC 25922, and *E. coli* strains from our collection resistant to the various antimicrobial agents used in the assay were always included for quality control purposes. Results were recorded after incubation at 37°C for 18 hours. A resistance phenotype was assigned when growth was observed on the medium containing an antimicrobial agent. An MDR phenotype was intended as resistance to ≥2 classes of antimicrobial agents. Isolates from the same study participant that exhibited the same resistance phenotype (the random sampling procedure did not initially consider the study participant source) were considered replicated isolates and were counted only once for data analysis. According to this criterion, data analysis was conducted with 1,080 isolates (Figure 1).

Data entry and analysis were calculated with the EpiInfo software package (version 2002, Centers for Disease Control and Prevention, Atlanta, GA, USA). Statistical differences in the prevalence of antimicrobial drug resistances were determined by the χ^2 test.

Molecular Analysis of Resistance Genes and Conjugation Assay

Acquired resistance genes and conjugative transfer of resistance traits were investigated in 78 isolates randomly selected from those representative of the 2 most common MDR phenotypes (Figure 1). Resistance genes bla_{TEM}, *catI*, *dfrA8*, *sul1*, and *sul2* and the *intI1* integrase gene were detected by colony blot hybridization (*22*) with specific probes generated by polymerase chain reaction (PCR), as described previously (*23*–*25*). Tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), and *tet*(D) were detected by PCR, as described previously (*26*). Conjugative transfer of resistance genes was assayed in MH broth using *E. coli* J53 (auxotropic for proline and methionine and resistant to rifampin and nalidixic acid) as a recipient and an initial donor/recipient ratio of 0.1. Mating tubes were incubated at 30°C for 14 h. Transconjugants were selected on MH agar containing rifampin (400 μg/mL) and nalidixic acid (32 μg/mL) plus one of the following antimicrobial agents: ampicillin (200 μg/mL), tetracycline (5 μg/mL), chloramphenicol (30 μg/mL), or trimethoprim-sulfamethoxazole (40/200 μg/mL). Under the above conditions, the detection sensitivity of the mating assay was $\geq 1 \times 10^8$ transconjugants/recipients.

Results

Antimicrobial Drug–Resistance Rates

Overall, high resistance rates were observed for ampicillin (95%), trimethoprim-sulfamethoxazole (94%), tetracycline (93%), streptomycin (82%), and chloramphenicol (70%). Lower resistance rates were observed for nalidixic acid (35%), kanamycin (28%), gentamicin (21%), and

ciprofloxacin (18%). Resistance to ceftriaxone and amikacin was uncommon (<0.5%) (Table 1). For first-line oral antimicrobial agents such as ampicillin, tetracycline, and trimethoprim-sulfamethoxazole, resistant strains were present as the dominant flora in >80% of carriers (Table 1).

Resistance rates for ampicillin, trimethoprim-sulfamethoxazole, kanamycin, and streptomycin were significantly higher in Bolivia than in Peru ($p<0.001$), whereas ciprofloxacin resistance rates were significantly higher in Peru than in Bolivia ($p<0.001$) (Table 1). Differences in the resistance rates were also observed within each country. In Bolivia, higher overall resistance rates were found in Camiri than in Villa Montes ($p<0.001$) for chloramphenicol, streptomycin, gentamicin, nalidixic acid, and ciprofloxacin (Table 1). In Peru, higher overall resistance rates were found in Moyobamba than in Yurimaguas. The most significant differences ($p<0.001$) were noted for streptomycin, nalidixic acid, and ciprofloxacin (Table 1).

Significantly higher ($p<0.05$) resistant rates were observed in boys, with some agents, and in some settings (ampicillin in both Peruvian cities, trimethoprim-sulfamethoxazole in Moyobamba, and chloramphenicol, kanamycin, and gentamicin in Camiri). Analysis by age showed that, with all agents (ceftriaxone and amikacin were not considered in this analysis because of the low numbers of resistant isolates), resistance rates were notably higher in the youngest age group, and an overall decreasing trend by age was observed (Figure 2). With some agents (kanamycin, gentamicin, and the quinolones), the decreasing trend was essentially limited to the younger age groups; with other agents (ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol); this phenomenon was more evident in the older age groups.

Combinations of Antimicrobial Drug–resistance Traits in Healthy Children

Of the 3,174 children evaluated in the study, only 84 (2.7%) carried an *E. coli* fecal population susceptible to all the antimicrobial agents tested, while 46 (1.5%), 73 (2.3%), 187 (5.9%), 537 (17%), 808 (26%), 624 (20%), and 816 (26%) had an *E. coli* population resistant to 1, 2, 3, 4, 5, 6, or >6 antimicrobial agents. No significant differences in this distribution were observed between the 2 countries. Of the 156 different combinations of resistance observed, 2 were most prevalent: 1) the pattern with 4 resistances, (ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin) in 361 (11.5%) children and 2) the pattern with 5 resistances, (ampicillin, tetracycline, trimethoprim-sulfamethoxazole, streptomycin, and chloramphenicol) in 567 (18%) children.

Patterns of Resistance Phenotypes of *E. coli* Isolates

Frequency and patterns of resistance phenotypes were determined on a random sample of 1,080 nonreplicate resistant *E. coli* isolates with 8 antimicrobial agents representative of 6 different classes (ampicillin for β-lactams, tetracycline for tetracyclines, chloramphenicol for phenicols, trimethoprim-sulfamethoxazole for folate inhibitors, kanamycin and gentamicin for aminoglycosides, and nalidixic acid and ciprofloxacin for quinolones). Only a few isolates were resistant to a single drug (9%) (Figure 3). Isolates showing resistance to 3 different drugs were the most prevalent (30%), followed by those showing resistance to 4 (22%) and 2 (20%) drugs, respectively. Seven isolates (<1%) showed resistance to all tested drugs (Figure 3). An MDR phenotype was common (90% of isolates). Resistance patterns were quite variable. Of 97 different patterns observed, some were highly prevalent. The

Table 1. Prevalence, expressed as percentage, of healthy children carrying antimicrobial drug–resistant *Escherichia coli* as part of their commensal flora and of children in whom resistant *E. coli* constituted the predominant flora*†

	Bolivia			Peru						
Drug	Camiri	Villa Montes	p value	Yurimaguas	Moyobamba	p value	Bolivia, subtotal	Peru, subtotal	Total	p value
Ampicillin	98 (95)	96 (87)	<0.05	92 (76)	93 (83)	NS	97 (91)	92 (80)	95 (85)	<0.001
SXT‡	98 (94)	95 (86)	<0.05	89 (72)	93 (83)	<0.05	96 (90)	91 (77)	94 (84)	<0.001
Tetracycline	96 (91)	92 (82)	<0.05	89 (71)	93 (81)	<0.05	94 (86)	91 (76)	93 (81)	<0.05
Streptomycin	96 (89)	88 (79)	<0.001	71 (50)	86 (66)	<0.001	92 (84)	79 (58)	82 (68)	<0.001
Chloramphenicol	74 (58)	65 (40)	<0.001	69 (42)	72 (48)	NS	70 (49)	71 (45)	70 (47)	NS
Nalidixic acid	44 (25)	29 (13)	<0.001	27 (8)	41 (18)	<0.001	36 (19)	38 (13)	35 (16)	NS
Kanamycin	37 (20)	31 (12)	<0.05	22 (8)	23 (9)	NS	34 (16)	22 (9)	28 (12)	<0.001
Gentamicin	28 (16)	18 (10)	<0.001	19 (9)	21 (10)	NS	23 (13)	20 (10)	21 (11)	<0.05
Ciprofloxacin	21 (10)	10 (4)	<0.001	16 (5)	25 (9)	<0.001	16 (7)	21 (7)	18 (7)	<0.001
Amikacin	0	0.1 (0)	NA	1 (0.4)	0.4 (0.3)	NA	0.1 (0.1)	1 (0.3)	0.4 (0.2)	NA
Ceftriaxone	0.1 (0)	0.1 (0.1)	NA	0.3 (0)	0.1 (0.1)	NA	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	NA

*Inside parentheses: proportion of carriers in whom resistant *E. coli* constituted the predominant flora. NS, not significant; NA, not applicable.
†Number of children from whom samples were obtained: Camiri = 794; Villa Montes = 790; Yurimaguas = 797; Moyobamba = 793; Bolivia = 1,584; Peru = 1,590; and total = 3,174.
‡SXT, trimethoprim-sulfamethoxazole.

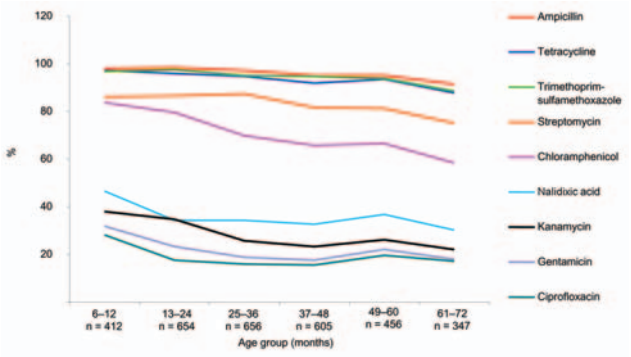

Figure 2. Total prevalence, by age group, of fecal carriage of antimicrobial drug–resistant *Escherichia coli* among 3,174 children in 4 urban areas of Bolivia and Peru. Ceftriaxone and amikacin were not considered in these analyses because their resistance rates were too low.

pattern that included ampicillin, tetracycline, and trimethoprim-sulfamethoxazole was the most prevalent, followed by that including ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol, as well as that including ampicillin and trimethoprim-sulfamethoxazole alone (22%, 15%, and 10% of the tested isolates, respectively) (Figure 3).

Acquired Resistance Genes and Transferability of Resistance Traits

The prevalence of several acquired resistance genes (bla_{TEM} for ampicillin resistance; *tet*(A)–(D) for tetracycline resistance; *dfrA8* for trimethoprim resistance; *sul1* and *sul2* for sulfonamide resistance; *catI* for chloramphenicol resistance) and class 1 integrons was investigated in 78 isolates randomly selected from representatives of the 2 most prevalent MDR phenotypes (ampicillin/tetracycline/trimethoprim-sulfamethoxazole, n = 45; ampicillin/tetracycline/trimethoprim-sulfamethoxazole/chloramphenicol, n = 33). In most cases the resistance phenotype could be accounted for by a combination of the above resistance genes, and some of them appeared to be highly prevalent (Table 2). The presence of the *intI1* integrase gene (associated with class 1 integrons) was detected in 35% of MDR isolates.

These 78 isolates were also tested for transferability of resistance traits in conjugation experiments. Overall, the transfer of at least 1 resistant trait was observed in 37 cases (47%). Transfer rates for each resistance trait were as follows: ampicillin 46%, tetracycline 40%, trimethoprim-sulfamethoxazole 41%, and chloramphenicol 27%. Cotransfer of all the resistance traits was observed in 26 of the 37 cases that gave positive results in the conjugation experiments. Molecular analysis of the transconjugants showed that, in all the cases, the resistance phenotype could be accounted for by the acquisition of resistance genes detected in the respective donors. Conjugative transfer of class 1 integrons was observed in 10 (37%) of the 27 *intI1*–positive isolates.

Discussion

Similar to pathogenic bacteria, commensals are exposed to the selective pressure of antimicrobial agents, and commensal *E. coli* have often been used as an indicator of the dissemination of acquired resistance genes (*7,8,11–13,15,27*). To our knowledge, this study is the first to address the status of antimicrobial drug resistance in commensal *E. coli* in a large population of preschool-age children from urban settings of low-resource countries. Results showed a high prevalence (70%–95%) of healthy carriers of *E. coli* resistant to a number of older antimicrobial agents (ampicillin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, and streptomycin). For these drugs, the small differences observed among localities, although sometimes statistically significant, are probably of limited clinical and epidemiologic relevance and presumably attributable to the large number of antimicrobial drug–resistant *E. coli* carriers. A relatively high prevalence (18%–35%) of carriers of *E. coli* resistant to other agents (kanamycin, gentamicin, nalidixic acid, and ciprofloxacin) was also observed, while the carriage of *E. coli* resistant to expanded-spectrum cephalosporins and amikacin was uncommon (<0.5%). Ranking patterns of resistance rates were similar overall in each of the 4 studied areas, which suggests a common scenario in urban

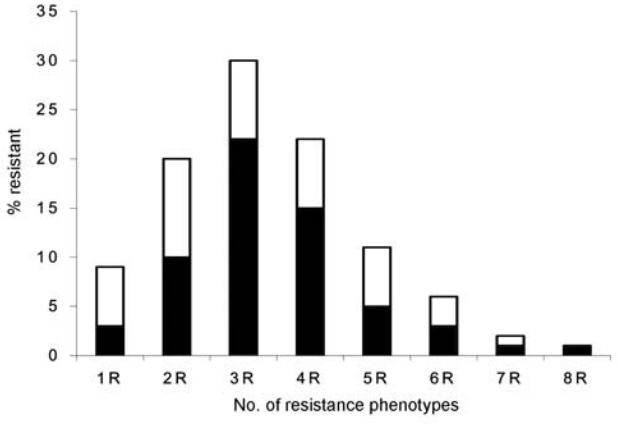

Figure 3. Frequency of resistance phenotypes in 1,080 randomly selected antimicrobial drug–resistant *Escherichia coli* isolates from 4 urban areas of Bolivia and Peru. Black bars indicate the most frequent resistance and multidrug-resistance phenotype within each category: 1R, TET; 2R, AMP-SXT; 3R, AMP-TET-SXT; 4R, AMP-TET-SXT-CHL; 5R, AMP-TET-SXT-CHL-KAN; 6R, AMP-TET-SXT-CHL-NAL-CIP; 7R, AMP-TET-SXT-CHL-GEN-NAL-CIP; 8R, AMP-TET-SXT-CHL-KAN-GEN-NAL-CIP. AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; KAN, kanamycin; GEN, gentamicin; NAL, nalidixic acid; CIP, ciprofloxacin.

Table 2. Acquired resistance genes in 78 MDR commensal *Escherichia coli**

Resistance trait	No. resistant isolates	Resistance gene	No. (%)† positive isolates
Ampicillin	78	bla_{TEM}	77 (99)
Tetracycline	78	*tet*(A)	27 (35)
		tet(B)	44 (56)
		tet(D)	4 (5)
		tet(A) and *tet*(B)	1 (1)
Trimethoprim	78	*dfrA8*	42 (54)
Sulfamethoxazole	78	*sul1*	7 (9)
		sul2	54 (69)
		sul1 and *sul2*	17 (22)
Chloramphenicol	33	*catI*	33 (100)

*MDR, multidrug resistant.
†Percentages refer to positive isolates among isolates resistant to each antimicrobial agent.

areas of these 2 Latin American countries. This view is further supported by data recently published for adults from Lima (Peru), where similar resistance rates were reported (*12*).

Our findings were in substantial agreement with previous reports of high resistance rates in commensals of study participants from low-resource settings (*7*,*8*,*11*–*13*,*15*,*28*). Comparative analysis with previous data available for 1 studied setting (Camiri, Bolivia) surveyed 10 years earlier (*7*) indicated a significant increase in the resistance rates to gentamicin and nalidixic acid and the de novo appearance of resistance to ciprofloxacin.

One aspect that was not previously reported among preschool children was the age-related differences in resistance rates; rates were significantly higher in the younger age groups. This phenomenon could reflect a larger use of antimicrobial drugs in younger children. However, this explanation cannot be the case for quinolones, which are not prescribed in this age group. Another influencing factor could be dietary changes related to weaning. In fact, a diet high in milk that contains abundant lactoferrin, which chelates dietary iron, has been hypothesized to favor the presence of strains expressing iron-uptake systems (such as aerobactin or enterobactin), which are often encoded by plasmids that also carry resistance genes (*29*). However, further investigation will be necessary to clarify the mechanism responsible for this phenomenon.

Multiple resistance traits in the commensal *E. coli* microbiota were apparently the rule, as they were detected in most study participants. The simultaneous presence of multiple strains expressing different resistance phenotypes and single strains expressing MDR phenotypes could contribute to this phenomenon. Although the relative contribution of the 2 mechanisms was not specifically investigated, the high prevalence of strains expressing MDR phenotypes probably provides a major contribution. Some MDR patterns (e.g., ampicillin/tetracycline/trimethoprim-sulfamethoxazole, ampicillin/tetracycline/ trimethoprim-sulfamethoxazole/chloramphenicol) were common and could be accounted for by a number of known acquired resistance genes. Resistance traits could be transferred by conjugation, often en bloc, suggesting a linkage of the corresponding resistance genes in self-transferable or mobilizable plasmids.

In conclusion, this study underscores the magnitude of the problem of antimicrobial drug resistance in low-resource settings and the urgent need for surveillance and control of this phenomenon. Inexpensive, sensitive, and simple methods to monitor antimicrobial drug resistance in commensal bacteria could be valuable tools for large-scale surveillance studies and to improve the efficacy of resistance control interventions. The direct plating method used in this study, which had previously shown high sensitivity and specificity in detecting resistant *E. coli* (*19*), was confirmed to be a valid tool to conduct such a large-scale survey.

Acknowledgments

We thank other members of the ANTRES Study Group for their support in this study: Ruth Arias, Vieri Boddi, Paolo Bonanni, Mirtha Camacho, Blanca Huapaya, Charlotte Kristiansson, Oscar Lanza Van den Berghe, Mattias Larsson, Luis Pacheco, Angela Pieri, Victor Suarez, Esteban Salazar, and Christian Trigoso. We are also grateful to the local health authorities for cooperation in carrying out the study, the laboratory staff for technical assistance, the field team members for valuable support in collecting samples, and all participating children and their parents for their willingness to take part to the survey.

The study was carried out within the research activities of the ANTRES project, supported by the European Commission, INCO-DEV programme, contract no. ICA4-CT-2001-10014.

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References

1. Byarugaba DK. A view on antimicrobial resistance in developing countries and responsible risk factors. Int J Antimicrob Agents. 2004;24:105–10.
2. World Health Organization. WHO report on infectious diseases 2000. Overcoming antimicrobial resistance. Geneva: The Organization; 2000.
3. World Health Organization. Global strategy for containment of antimicrobial resistance. Geneva: The Organization; 2001 [cited 2006 Jan 27]. Available from http://www.who.int/drugresistance/en/
4. Okeke IN, Lamikanra A, Edelman R. Socio-economic and behavioral factors leading to acquired bacterial resistance to antimicrobrial agents in developing countries. Emerg Infect Dis. 1999;5:18–27.
5. Shears P. Antibiotic resistance in the tropics. Epidemiology and surveillance of antimicrobial resistance in the tropics. Trans R Soc Trop Med Hyg. 2001;95:127–30.

6. Bartoloni A, Colao MG, Roselli M, Orsi A, Aquilini D, Corti G, et al. Antimicrobial agent susceptibility patterns of staphylococci isolated in urban and rural areas of Bolivia. J Trop Med Hyg. 1990;93:360–4.
7. Bartoloni A, Cutts F, Leoni S, Austin CC, Mantella A, Guglielmetti P, et al. Patterns of antimicrobial use and antimicrobial resistance among healthy children in Bolivia. Trop Med Int Health. 1998;3:116–23.
8. Bartoloni A, Bartalesi F, Mantella A, Dell'Amico E, Roselli M, Strohmeyer M, et al. High prevalence of acquired antimicrobial resistance unrelated to heavy antimicrobial consumption. J Infect Dis. 2004;189:1291–4.
9. Walson JL, Marshall B, Pokhrel BM, Kafle KK, Levy SB. Carriage of antibiotic-resistant fecal bacteria in Nepal reflects proximity to Kathmandu. J Infect Dis. 2001;184:1163–9.
10. Calva JJ, Sifuentes-Osornio J, Cerón C. Antimicrobial resistance in faecal flora: longitudinal community-based surveillance of children from urban Mexico. Antimicrob Agents Chemother. 1996;40: 1699–702.
11. Lester SC, del Pilar Pla M, Wang F, Pere Schael I, Jiang H, O'Brien TF. The carriage of *Escherichia coli* resistant to antibiotic agents by healthy children in Boston, in Caracas, Venezuela and in Qin Pu, China. N Engl J Med. 1990;323:285–9.
12. Nys S, Okeke IN, Kariuki S, Dinant GJ, Driessen C, Stobberingh EE. Antibiotic resistance of faecal *Escherichia coli* from healthy volunteers from eight developing countries. J Antimicrob Chemother. 2004;54:952–5.
13. Okeke IN, Fayinka ST, Lamikanra A. Antibiotic resistance in *Escherichia coli* from Nigerian students, 1986–1998. Emerg Infect Dis. 2000;6:393–6.
14. Shanahan PM, Wylie BA, Adrian PV, Koornhof HJ, Thomson CJ, Amyes GB. The prevalence of antimicrobial resistance in human faecal flora in South Africa. Epidemiol Infect. 1993;111:221–8.
15. van de Mortel HJ, Jansen EJ, Dinant GJ, London N, Palacios Pru E, Stobberingh EE. The prevalence of antibiotic-resistant faecal *Escherichia coli* in healthy volunteers in Venezuela. Infection. 1998;26:292–7.
16. Col NF, O'Connor RW. Estimating worldwide current antibiotic usage: report of Task Force 1. Rev Infect Dis. 1987;9(Suppl 3)3:232–43.
17. O'Brien TF. Global surveillance of antibiotic resistance. N Engl J Med. 1992;326:339–40.
18. World Health Organization. Management and prevention of diarrhoea: practical guidelines. 3rd ed. Geneva: The Organization; 1993.
19. Bartoloni A, Benedetti M, Pallecchi L, Larsson M, Mantella A, Strohmeyer M, et al. Evaluation of a rapid screening method for detection of antimicrobial resistance in the commensal microbiota of the gut. Trans R Soc Trop Med Hyg. 2006;100:119–25.
20. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Approved standard M07-A6. Wayne (PA): The Committee; 2003.
21. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. 13th informational supplement M100-S13. Wayne (PA); The Committee; 2004.
22. Sambrook J, Russel DW. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2001.
23. Maynard C, Bekal S, Sanschagrin F, Levesque RC, Brousseau R, Masson L, et al. Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin. J Clin Microbiol. 2004;42:5444–52.
24. Lee JC, Oh JY, Cho JW, Park JC, Kim JM, Seol SY, et al. The prevalence of trimethoprim-resistance-conferring dihydrofolate reductase genes in urinary isolates of *Escherichia coli* in Korea. J Antimicrob Chemother. 2001;47:599–604.
25. Infante B, Grape M, Larsson M, Kristiansson C, Pallecchi L, Rossolini GM, et al. Acquired sulfaonamide resistance genes in faecal *Escherichia coli* from healthy children in Bolivia and Peru. Int J Antimicrob Agents. 2005;25:308–12.
26. Hartman AB, Essiet II, Isenbarger DW, Lindler LE. Epidemiology of tetracycline resistance determinants in *Shigella* spp. and enteroinvasive *Escherichia coli*: characterization and dissemination of tet(A)-1. J Clin Microbiol. 2003;41:1023–32.
27. Alliance for Prudent Use of Antibiotics (APUA). Reservoirs of Antibiotic Resistance Network (ROAR). Commensal bacteria are reservoirs of resistance. 2004 [cited 2006 Jan 27]. Available from http://www.tufts.edu/med/apua/ROAR/project.htm
28. Grenet K, Guillemot D, Jarlier V, Moreau B, Dubourdieu S, Ruimy R, et al. Antibacterial resistance, Wayampis amerindians, French Guyana. Emerg Infect Dis. 2004;10:1150–3.
29. Khachatryan AR , Hancock DD, Besser TE, Call DR. Role of calf–adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl Environ Microbiol. 2004;70:752–7.

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Human *Streptococcus suis* Outbreak, Sichuan, China

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From mid-July to the end of August 2005, a total of 215 cases of human *Streptococcus suis* infections, 66 of which were laboratory confirmed, were reported in Sichuan, China. All infections occurred in backyard farmers who were directly exposed to infection during the slaughtering process of pigs that had died of unknown causes or been killed for food because they were ill. Sixty-one (28%) of the farmers had streptococcal toxic shock syndrome; 38 (62%) of them died. The other illnesses reported were sepsis (24%) and meningitis (48%) or both. All isolates tested positive for genes for *tuf*, species-specific 16S rRNA, *cps2J*, *mrp*, *ef*, and *sly*. A single strain of *S. suis* caused the outbreak, as shown by the identification of a single ribotype. The high death ratio was of concern; prohibiting backyard slaughtering ended the outbreak.

Streptococcus suis is a zoonotic microbe that can exist in pigs without causing illness but can occasionally cause disease. Serotype 2 is a dominant pathogenic serotype (*1*). Types 2 and 5 have been isolated from purulent lesions in the lungs and other extramammary sites in cattle, sheep, and goats (*2*). Infection may cause death in weaning piglets as well as growing pigs (*3*). The bacterium is isolated from an increasingly wide range of mammalian species, including horses, dogs, cats, and birds (*4*).

Sporadic cases of *S. suis* infection may occur in humans, the most common clinical manifestations included purulent meningitis, septicemia, arthritis, and endocarditis; some infections lead to sequelae such as deafness and ataxia (*5*–*7*). To date, ≈200 human cases have been reported in areas of intensive pig rearing (the Netherlands, Denmark) or areas where large quantities of pork are eaten (Hong Kong, Thailand, Vietnam) (*8*). Most reported cases of human *S. suis* infections were associated with contact with pigs or pork products (*5*). Human *S. suis* infections do not normally cause major outbreaks.

On July 11, 2005, a local hospital in Ziyang Prefecture of Sichuan Province reported a suspected case of hemorrhagic fever with renal syndrome. The patient was a 46-year-old male farmer with acute onset of high fever, lethargy, vomiting, and generalized purpura. The day before illness onset, the farmer slaughtered a pig that had died of an unknown cause. The farmer rapidly lapsed into coma. On further investigation, we identified 4 other patients with similar circumstances in the same hospital and more patients from other hospitals in the area. *S. suis* was isolated from blood cultures in some of these cases. We began an investigation of this outbreak to describe its epidemiologic, clinical, and microbiologic characteristics (*9*).

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Methods

Epidemiologic Investigation

We reviewed medical records in all health care facilities in Ziyang Prefecture for patients admitted since June 10, 2005 (2 weeks before the onset of the first known case), with a diagnosis of septic shock or meningitis. On July 19, enhanced surveillance was introduced to include healthcare facilities in Ziyang and the 5 surrounding prefectures. We ordered all healthcare facilities to immediately report all new patients with clinical sepsis, meningitis, arthritis, or endocarditis and fever >37.3°C, who had epidemiologic risk factors (contact with sick pigs or any part, such as meat, skin, organs, or tissue, of pigs that had died of undetermined causes). Beginning on July 21, we made public announcements to encourage reporting. Public health workers interviewed patients, or surrogates for deceased patients, by using a questionnaire to collect demographic, clinical, and exposure information. Specimens (blood, cerebrospinal fluid [CSF], or postmortem tissue) were collected for laboratory investigation. We reviewed medical records to obtain supplementary clinical information. We placed all close contacts of case-patients, including family members and attending healthcare workers, under medical surveillance. We extended these surveillance procedures to all of Sichuan Province on July 25.

A probable case of *S. suis* infection was a compatible clinical illness (sepsis, meningitis, arthritis, or endocarditis), without laboratory evidence of infection by another organism, with history of contact with sick or dead domestic livestock (pigs, goats, or sheep) or another case-patient within 7 days before onset of symptoms. A confirmed case was defined as a compatible clinical illness regardless of exposure and the verification of *S. suis* isolated from a normally sterile site. We stopped the enhanced surveillance on August 18, two weeks after onset of the last case.

To assess the extent of underreporting, notification statistics on meningococcal meningitis were reviewed. Meningococcal meningitis is a statutorily notifiable disease in China. Because laboratory diagnosis was not routinely performed for all meningitis patients, the figures on suspected meningococcal cases could reflect nonmeningococcal meningitis caused by other bacteria, including *S. suis.* In this investigation, statistics in the 12 affected prefectures of Sichuan Province from January 2003 to August 2005 were reviewed.

Laboratory Investigation

Specimens of blood, CSF, or postmortem tissue from human patients and blood or postmortem tissue from affected pigs were injected onto sheep blood agar and infusion broth (REF 237500, Oxoid, Basingstoke, UK) in 0.5% CO_2 at 37°C. *S. suis* strains were identified by examining growth colony shape, followed by biochemical reactions with Vitek2 compact and API 20 strep (bioMérieux, Inc., Beijing, China) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was used to characterize selected genes of *S. suis* serotype 2. PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol, and sequenced with an ABI Prism 3700 DNA instrument (Applied BioSystems, Foster City, CA, USA). The following genes were sequenced, in accordance with methods published elsewhere: 1) genus-specific gene segments, *tuf* sequence, and 2) the species-specific gene coding for 16S rRNA of *S. suis*, the gene coding for the capsule of *S. suis* serotypes 2 (*cps2J*) and 1/2, the muramidase-released protein gene (*mrp*), suilysin (*sly*), and the extracellular factor gene (*ef*) (*10–12*). Sequence data were analyzed by using a basic local alignment search tool (BLAST) search performed against sequences published by the National Center for Biotechnology Information (Bethesda, MD, USA).

Automated *Pvu*II and *Pst*I ribotyping was performed by using the RiboPrinter microbial characterization system (DuPont China, Shenzhen, China), with bacterial isolates grown overnight on brain-heart infusion blood agar. Template preparation, restriction enzyme digestion, gel electrophoresis, and Southern hybridization with an *Escherichia coli* rrnB rRNA operon probe were carried out with the RiboPrinter system. Images were developed with a charge-coupled-device camera and analyzed by using the RiboPrinter's customized software.

Statistical Analysis

Percentage, proportion, and case-fatality ratios were calculated. The χ^2 and rank sum tests were used to compare the case-fatality ratios and the median duration from exposure to onset between 2 groups, respectively, by using Stata version 8 (StataCorp LP, College Station, TX, USA). All probabilities were 2-tailed, and $p<0.05$ indicated significance.

Results

The first case-patient was a 52-year-old male farmer from Yanjiang District of Ziyang Prefecture. On June 24, 2005, three days after he slaughtered and dressed a goat that had died of an unknown cause, fever (38.2°C), chills, abdominal pain, vomiting, generalized aching, and generalized purpura developed. Ten hours later, he died on the way to the hospital. The second case-patient was his neighbor who had similar symptoms on June 26 and died shortly after hospital admission. He had helped slaughter the goat with the first case-patient. Blood, tissue, and other specimens for culture were not available from these patients.

The Outbreak

Other cases of *S. suis* infection occurred in early July. The number of cases gradually increased, peaked during the second half of July, and dwindled rapidly thereafter (Figure 1). The decline coincided with new measures that prohibited domestic slaughter of sick pigs or pigs that died of any illness. These measures were implemented through provincial legislation and enforced with prosecution. The last case-patient had onset of illness on August 4. By August 18, two weeks after the date of onset of the last case, we had identified 215 cases (66 laboratory confirmed, 149 probable); 39 persons died in Sichuan Province. This finding compares to an expected number of suspected meningococcal disease cases of 10 per month reported through routine surveillance during the summer months (June to August) from 2003 to 2005 (Figure 2).

Cases of *S. suis* infection predominantly involved adult male farmers with recent exposure to sick pigs or carcasses of pigs that had died of unknown illnesses (Table 1). The most common methods of exposures were slaughtering a sick pig or preparing a pig carcass for meat, hides, and other pig products. Forty-eight percent of case-patients had wounds on their hands at the time of slaughter or carcass preparation. Illnesses that fit the case definition did not develop in close contacts of case-patients who did not participate in the slaughter, carcass preparation, or carcass disposal. None of the 417 healthcare workers who cared for case-patients became infected, and no similar clinical illness developed.

The residences of *S. suis* case-patients were widely distributed in 203 villages of 12 prefectures in Sichuan Province. In 194 villages, only 1 case was identified (Figure 3). In Ziyang, Yibin, and Chengdu, 2 case-patients in 1 village each were identified. The Ziyang cluster was composed of the first 2 case-patients in the outbreak. The Yibin cluster consisted of 1 farmer who slaughtered a sick pig and became ill with streptococcal toxic shock syndrome (STSS); another person who processed meat from the same pig became ill with meningitis and recovered. In Chengdu, 2 persons slaughtered a pig that had died of an unexplained illness; *S. suis* infection developed in both persons and they recovered. In 6 additional villages, ≥2 cases of *S. suis* infection occurred in patients who did not know each other and had no common exposure to a pig.

Clinical Patterns

All 215 affected persons were previously healthy adults. We observed 3 distinct clinical symptom settings (Table 2). First, 28% of patients had sepsis characterized by acute onset of fever, chills, headaches, dizziness, malaise, abdominal pain, and diarrhea. In some severe cases, patients became comatose. Second, 48% of patients had meningitis characterized by headache, stiff neck, and other signs of acute meningitis. Some meningitis patients also had disseminated intravascular coagulation and coma. Third, 28% of patients had STSS that has been described in other forms of streptococcal infections (*13*) and met the criteria established by Centers for Disease Control and Prevention (*14*). Patients with STSS had a 62% case-fatality ratio compared to 0.6% for other clinical forms of streptococcal infections. Fatal STSS cases progressed from onset to death in a median of 25 hours (range 8 hours to 10.5 days). Discharge records available on 85 survivors in all 3 clinical symptom settings showed a median interval from onset to recovery of 15 days (range 5–36 days).

We determined exposure and onset times for 63 of the 66 laboratory-confirmed cases. Of the 215 cases, we could determine exposure time and onset times for 203. The median interval between exposure and onset was 2.2 days (range 3 hours to 14 days). The patients with STSS had a shorter incubation period and a higher frequency of gastrointestinal symptoms, coma, and petechiae and ecchymoses than did other patients (Table 3). Eight percent of non-STSS cases had petechiae or ecchymoses, and 9% had hypotension but did not fully fit the criteria for STSS.

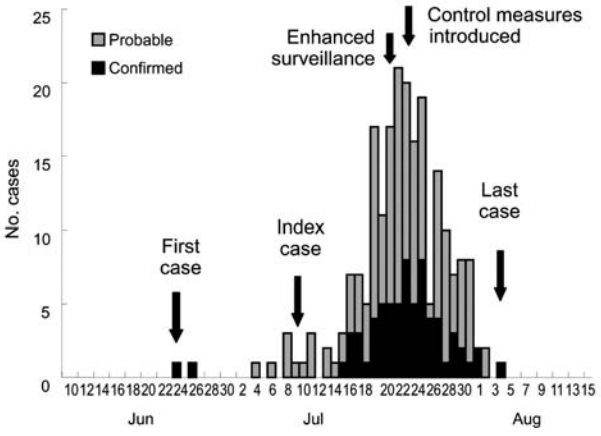

Figure 1. Epidemic curve showing the dates of onset for 215 human cases of *Streptococcus suis* infection, Sichuan, China (as of August 18, 2005).

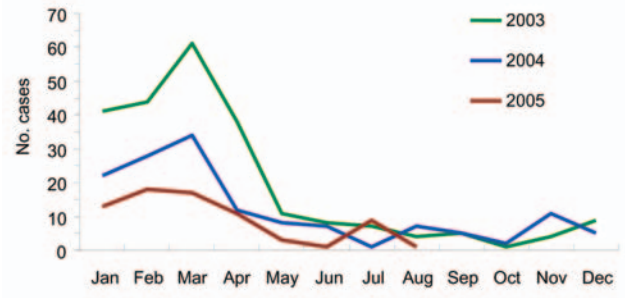

Figure 2. Suspected cases of meningococcal meningitis in the 12 affected prefectures in Sichuan.

Table 1. Demographic features and exposure history of 215 *Streptococcus suis* patients in Sichuan, China, 2005

Characteristic	Probable cases, n = 149	Confirmed cases, n = 66	Total, N = 215
Demographic features			
Male (%)	120 (81)	60 (91)	180 (84)
Median age, y (range)	54 (26–82)	57 (33–81)	54 (26–82)
Farmer (%)	145 (97)	62 (94)	207 (96)
Other occupation (%)*	4 (3)	4 (6)	8 (4)
Wounds on hands during pig exposure (%)	74 (50)	30 (45)	104 (48)
Exposure history 7 days before onset of symptom, no. (%)			
Slaughtered sick pig or goat	93 (62)	47 (71)	140 (65)
Prepared carcasses of pig or goat without slaughtering	44 (30)	16 (24)	60 (28)
Other exposure to sick pigs	12 (8)	3 (5)	15 (7)
Only ate meat from sick/dead pig/goat	0	0	0
Only contact with known case-patients	0	0	0

*Fed sick pigs or handled carcasses (e.g., sold carcasses, buried carcasses) of pigs that died from unexplained illness.

Typical skin manifestations are shown in Figure 4. Leukocytosis and thrombocytopenia were found in more than half of the patients (Table 4). Liver impairment was found in 74%, and renal function impairment was found in 19% of the patients who had been tested. CSF abnormalities compatible with purulent meningitis were found in 31 (40%) of 77 patients who had a lumbar puncture.

Postmortem examination of 4 STSS patients (2 confirmed and 2 probable infections) showed features of disseminated intravascular coagulation. Evidence of multiple organ damage was observed, primarily involving kidneys, adrenal glands, lungs, liver, pancreas, and heart. Histologic findings included microthrombosis (hyaline thrombus) in organ capillaries; necrosis of parenchymal cells; and congestion, exudate, and hemorrhage of interstitial vessels of kidneys, lungs, and other organs.

Microbiologic Investigations

We collected 348 specimens of blood (271), CSF (53), and tissue (24) from postmortem examination of 172 case-patients. We isolated *S. suis* from blood (36), CSF (27), and postmortem liver, spleen, and heart tissues (3). Fifty-five (83%) of the confirmed patients were diagnosed after July 23, 2005, representing 42% (55/130) (data not shown) of all tested samples, compared to that of 26% (11/42) (data not shown) before enhancement of surveillance.

Isolates from 66 patients and 3 diseased pigs featured pure growth of tiny α-hemolytic colonies on sheep blood agar. The colony shape and biochemical reactions by Vitek2 compact and API-Strep were all compatible with that of *S. suis*. In the investigations after July 23, 2005, the results matched the suggested key indicators for the pathogen, including Voges-Proskauer negativity, hydrolysis of esculin, trehalose positivity, negativity for growth in 6.5% NaCl, and absence of β-hemolysis on sheep blood agar. PCR on all isolates showed gene coding for *tuf*, species-specific 16S rRNA of *S. suis*, genes coding for the capsule of *S. suis* serotypes 2 (*cps2J*) and 1/2, and *mrp*, *sly*, and *ef*. The PCR products of virulence genes of all 69 strains were sequenced. These were identical to the sequences published by the National Center for Biotechnology Information. On further investigation, only a single ribotype for either *Pvu*II or *Pst*I restriction was identified among the 25 *S. suis* type 2 isolates, including 22 from patients and 3 from diseased pigs (Figure 5), indicating a single clonal strain as the source of the infection (*1*).

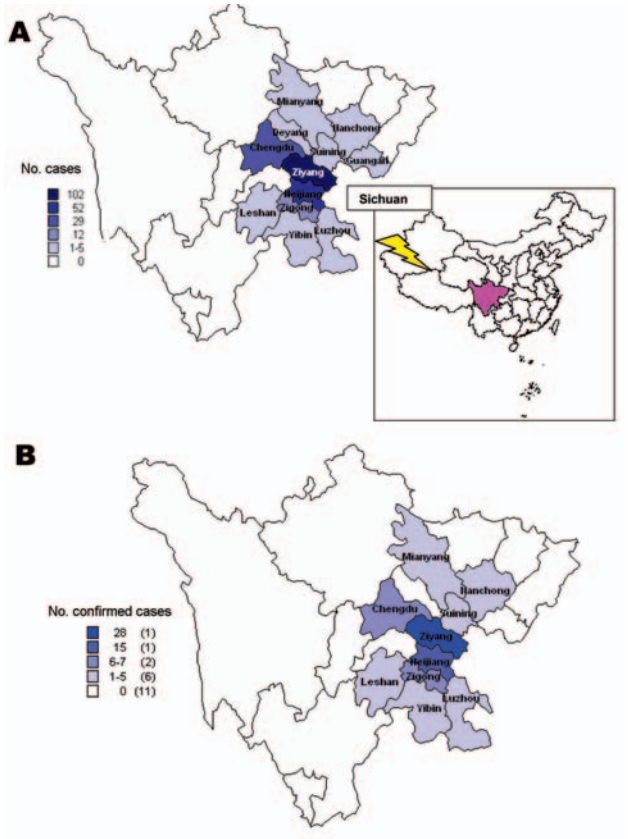

Figure 3. Geographic distribution of *Streptococcus suis* cases in Sichuan Province, China, relating to (A) all reports, and (B) 66 laboratory-confirmed cases alone (as of August 18, 2005).

Table 2. Clinical symptoms and case-fatality ratio of 215 *Streptococcus suis* patients in Sichuan Province, China, 2005*

	Probable		Confirmed		Total	
Clinical symptom	No. (%)	No. deaths (case-fatality %)	No. (%)	No. deaths (case-fatality %)	No. (%)	No. deaths (case-fatality %)
Sepsis	45 (30)	0	7 (11)	0	52 (24)	0
Meningitis	69 (46)	1 (1)	33 (50)	0	102 (48)	1 (1)
STSS	35 (24)	23 (66)	26 (39)	15 (58)	61 (28)	38 (62)
Total	149 (100)	24 (16)	66 (100)	15 (23)	215 (100)	39 (18)

*Streptococcal toxic shock syndrome (STSS) is defined according to the 1996 criteria established by the Centers for Disease Control and Prevention, Atlanta, GA, USA, which include hypotension (systolic blood pressure ≤90 mm Hg for adults) and multiorgan involvement characterized by ≥2 of the following: renal impairment, coagulopathy, liver involvement, acute respiratory distress syndrome, generalized erythematous macular rash that may desquamate, soft-tissue necrosis, including necrotizing fasciitis or myositis, or gangrene. Difference in case-fatality ratio between STSS and other clinical symptoms $p<0.001$ by χ^2 test.

Discussion

We report an unprecedented outbreak of human *S. suis* serotype 2 infection involving 215 patients in Sichuan Province during the summer of 2005. This outbreak was characterized by the large number of patients involved, distinctive clinical manifestations, and the major challenges facing public health authorities in both surveillance and control.

This is the largest recorded outbreak of *S. suis* infection in humans. From late July to early August 1998, an outbreak of 25 cases with 14 deaths occurred in Jiangsu Province, China, from serotype 2 infection (*15*). The size of the outbreak in our study was probably related to a local farming practice. In Sichuan, a sizable swine population was found in small backyard farms; each family kept only a few animals. Farmers habitually slaughtered sick pigs for human consumption. Beginning mid-June 2005, a major outbreak of *S. suis* killed 647 pigs in almost the same areas as the outbreak in humans (*16*). The outbreak in swine peaked around July 20 with ≈4 dead pigs in each affected village. *S. suis* caused 98% of the deaths of these pigs in Sichuan during this period (*17*). All infected pigs came from backyard farms, usually with 1 sick pig in the herd. A pathogenic strain could have spread by distributing infected piglets to the backyard pig farms and then propagated among healthy pigs. The single ribotype identified in the investigation lends support to this theory.

The main risk factor for *S. suis* infection in the outbreak was direct involvement in slaughtering sick pigs and preparing carcasses of pigs that died of unknown causes. Unlike professionals in modern abattoirs, the local farmers did not wear protective gear or gloves. Normally 1–2 persons carried out the procedure, which involved bloodletting though a neck artery, manually inflating the carcasses, scalding the pigskin with ≈80°C water, and splitting and shaving the skin with large knives. Scalding and shaving were often performed together. The farmers then sliced the meat into smaller pieces before cooking for food. The complete process of slaughtering could take >1 hour. Our study demonstrated that all patients had been infected during direct contact with blood or tissues of sick or dead pigs. Often this may have occurred through direct exposure of skin wounds. Droplet exposure may also have occurred

Table 3. Clinical symptoms of human cases of *Streptococcus suis* infection in Sichuan, China, 2005*

Symptoms and signs	STSS, no. (%) (n = 61)	Non-STSS, no. (%) (n = 154)	Total, no. (%) (N = 215)
Fever (temperature >37.3°C)	61 (100)	154 (100)	215 (100)
Chills	48 (79)	128 (83)	176 (82)
Headache	38 (62)	110 (71)	148 (69)
Myalgia	30 (49)	73 (47)	103 (48)
Vomiting	41 (67)	80 (52)	121 (56)
Abdominal pain	24 (39)	33 (21)	57 (27)
Diarrhea	28 (46)	22 (14)	50 (23)
Coma	16 (26)	26 (17)	42 (20)
Petechiae, ecchymosis†	37 (61)	12 (8)	49 (23)
Neck rigidity	4 (7)	50 (32)	54 (25)
Kernig positive	1 (2)	27 (18)	28 (13)
Brudzinski positive	2 (3)	17 (11)	19 (9)
Hypotension (blood pressure <90 mm Hg)†‡	25 (93)	2 (9)	27 (55)

*STSS, streptococcal toxic shock syndrome. Median duration between exposure and onset (range) was 1.6 d (9 h–9 d) for STSS, 2.5 d (6 h–14 d) for non-STSS, and 2.2 d (3 h–14 d) for total sample. Difference between median incubation periods of STSS and non-STSS, $p<0.05$, rank sum test. †Difference in frequency between STSS and non-STSS, $p<0.001$, χ^2. ‡Not conducted in every patient.

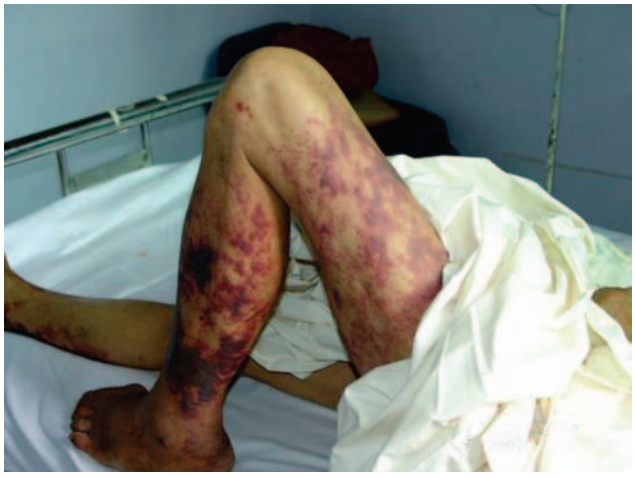

Figure 4. Photograph of a *Streptococcus suis* patient's legs with streptococcal toxic shock syndrome, featuring purpura and evidence of gangrenous changes in the calf extending down to the foot.

Table 4. Initial laboratory test results of human cases of *Streptococcus suis* infection in Sichuan, China, 2005*

Result	STSS (n = 61)	Non-STSS (n = 154)	Total (N = 215)
Leukocytosis, >10 × 10^9/L, no. (%)†	26 (52)	97 (71)	123 (66)
Mean leukocyte count (range)	12.3 (1.0–47.8)	14.5 (2.0–64.0)	13.9 (1.0–64.0)
Thrombocytopenia, <100 × 10^6/L, no. (%)†	34 (74)	53 (45)	87 (53)
Mean platelet count (range)	94.7 (10.0–287.0)	118.5 (13.3–689.0)	115.0 (4.4–689.0)
Liver function impairment, no. (%)†‡	27 (90)	50 (68)	77 (74)
Renal impairment, no. (%)†§	20 (59)	3 (3)	23 (19)
CSF abnormality, no. (%)†¶	0	31 (46)	31 (40)

*STSS, streptococcal toxic shock syndrome.
†Not all tests were conducted on all patients.
‡Alanine aminotransferase >2× upper limit of normal.
§Creatinine ≥177 μmol/L for adults or ≥2× upper limit of normal for age.
¶CSF, cerebrospinal fluid, protein >0.45 g/L, glucose <2.5 mmol/L, and >8 leukocytes × 10^6/L.

during slaughter or processing of carcasses, but we could not document this occurrence. The observed risk factors were consistent with those reported in other studies (*5*,*6*). No evidence of infection from eating cooked pork from these pigs was observed. The uncooked meat was shared with neighboring families, but these villagers normally do not eat raw meat or raw animal viscera. Person-to-person transmission was highly unlikely since we found no disease in family members, neighbors, or healthcare workers who had not been exposed to sick or dead pigs.

One missing link in the outbreak is the exact relationship between the dead goat in the early cluster of patients and the subsequent propagation of the *S. suis* infections. We could not confirm these 2 cases microbiologically. We speculate that *S. suis* caused these 2 early human infections because they occurred shortly after exposure to the dead goat, and because clinical manifestations in the 2 patients were similar to those of others in the outbreak. Human *S. suis* infection after exposure to sick goats has not been reported, despite isolation of the organism from these animals (*2*). In backyard farms where different animals are kept together, *S. suis* infection could have been transmitted between pigs and goats. Animal surveillance would help establish the role of animals other than pigs in carriage of the bacteria and the potential for causing human infections.

Clinically, 3 distinctive forms of human *S. suis* infection occurred, namely, STSS, sepsis, and meningitis. STSS has not been reported from *S. suis* infection, although it has been previously described in other streptococcal infections (*13*) and *Staphylococcus aureus* infections (*18*). Unusual STSS-like illnesses brought the outbreak to the attention of local health authorities. A dose effect may explain the relatively high proportion of STSS in this outbreak. While other explanations like comorbid conditions, e.g., asplenia, diabetes mellitus, alcoholism, and malignancy (*19*), have been reported, this was not the case for the outbreak in our study, which involved previously healthy adults.

Laboratory examination confirmed virulence factors in the *S. suis* isolates from this outbreak. They include *mrp*, *sly*, and *ef*, although their precise clinical role has not yet been shown. These isolates (*mrp*+, *ef*+, *sly*+) are related to European strains that are considered to be more virulent than North American strains (*1*). Genome analysis could determine if novel virulence genes were involved.

Finally, the high number of deaths due to STSS is a cause for concern. Prompt institution of effective responses to human *S. suis* outbreaks is a public health challenge, especially in rural China. Timely diagnosis is difficult. In our study, only 31% of the cases were laboratory confirmed. Suboptimal access to health services, personal delay in seeking treatment, underutilization of blood cultures in local hospitals, and self-administration of antimicrobial drugs may explain the relatively low proportion of culture-positive human cases, especially during the early phase of the outbreak. Many patients died without having sought treatment from any health facility. As for the investigation process, the existing surveillance system that covers meningitis is not robust enough to alert public health personnel of the impending threat of *S. suis*. Control measures included prohibiting by law of slaughtering, eating, selling, and transporting deceased or sick pigs. Subsidies were offered to families to support hygienic handling of

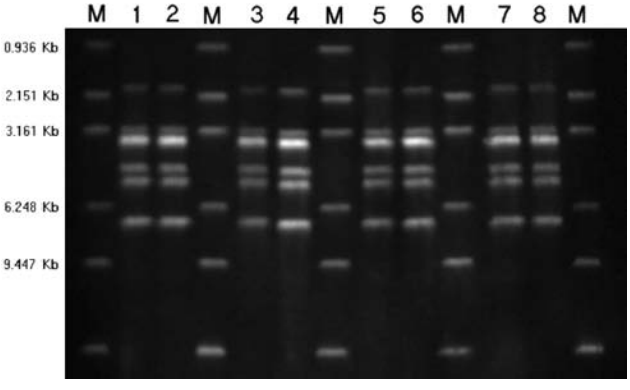

Figure 5. Ribotyping of *Streptococcus suis* serotype 2 isolates by *Pvu*II restriction. Lane 1, deceased pig isolate SC5; lane 2, deceased pig isolate SC16; lane 3, patient isolate SC154; lane 4, patient isolate SC160; lane 5, patient isolate SC175; lane 6, patient isolate SC179; lane 7, patient isolate SC204; lane 8, patient isolate SC206; M, molecular size standard.

deceased or sick pigs and to patients for medical care. Village heads were held accountable for illegal slaughtering in their village. These measures were supplemented with disinfection of affected backyard farms. Public education campaigns were staged to increase awareness of how to prevent and control human *S. suis* infections. In the long run, the prevention and control of swine infection should form the more strategic component of the public health program. Surveillance systems should be established to alert farmers and the general public if an infection outbreak in pigs is recognized (*19*).

Acknowledgments

We thank the Sichuan Health Bureau, the government of Ziyang, the Center for Disease Control and Prevention of Chengdu, and Ziyang, Neijiang, Suining, Mianyang, Zigong, Luzhou, Yibing, Deyang, Leshan, Nanchong, and Guangan Prefectures for assisting in coordinating field investigations and providing logistics support. The Ministry of Health in China has generously facilitated this study. We thank Lee Chin-Kei and Robert E. Fontaine for helping us prepare the article.

The Chinese Center for Disease Control and Prevention supported this study both technically and administratively.

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References

1. Berthelot-Herault F, Gottschalk M, Morvan H, Kobisch M. Dilemma of virulence of *Streptococcus suis*: Canadian isolate 89-1591 characterized as a virulent strain using a standardized experimental model in pigs. Can J Vet Res. 2005;69:236–40.
2. Acha PN, Szyfres B. Zoonoses and communicable diseases common to man and animals. Vol 1. Bacterioses and mycoses. 3rd ed. Washington: Pan American Health Organization; 2003. p. 257–65.
3. Higgins R, Gottschalk M. Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. Ames (IA):Iowa State University Press; 1999. p. 563–78.
4. Krauss H, Weber A, Appel M, editors. Zoonoses—infectious diseases transmissible from animals to humans, 3rd ed. Washington: ASM Press; 2003. p. 239-40.
5. Kay R, Cheng AF, Tse CY. *Streptococcus suis* infection in Hong Kong. QJM. 1995;88:39–47.
6. Suankratay C, Intalapaporn P, Nunthapisud P, Anmyingmongkol K, Wilde H. *Streptococcus suis* meningitis in Thailand. Southeast Asian J Trop Med Public Health. 2004;35:868–76.
7. Arends JP, Zanen HC. Meningitis caused by *Streptococcus suis* in humans. Rev Infect Dis. 1988;10:131–7.
8. Strangmann E, Froleke H, Kohse KP. Septic shock caused by *Streptococcus suis*: case report and investigation of a risk group. Int J Hyg Environ Health. 2002;205:385–92.
9. World Health Organization. Outbreak associated with *Streptococcus suis* in pigs, China. Wkly Epidemiol Rec. 2005;80:269–76.
10. Tarradas C, Arenas A, Maldonado A, Luque I, Miranda A, Perea A. Identification of *Streptococcus suis* isolated from swine: proposal for biochemical parameters. J Clin Microbiol. 1994;32:578–80.
11. 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;47:3169–75.
12. Picard FJ, Ke D, Boudreau DK, Boissinot M, Huletsky A, Richard D, et al. Use of *tuf* sequences for genus-specific PCR detection and phylogenetic analysis of 28 streptococcal species. J Clin Microbiol. 2004;42:3686–95.
13. Hashikawa S, Iinuma Y, Furushita M, Ohkura T, Nada T, Torii K, et al. Characterization of group C and G streptococcal strains that cause streptococcal toxic shock syndrome. J Clin Microbiol. 2004;42:186–92.
14. Centers for Disease Control and Prevention. Streptococcal toxic-shock syndrome (STSS), 1996 case definition [monograph on the Internet]. 2004 Apr [cited 2005 Aug 20]. Available from http://www.cdc.gov/epo/dphsi/casedef/streptococcalcurrent.htm
15. Hu X, Zhu F, Wang H, Chen S, Wang G, Sun J, et al. Studies on human streptococcal infectious syndrome caused by infected pigs [article in Chinese]. Zhonghua Yu Fang Yi Xue Za Zhi. 2000;34:150–2.
16. Office International des Epizooties. The People's Republic of China notifies officially to the OIE an outbreak of *Streptococcus suis* in pigs, including laboratory investigation research in animals [monograph on the Internet]. 2005 Aug [cited 2005 Aug 20]. Available from http://www.oie.int/eng/press/en050805b.htm
17. Ministry of Health, People's Republic of China. Estimation report of *Streptococcus suis* infection in Sichuan province [monograph on the Internet]. 2005 Aug [cited 2006 Feb 5]. Available from http://www.moh.gov.cn/public/open.aspx?n_id=10317&seq=0
18. Shands KN, Schmid GP, Dan BB, Blum D, Guidotti RJ, Hargrett NT, et al. Toxic-shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases. N Engl J Med. 1980;303:1436–42.
19. Huang YT, Teng LJ, Ho SW, Hsueh PR. *Streptococcus suis* infection. J Microbiol Immunol Infect. 2005;38:306–13.

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Co-infections of Adenovirus Species in Previously Vaccinated Patients

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Despite the success of the adenovirus vaccine administered to US military trainees, acute respiratory disease (ARD) surveillance still detected breakthrough infections (respiratory illnesses associated with the adenovirus serotypes specifically targeted by the vaccine). To explore the role of adenoviral co-infection (simultaneous infection by multiple pathogenic adenovirus species) in breakthrough disease, we examined specimens from patients with ARD by using 3 methods to detect multiple adenoviral species: a DNA microarray, a polymerase chain reaction (PCR)–enzyme-linked immunosorbent assay, and a multiplex PCR assay. Analysis of 52 samples (21 vaccinated, 31 unvaccinated) collected from 1996 to 2000 showed that all vaccinated samples had co-infections. Most of these co-infections were community-acquired serotypes of species B1 and E. Unvaccinated samples primarily contained only 1 species (species E) associated with adult respiratory illness. This study highlights the rarely reported phenomenon of adenoviral co-infections in a clinically relevant environment suitable for the generation of new recombinational variants.

Adenoviruses cause an estimated 8% of clinically relevant viral disease globally (*1*). Human adenoviruses (HAdVs) are divided into 51 serotypes (HAdV-1–HAdV-51) on the basis of type-specific antiserum-mediated neutralization of infectivity (determined primarily by the hexon coat protein and terminal knob portion of the fiber protein) (*2*) and into 6 species, also referred to as subgenera or subgroups (HAdV-A, B, C, D, E, and F) on the basis of hemagglutination inhibition and biochemical criteria (*3–5*). Species HAdV-B is further classified into subspecies B1 and B2 (*3*). In civilian populations, HAdV-B1 serotypes 3, 7, 16, and 21; HAdV-E serotype 4; and 1 member of subspecies HAdV-B2, serotype 14, cause outbreaks of illness ranging from mild febrile respiratory infections and conjunctivitis to potentially lethal disseminated infections in both adults and children (*1,6*). HAdV-C serotypes 1, 2, 5, and 6 cause locally endemic upper respiratory infections in infants and children (*7,8*) and occasional outbreaks in adults. Other HAdV species are usually not associated with respiratory disease in otherwise healthy humans.

HAdV seems to have found a particularly destructive niche in military training camps. HAdV-B1 serotypes 3, 7, and 21; HAdV-E serotype 4; and HAdV-B2 serotype 14 have caused severe outbreaks of acute respiratory disease (ARD) among military recruits in training centers (*9,10*). Before initiation of an HAdV vaccination program in 1971, outbreaks occurred regularly, and ≈1 of 6 recruits in affected camps required hospitalization (*1*). Systematic vaccination of recruits against the 2 most common agents of ARD in the military, HAdV serotypes 4 and 7, decreased HAdV-specific respiratory illness by 95% to 99% and overall respiratory illness rates by 50% to 60% (*11–13*). Despite this general efficacy, breakthrough infection (infection of vaccinated persons by the vaccine-targeted adenoviral serotypes) was still regularly reported (*14*). Production of the vaccine was suspended in 1996, at which point vaccination became sporadic until the existing stocks ran out in 1999. ARD rates quickly returned to prevaccine levels, with HAdV as the apparent causal agent. As a result, reintroduction of the vaccine is being actively pursued (*15*).

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To explore the possibility that unique HAdV strains were causing ARD in vaccinated persons, throat swab samples were selected from the Naval Health Research Center population-based febrile respiratory illness surveillance collection from vaccinated (n = 21) and unvaccinated (n = 31) recruits who reported ARD from 1996 to 2000. Samples were chosen that had tested positive for serotypes 4 or 7 by culture and serotypic antibody neutralization. The gene coding for the primary adenoviral antigen, the hexon coat protein, was sequenced from these isolates. The sequence data suggested that the detectable serotype 4 and 7 strains apparently responsible for breakthrough infection were the same as those circulating in unvaccinated military and civilian populations (*16*). In this study, we reanalyze the same set of samples to identify co-infections with multiple HAdV strains and to address what role co-infections may play in breakthrough infection.

Materials and Methods

Sample Collection and Preparation

Samples were collected as throat swabs into viral transport medium from military recruits with ARD at a variety of training camps as previously described (*16*). The throat swab samples were cultured on A549 cells and tested by using standard serologic methods. Both original swabs and in vitro tissue culture fluid (ITCF) samples were stored at –80°C. Samples that initially tested positive for serotypes 4 or 7 by culture and microneutralization were chosen for analysis and grouped by previous vaccination status. DNA extracts from ITCF samples were collected and used in molecular assays. Collection details and symptom definitions were previously reported (*16*), and sample details are shown in Tables 1 and 2. Initially, 13 unidentified (blinded) samples were sent by the Naval Health Research Center to the Naval Research Laboratory personnel for testing. After the initial 13 samples showed a high rate of respiratory HAdV co-infection, primarily in vaccinated persons, an additional 39 samples were tested in an unblinded fashion.

Microarray-based Genotyping

One microliter of purified DNA extract from each of the 52 ITCF samples was used as the template in 50-μL degenerate PCR amplifications targeting portions of the *E1A*, hexon, and fiber genes. The primers, degenerate polymerase chain reaction (PCR) amplification protocol, probes, and microarray fabrication techniques have been previously described (*18*). Once constructed, the spotted microarrays were blocked with a 3% bovine serum albumin–casein solution (BSA-C) for 15 min at room temperature, and the slides were outfitted with MAUI Mixer DC hybridization chambers (BioMicro Systems, Salt Lake City, UT, USA). Twenty-microliter hybridization reactions (13.6 μL biotinylated degenerate PCR amplicons, 2 μL 3% BSA-C, 4 μL 20× SSC (0.3 mol/L sodium citrate, 3.0 mol/L NaCl, pH 7.0), and 0.4 μL 10% sodium dodecyl sulfate [SDS]) were denatured for 3 min at 98°C and immediately applied to the microarrays. Hybridizations were performed for 2 h at 63°C in a MAUI Hybridization System (BioMicro Systems). Slides were then washed twice with 4× SSC-0.2% SDS buffer and 2× SSC buffer, and hybridization was detected by the sequential addition of Cy5-conjugated mouse antibiotin immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA, USA) and Cy5-conjugated goat antimouse IgG (Jackson ImmunoResearch). Images were obtained with a ScanArray Lite confocal laser scanning system (Perkin-Elmer, Torrance, CA, USA) at a laser power of 60 to 80 and a photomultiplier tube gain of 60 to 80. The fluorescent signal from each microarray element was considered positive only when its quantified intensity was >3× that of known internal negative control elements. Each ITCF sample was subjected to 2 to 5 independent amplification and hybridization experiments. Hybridization patterns unique to specific serotypes were determined empirically with prototype strains (*18*). Although members of species HAdV-B1 often produced complex hybridization profiles (*18*), these profiles were unique, reproducible, and readily identifiable in both single infections and co-infections.

Adenovirus Consensus PCR–Enzyme-linked Immunosorbent Assay

We used a commercially available kit capable of typing adenoviruses to the species level to confirm the results obtained with microarray analyses. Briefly, the Adenovirus Consensus kit (Argene, North Massapequa, NY, USA) uses a PCR–enzyme-linked immunosorbent assay that amplifies a fragment from the adenovirus virus-associated (VA) RNA gene and subsequently detects and types the amplicon with species-specific biotinylated oligonucleotide probes in a colorimetric microwell format (*19*). Results obtained with the kit were interpreted according to the manufacturer's adenovirus typing protocol.

Adenovirus-specific PCR

The species-specific PCR amplification was performed with previously published primers (*20*) and a Multiplex PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions (with 0.5× Q solution). These amplifications were performed in 25-μL reaction volumes at an annealing temperature of 52°C. In general, the PCRs were performed in an iCycler (Bio-Rad, Hercules, CA, USA) and analyzed by electrophoresis on 1.5% agarose gels. Monoplex PCR was performed under identical reaction conditions, except that the same primers were used in

Table 1. Naval Health Research Center data for molecular detection of adenoviral co-infections in vaccinated and unvaccinated patients with febrile respiratory illness*

Original designation†	Vaccination date	Microneu-tralization‡	Multiplex PCR	Species-specific PCR (B, C, E)	Sequencing§	GenBank accession no.
7151.AV5.V.98.FJ	5 Nov 1997	4	B, C	B, C, E	5, 21	
7137.AV4.V.97.FJ	1 Dec 1997	4	E	B, E	4 variant	AY337237
7274.AV4.V.98.FJ	11 Feb 1998	4	E, B	E, B	4 vaccine (Δ = 2)	AF065062
7307.AV5.V.98.FJ	9 Feb 1998	4	C, B	B, C, E	5	
7333.AV4.V.98.FJ	25 Mar 1998	4	E	E, **B**	4 variant	AY337242
4185.AV4.V.97.FLW	24 Mar 1997	4	E	B, E	4 variant, 7h	AY337252
4476.AV4.V.97.FLW	24 Oct 1997	4	E	B, E	4 variant	AY337249
79.AV4.V.96.GL	7 Oct 1996	4	E	E	4 vaccine (Δ = 3)	AF065062
141.AV7.V.96.GL	12 Nov 1996	7	B	B	7d2 (prototype)	AY337258
275.AV4.V.97.GL	31 Jan 1997	4	E	E	4 vaccine (Δ = 3)	AY337239
1212.AV7.V.97.GL	29 Sep 1997	7	B	B, E	7d2 (Δ = 2)	AY337255
1108.AV7.V..97.GL	8 Oct 1997	7	E, B	B, E	7 vaccine (Δ = 0)	AF065067
1122.AV7.V.97.GL	8 Oct 1997	7	B	B	7d2 (Δ = 2)	AF321311
1150.AV7.V.97.GL	8 Oct 1997	7	B	B, E	7 vaccine (Δ = 2)	AY337254
1152.AV7.V.97.GL	8 Oct 1997	7	B	B	7 vaccine (Δ = 1)	AY337253
1186.AV7.V.97.GL	8 Oct 1997	7	B	B, E	7d2 (Δ = 2)	AF321311
1251.AV7.V.97.GL	8 Oct 1997	7	B	B	7d2 (Δ = 2)	AF321311
1275.AV7.V.97.GL	8 Oct 1997	7	B	B, E	7 vaccine (Δ = 1)	AY337257
1302.AV7.V.97.GL	8 Oct 1997	7	B	B, E	7 vaccine (Δ = 2)	AY337256
1649.AV7.V.98.GL	13 Jan 1998	7	B	B	7d2 (Δ = 2)	AF321311
1856.AV5.V.98.GL	25 Mar 1998	4	C	B, C, **E**	5, 7h	
60406.AV7.99.FB		7	B	B	7 vaccine (Δ = 2)	AY337256
60673.AV4.00.FB		4	E	E	4 variant	AY337237
60691.AV4.00.FB		4	E	E, **B**	4 variant	AY337238
60697.AV4.00.FB		4	E	E	4 variant	AY337246
60708.AV4.00.FB		4	E	E	4 variant	AY337237
60716.AV4.00.FB		4	E	E	4 variant	AY337247
CHPPM2.AV4.00.FB			E	E, **B**	4 variant	AY337237
CHPPM9.AV4.00.FB		4	E	E, **B**	4 variant	AY337237
CHPPM13.AV4.00.FB			E	E, **B**	4 variant	AY337237
CHPPM29.AV4.00.FB		4	E	E	4 variant	AY337237
CHPPM44.AV4.00.FB		4	E	E	4 variant	AY337237
7372.AV5.98.FJ		4	C	B, C, E	5, 7h	
40098.AV4.98.FJ		4	E	E	4 variant	AY337241
40160.AV4.98.FJ		4	E	E, **B**	4 variant	AY337237
40183.AV4.98.FJ		4	E	E	4 variant	AY337237
40781.AV4.99.FJ		4	E	E	4 variant	AY337238
40844.AV4.99.FJ		4	E	E	4 variant	AY337237
41059.AV4.99.FJ		4	E	E	4 variant	AY337237
10060.AV4.98.GL			E	E	4 variant	AY337237
10190.AV4.98.GL		4	E	E, **B**	4 variant	AY337237
10206.AV4.98.GL		4	E	E	4 variant	AY337244
10213.AV4.98.GL		4	E	E	4 variant	AY337240
10257.AV4.98.GL		4	E	E	4 variant	AY337237
10258.AV4.98.GL		4	E	E	4 variant	AY337237
10756.AV4.00.GL		4	E	E	4 variant	AY337243
50108.AV4.00.LAC			E	B, E	4 variant	AY337251
20044.AV4.98.MCRD		4	E	B, E	4 variant	AY337248
20139.AV4.98.MCRD			E	E	4 variant	AY337237
20142.AV4.98.MCRD			E	E	4 variant	AY337250
20143.AV4.98.MCRD			E	E, **B**	4 variant	AY337237
20145.AV4.98.MCRD			E	E	4 variant	AY337245

*PCR, polymerase chain reaction. Letters or numbers in **boldface** indicate weak positives.
†Acquisition number, serotype, isolation year, and isolation location.
‡Results are listed as serotypes. Species B1 includes serotypes 3, 7, 16, 21; species C includes serotypes 1, 2, 5, 6; and species E includes serotype 4.
§Variant/vaccine grouping based on the hexon gene sequence defined by Blasiole et al. (*16*). Δ = # reflects number of base substitutions from vaccine strain in 1,490 bp of the hexon sequence (*16*). The 7d2 designation is based on that of Blasiole et al. (*16*). The 7h designation based on fiber gene sequence is as defined by Kajon and Wadell (*17*).

Table 2. Naval Research Laboratory data for molecular detection of adenoviral co-infections in vaccinated and unvaccinated patients with febrile respiratory illness*

Original designation†	Vaccination date	Microarray‡	Adenovirus Consensus kit	PCR determination‡	
				Positive	Negative
7151.AV5.V.98.FJ	5 Nov 1997	C, 21	C, B1	5, 21	
7137.AV4.V.97.FJ	1 Dec 1997	4, C, B2	E	4, 1	B2
7274.AV4.V.98.FJ	11 Feb 1998	4, 21, C, B2	E, B1, B2	4, 21, B2	C
7307.AV5.V.98.FJ	9 Feb 1998	C, 21	C	C	21
7333.AV4.V.98.FJ	25 Mar 1998	4, C, B2	E	4, 1, 5, B2	
4185.AV4.V.97.FLW	24 Mar 1997	4, C, B2	E, B2, F, B1	4, B2	C
4476.AV4.V.97.FLW	24 Oct 1997	4, C, B2	E, B2, F, B1	4, 5, B2	
79.AV4.V.96.GL	7 Oct 1996	4, C, 7	E	4, C, B2	7
141.AV7.V.96.GL	12 Nov 1996	7, 4, **3**	B1, B2, E	7, 4, B2	3
275.AV4.V.97.GL	31 Jan 1997	4, C, 7	E, B2, F, B1	4, C, B2	7
1212.AV7.V.97.GL	29 Sep 1997	7, 4, **3**	B1, E, F	7, 4, 3, F	
1108.AV7.V..97.GL	8 Oct 1997	7, 4, C, **3**	B1, E, F	7, 4, C	3
1122.AV7.V.97.GL	8 Oct 1997	7, C, **3**	B1	7, C	3
1150.AV7.V.97.GL	8 Oct 1997	7, 4, **3**	B1, E, F	7, 3, F	4
1152.AV7.V.97.GL	8 Oct 1997	7, 4, **3**	B1	7, 4	3
1186.AV7.V.97.GL	8 Oct 1997	7, 4	B1, E, F	7	4
1251.AV7.V.97.GL	8 Oct 1997	7, 4, **3**	B1, E, F	7	4, 3
1275.AV7.V.97.GL	8 Oct 1997	7, 4, **3**	B1, E	7, 4, 3	
1302.AV7.V.97.GL	8 Oct 1997	7, 4, **3**	B1, E, F	7, 4	3
1649.AV7.V.98.GL	13 Jan 1998	7, 3, 4	B1	7, 3	4
1856.AV5.V.98.GL	25 Mar 1998	C, 7	C	C	7
60406.AV7.99.FB		7	B1	7	
60673.AV4.00.FB		4, C	E	4	C
60691.AV4.00.FB		4, C	E	4	C
60697.AV4.00.FB		4, C	E	4, 1	
60708.AV4.00.FB		4, C	E	4	C
60716.AV4.00.FB		4, C	E	4	C
CHPPM2.AV4.00.FB		4, C	E	4	C
CHPPM9.AV4.00.FB		4, C	E	4	C
CHPPM13.AV4.00.FB		4, C	E	4	C
CHPPM29.AV4.00.FB		4, C	E	4	C
CHPPM44.AV4.00.FB		4, C	E	4	C
7372.AV5.98.FJ		C, **7**	C	C	7
40098.AV4.98.FJ		4	E, F	4, F	
40160.AV4.98.FJ		4	E	4	
40183.AV4.98.FJ		4	E	4	
40781.AV4.99.FJ		4, C	E, B2	4, B2	C
40844.AV4.99.FJ		4, C	E, B2	4, B2	C
41059.AV4.99.FJ		4, C	E, B2, F	4, C, B2, F	
10060.AV4.98.GL		4, C, B2	E, B2	4, B2	C
10190.AV4.98.GL		4	E	4	
10206.AV4.98.GL		4, C, B2	E, B2	4, B2	C
10213.AV4.98.GL		4, C, **B2**	E, B2, F	4, B2	
10257.AV4.98.GL		4, B2	E	4, B2	
10258.AV4.98.GL		4	E	4	
10756.AV4.00.GL		4	E	4	
50108.AV4.00.LAC		4, B2	E	4, B2	
20044.AV4.98.MCRD		4, C, 7, **3**	E	4, 1, B2	7, 3
20139.AV4.98.MCRD		4	E	4	
20142.AV4.98.MCRD		4	E	4	
20143.AV4.98.MCRD		4, C, B2	E, B2, F	4, C, B2, F	
20145.AV4.98.MCRD		4, C, B2	E, B2, F	4, B2	C

*PCR, polymerase chain reaction. Species and serotype are listed in order of predominance. Letters or numbers in **boldface** indicate weak positives.
†Acquisition number, serotype, isolation year, and isolation location.
‡Results are listed as serotypes or species. Species B1 includes serotypes 3, 7, 16, 21; species C includes serotypes 1, 2, 5, 6; and species E includes serotype 4.

independent reactions. Sequencing reactions and microneutralization assays were performed as previously described (*21*,*22*). Serotype-specific PCR assays (Tables 1 and 2) were verified as described (*20*,*23*–*26*), with occasional substitutions of polymerase type and annealing temperature adjustments.

Co-infection Separation

Limiting dilutions of ITCF sample 7151 were plated on A549 cells and allowed to adsorb for 16 hours, after which agarose overlays (0.4% agarose in Dulbecco minimal essential medium, 2% fetal bovine serum, 4 mmol/L glutamine) were added to each infected monolayer. Well-separated virus plaques were picked 5 days postinfection, placed into viral transport medium, and tested by PCR for HAdV-B and HAdV-C. A second round of plaque purification was performed on several plaque isolates that were treated with 0.05% Triton-X 100 to potentially disrupt virus clumps before their dilution and plating. After 6 hours of adsorption, the original inoculum was removed, and the monolayers were overlayed with agarose solution. The newly formed plaques were tested as described above.

Results

By using a new 70-mer spotted microarray (*18*), a PCR–enzyme-linked immunosorbent assay (*19*), and a species-specific multiplex PCR assay (*20*), we generated data profiles for each of the 52 tissue culture-amplified samples; the raw data from 2 of these samples are shown as representative examples (Figure). The microarray profile of vaccinated sample 7274 detected HAdV-4 (species E), HAdV-21 (species B1), HAdV-C, and HAdV-B2 according to previously validated hybridization patterns (*18*) (Figure, panel A). Except for detection of an apparent low-level HAdV-C co-infectant, the results of the Adenovirus Consensus kit (HAdV-B1, HAdV-B2, and HAdV-E) (Figure, panel B), multiplex and monoplex species-specific PCR (HAdV-B and HAdV-E) (Figure, panel C), and serotype-specific PCR (HAdV-4, HAdV-21, and HAdV-B2) (Figure, panel D) confirmed the microarray-based identification of multiple adenoviral strains in sample 7274.

In contrast, the microarray profile of unvaccinated sample 10756 detected a single serotype, HAdV-4 (Figure, panel E). The microarray-based finding was verified by results of the Adenovirus Consensus kit (Figure, panel F),

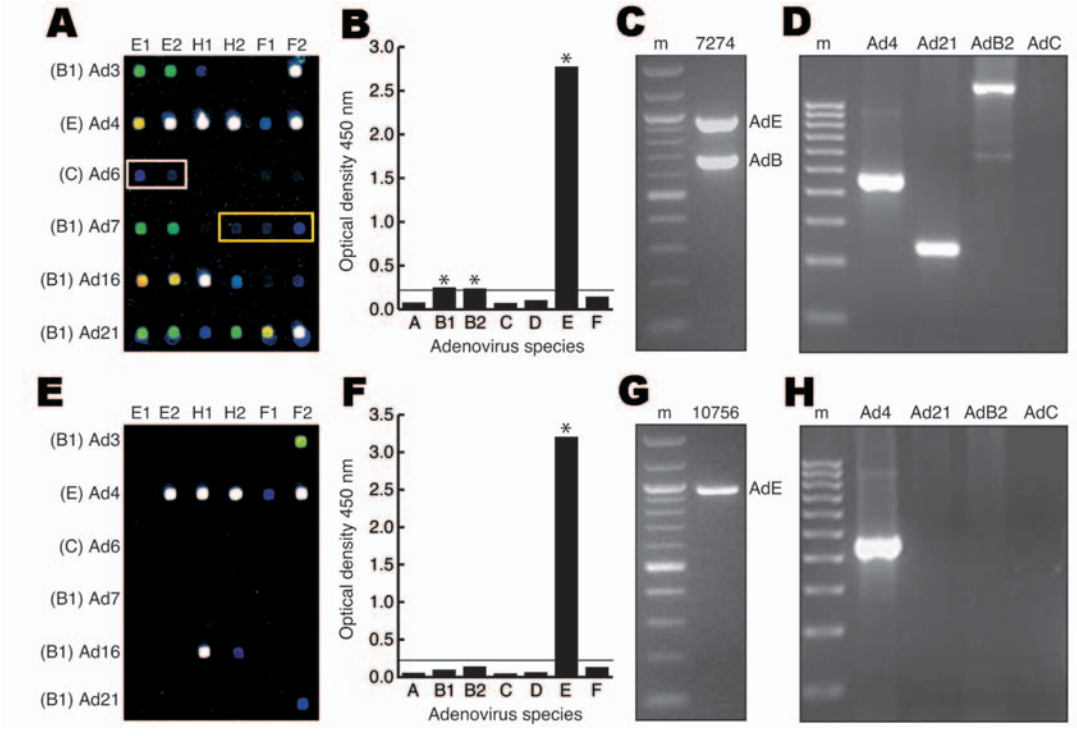

Figure. Molecular methods used to identify human adenovirus (HAdV) co-infections. A–D) Vaccinated sample 7274. A) Microarray hybridization profile. White and yellow rectangles indicate low-positive HAdV-C and HAdV-B2 targets, respectively. Spot colors denote hybridization signal intensity (white > yellow > green > blue). Species and corresponding serotype designations are indicated on the left. Probe designations (E1, E2 = serotype-specific E1A probes; H1, H2 = serotype-specific hexon probes; F1, F2 = serotype-specific fiber probes) are indicated above each array. B) Adenovirus Consensus kit optical density values. *, amplification positive. The horizontal line is the manufacturer's significance threshhold. C) Multiplex species-specific polymerase chain reaction (PCR). m, molecular mass marker. Species designations are to the right of the corresponding band. D) PCR verification with independent serotype or species-specific primers. E–H) Unvaccinated sample 10756. E) Microarray hybridization profile. F) Adenovirus Consensus kit optical density values. G) Multiplex species-specific PCR. H) PCR verification with independent serotype-specific primers.

multiplex species-specific PCR (Figure, panel G), and HAdV-4 serotype-specific PCR (Figure, panel H). The data profiles for all 52 samples assembled and compared in this manner are shown in Tables 1 and 2. Dual, triple, and quadruple infections were found in all 21 of the vaccinated samples and in 14 of the 31 unvaccinated samples tested (Tables 1 and 2).

Previously vaccinated persons showed a high rate of co-infection with both species commonly associated with ARD (HAdV-B1 and HAdV-E), whereas unvaccinated persons were primarily infected with HAdV-E. Since HAdV-4 and HAdV-7 are the 2 most common ARD-associated serotypes, that they were also the most commonly paired respiratory pathogenic co-infectants detected in vaccinated persons is not surprising. When the vaccine was used, the rates of other respiratory adenoviruses were much higher than when the vaccine was not used (*16*). However, these isolates were chosen for study because they yielded antigenic signals consistent with either HAdV-4 or HAdV-7 and were therefore expected to contain at least 1 of these 2 viruses as the highest titer adenoviral components (Table 1) (*16*).

The ability of the microarray to identify to the serotype level resulted in the detection of the greatest number of co-infections, despite its inability to detect members of species B2 when a co-infecting HAdV-7 was present (hybridization pattern interference) and members of species F that were not targeted (Table 3). Microarray-based identification of multiple ARD-associated serotypes from diverse HAdV-B1 species (serotypes 3, 7, and 21) was necessary because co-infections with these serotypes would not have been indicated or resolved by methods limited to species-level identification.

Although most apparent co-infections could be verified by each of the primary methods tested and by serotype-specific PCR (e.g., single infections: 10756, 60406, 20142; co-infections: 1212, 7151, 7274), some could not be verified (e.g., 60691, CHPPM2). Those co-infectant signals that could not be verified were usually weak positives. The strains responsible for these signals appeared to be subordinate co-infectants because the predominant serotype or species signals generated for the associated samples by the microarray, Adenovirus Consensus kit, and serotype-specific PCR were corroborated in every case and matched the results obtained from the sequencing experiments previously reported (*16*).

The microarray and Adenovirus Consensus kit use detection and signal amplification techniques that enhance assay sensitivity and thus render them more sensitive than traditional PCR/agarose gel visualization techniques, as shown by the number of triple and quadruple co-infections detected with these techniques (Table 3). Thus, attempting to corroborate these methods with the 3 PCR-based methods used was not completely successful. Nevertheless, most of the positive results from these tests were verified by comparing the microarray and Adenovirus Consensus kit results or by comparison with the results from independent methods such as microneutralization, hexon sequence analysis, serotype-specific PCR that uses primers not used in the multiplex tests, and PCR amplicon sequencing (Tables 1, 2, and 4). These results suggest that these methods can identify and corroborate HAdV co-infections and that, in general, the HAdV load in ARD patients is more complex than previously thought.

To determine whether >1 replication-competent serotype or strain was present in the samples with evidence of co-infection, we attempted to physically separate the paired co-infectants in sample 7151 by plaque purification. Of 92 plaques picked from the initial plate, all tested positive for HAdV-C by PCR and 12 of 92 also tested positive for HAdV-B. Several of the plaques that retained both HAdV-B and HAdV-C signals were replaqued, and PCR testing of these plaques yielded only HAdV-C isolates. Further efforts that used a detergent to increase separation within the original ITCF sample 7151 and applied the agarose overlay more quickly (6 hours) to prevent interplaque contamination also yielded only HAdV-C plaques (data not shown).

Discussion

We demonstrate the rarely reported phenomenon of co-infections with multiple adenoviral species. Two previous

Table 3. Human adenovirus load detected with molecular identification methods*

Method	Status	No. samples with X co-infectant strains			
		X = 1	X = 2	X = 3	X = 4
Microarray	Vaccinated	0	4	15	2
	Unvaccinated	9	16	5	1
Adenovirus Consensus kit	Vaccinated	8	2	8	3
	Unvaccinated	22	5	4	0
Multiplex PCR	Vaccinated	17	4	0	0
	Unvaccinated	31	0	0	0
Monoplex PCR†	Vaccinated	7	11	3	0
	Unvaccinated	21	9	1	0

*PCR, polymerase chain reaction.
†Species-specific PCR from Table 1, Naval Health Research Center data.

Table 4. Human adenovirus (HAdV) species and serotype-specific primers

Name	Sequence	Target gene	Reference
Primer 1	CTT GGT CTA CGA CCA GAC GG		
Primer 3	GTT TGC TCA TGA ACA TGG CCA GAT CGC AC	Species B2 E3	(*26*)
F30	CTT CAA CCC TGT CTA CCC TAT GAA		
F969	TTC TCT AAT GTA GTA AAA GG	HAdV11 fiber	(*25*)
HsgF1	ATT TCT ATT CCT TCG CG		
HsgF2	TCA GGC TTG GTA CGG CC	Species F hexon	(*24*)
HsgC1	ACC TTT GAC TCT TCT GT		
HsgC2	TCC TTG TAT TTA GTA TC	Species C hexon	(*24*)
Ad3F	GGT AGA GAT GCT GTT GCA GGA		
Ad3R	CCC ATC CAT TAG TGT CAT CGG T	HAdV3 hexon	(*23*)
Ad7F	GGA AAG ACA TTA CTG CAG ACA		
Ad7R	AAT TTC AGG CGA AAA AGC GTC A	HAdV7 hexon	(*23*)
Ad21F	GAA ATT ACA GAC GGC GAA GCC		
Ad21R	AAC CTG CTG GTT TTG CGG TTG	HAdV21 hexon	(*23*)
Ad4F5	GTT GCT AAC TAC GAT CCA GAT ATT G		
Ad4R4	CCT GGT AAG TGT CTG TCA ATC C	HAdV4 hexon	This study
Ad7F-F	ACA ACT GCC TAT CCT TTC AAT G		
Ad7F-R	GAC CAA GTT ACA CGA ATA CAA TAT G	HAdV7 fiber	This study
Ad5 E1A-F1	CCT AAA ATG GCG CCT GCT ATC CTG		
Ad5 E1A-R1	GCG ACG CCC ACC AAC TCT CAC	HAdV5 E1A	This study
Ad5 E1A-F2	GAG CCT TGG GTC CGG TTT CTA TG		
Ad5 E1A-R2	CCA TTT TAG GAC GGC GGG TAG	HAdV5 E1A	This study
Ad5 hexon-F1	GAC GGA GCC AGC ATT AAG TTT GAT		
Ad5 hexon-R1	GTT GGC GGG TAT AGG GTA GAG CAT	HAdV5 hexon	This study
Ad5 fiber-F1	TAT TCA GCA TCA CCT CCT TTC C		
Ad5 fiber-R1	AAG CTA TGT GGT GGT GGG GC	HAdV5 fiber	This study
AdA1	GCT GAA GAA MCW GAA GAA AAT GA		
AdA2	CRT TTG GTC TAG GGT AAG CAC	Species A fiber	(*20*)
AdB1	TST ACC CYT ATG AAG ATG AAA GC		
AdB2	GGA TAA GCT GTA GTR CTK GGC AT	Species B fiber	(*20*)
AdC1	TAT TCA GCA TCA CCT CCT TTC C		
AdC2	AAG CTA TGT GGT GGT GGG GC	Species C fiber	(*20*)
AdD1	GAT GTC AAA TTC CTG GTC CAC		
AdD2	TAC CCG TGC TGG TGT AAA AAT C	Species D fiber	(*20*)
AdE1	TCC CTA CGA TGC AGA CAA CG		
AdE2	AGT GCC ATC TAT GCT ATC TCC	Species E fiber	(*20*)
AdF1	ACT TAA TGC TGA CAC GGG CAC		
AdF2	TAA TGT TTG TGT TAC TCC GCT C	Species F fiber	(*20*)

studies have noted rare instances of HAdV-C dual infections in small numbers (*27*,*28*). HAdV-C, although rarely associated with pharyngitis outbreaks in recruits (*10*), is usually seen in children (*7*,*8*) and can produce latent infections that last into young adulthood. This fact, combined with low incidence of co-infection (*27*,*28*), has led to the assertion that multistrain adenovirus co-infections are not common (*28*) or clinically relevant. The results from the population tested in this study suggest otherwise. Samples from vaccinated recruits showed a high rate of co-infection with multiple species of adenovirus associated with adult ARD (HAdV-E and HAdV-B1).

Many of the identified co-infectants in this study were species not generally associated with ARD in the military (HAdV-C, HAdV-B2, and HAdV-F). Although these species were not likely the cause of ARD observed in these patients, since they are not believed to cause ARD in adults and because they have a high potential for latent carriage (*1*,*7*,*8*,*29*), their presence sheds new light on the general complexity of the human adenoviral load. In addition, they remain viable reservoirs capable of genetic complementation or recombination with upper respiratory strains.

Recombination can generate new strains with unique and stable phenotypes. Intraspecies adenovirus recombination has been demonstrated in laboratory cell-culture co-infection studies (*30*–*32*). These recombination events can generate viable hybrids with intermediate or unique immunogenic and tropic properties. Evidence suggests recombination can generate hybrids in immunocompromised patients (*29*,*33*,*34*), possibly as a result of co-infection with normally isolated serotypes. Recombination, particularly intraspecies, seems to play a major role in the evolution of new, virulent strains of HAdV (*1*,*17*,*35*,*36*). The currently dominant pathogenic HAdV in US military

recruits, a considerably diverged variant HAdV-4 strain (*16*), appears to be a recent recombinant between HAdV-4 and an HAdV-B1 serotype, probably HAdV-7 (*37*). Given that these 2 are the most common co-infectants seen in our sample set, this finding suggests that the observed dominance of co-infections in vaccinated persons may have contributed to the emergence of the new variant. In general, the understanding and control of situations that create or promote co-infection may be important considerations.

The HAdV vaccine, an enteric-coated live-virus tablet designed to transiently and selectively infect the gastrointestinal tract with normal respiratory HAdV strains, contains viable HAdV-4 and HAdV-7. Thus, we cannot assume whether the detected co-infectants arose from the vaccine itself or from community acquisition of circulating strains. Most HAdV-4 strains in this study are not the vaccine strain but rather a highly divergent variant that has recently been dominant in military training centers (GenBank strain Z-G 95-873). This identity was shown by sequence analysis of 1,500 bp of the hexon gene from many primary infectants identified in the same sample set that was analyzed here (*16*). The variant HAdV-4 isolates consistently differ from the vaccine strain by 32 base substitutions, including 9 coding changes, in this region (*16*) (Table 1). Hexon sequence analysis showed that many HAdV-7 co-infectants are HAdV-7d2. HAdV-7d2 is distinguished from the HAdV-7 vaccine strain (HAdV-7a) by a single coding polymorphism in the hexon sequence, but this polymorphism (protein L443Q or nucleotide T1328A in GenBank [*16*]) is specific to HAdV-7d and HAdV-7d2 and is not found in HAdV-7a, b, c, g, or h or in the vaccine strain (*16*,*38*,*39*). Three of the other HAdV-7 co-infectants (1856, 4185, and 7372) were shown to be HAdV-7h by fiber gene sequencing. The fiber gene of HAdV-7h appears to have been horizontally transferred from HAdV-3 and thus is highly diverged from the usual HAdV-7 fiber gene, as found in the vaccine strain (*17*). Thus, sequence analyses show that most, if not all, co-infectants are currently circulating HAdV-4 and HAdV-7 strains that are distinct from the vaccine strains (*16*) (Table 1).

Four lines of evidence support the idea that most of the apparent genetic complexity in the throat swab samples comes from multiple strains, as opposed to recombinants with mixed genetic characteristics. The first comes from the microarray data. The microarray tests for hybridization of 6 independent probes designed to match serotype-specific sequences in 3 genes (*18*). Since different species do not cross-react among the microarray probes, hybridization of genes from 1 species to the identifying probes for 2 species would require redundant presence of 2 different alleles in all 3 genes. Since both natural recombination in hosts (*17*) and artificially encouraged recombination in cell culture (*30*,*32*) strongly favor homologous recombination and generation of nonredundant hybrid strains, redundant characterization of paired, divergent alleles is inconsistent with a single recombinant genome.

The second line of evidence supporting co-infection with independent genomes comes from comparisons of relative co-infectant titers before and after potentially selective events, such as growth in tissue culture. PCR amplification of fiber gene sequences using species B- and E-specific primers was performed on serial dilutions of vaccinated sample 7274 before and after passage of the original ITCF through 2 additional cycles of growth in A549 cells. In this instance, the relative titers of HAdV-4 and HAdV-7, as measured by serial-dilution PCR, changed by 2 orders of magnitude (data not shown). The rapid drift in relative concentrations of PCR targets from paired co-infecting strains strongly suggests that the co-infectants' genomes are replicating independently and thus likely to be physically separate entities.

The third line of evidence supporting co-infectant independence comes from whole-genome sequencing efforts. Several molecular methods indicated that vaccinated sample 7151 harbored an HAdV-5/HAdV-21 co-infection (Tables 1 and 2). The genome of the HAdV-5 co-infecting strain was sequenced and assembled into a contiguous sequence (GenBank no. AY601635) consistent with a published HAdV-5 genome (GenBank accession no. AY339865) (*40*), which suggested no recombination of foreign DNA. However, this effort also generated several orphan sequences that did not fit into the assembled sequence and were subsequently identified as genetically redundant HAdV-21 regions. Further amplification and sequencing of several genetically distant fragments from the same sample using HAdV-21-specific primers yielded ≈2 kb of HAdV-21 sequence. On the basis of the entire genome and partial PCR sequencing analyses, ≥2 co-infecting HAdV genomes are contained in sample 7151.

The fourth line of evidence comes from our attempts to physically separate paired co-infectants by plaque purification. Sample 7151, which contained the HAdV-5/HAdV-21 co-infection, was used initially because it contained relatively equal titers of both co-infectants. Although most of the plaques tested contained HAdV-5, some contained both HAdV-5 and HAdV-21. Although we were unable to identify plaques that contained only HAdV-21, our results demonstrate the physical independence of the co-infecting entities and the functional independence of HAdV-5. Our results also suggest that either the HAdV-21 co-infectant is functionally dependent on HAdV-5 or is effectively outgrown by HAdV-5 to a degree that prevents independent isolation. Similar attempts were made with a few samples that had HAdV-4/HAdV-7 co-infections, but these were generally biased in titer (10^4 in favor of HAdV-7) and, as expected, yielded only HAdV-7 in >300 plaques tested.

The data demonstrated the functional independence of 1 co-infectant (HAdV-7) and physical independence of the co-infecting entities but could not conclusively demonstrate functional independence of the minor co-infectant.

Conventional clinical microbiologic methods, including microneutralization and hemagglutination inhibition, are comparative and designed to identify the primary HAdV serotype (or species) in a sample. Secondary infections are masked in these methods by the tests (e.g., microneutralization is reported as the strongest reaction, not the spectrum of reactions across all serotypes). Likewise, direct sequencing (*16*) may restrict identification to a single strain, particularly if 1 co-infectant is dominant. Restriction enzyme analysis methods are capable of resolving HAdV-C dual infections in which both serotypes are present in similar numbers (*27*). In contrast, when using sensitive molecular methods that can yield measurable signals from secondary (less numerous) co-infectants against the background of stronger signals produced by primary infecting strains, these methods may identify co-infections more than do conventional methods. In the case of respiratory infections, this finding has previously been documented (*41*).

Finally, each of the methods designed to test for multiple species or serotypes showed a higher number of HAdV (and accepted virulent HAdV species and serotypes) in vaccinated persons than in unvaccinated persons. HAdV vaccine was administered routinely to all trainees until supplies were exhausted, at which point adenovirus vaccination was stopped. Since trainees were vaccinated systematically, persons tended to be sampled at times when either all or no recruits were being vaccinated. Therefore, vaccinated samples collected and tested (from 1996 to 1998) are not concurrent with unvaccinated samples (collected from 1998 to 2000). Because of this sampling limitation, we could not confidently correlate HAdV co-infection with breakthrough infections in previously vaccinated persons. Thus, although this study highlights the previously underappreciated phenomenon of adenoviral co-infection, the conclusive examination of its relationship to vaccination must await reintroduction of HAdV vaccine (*15*).

Acknowledgments

We thank Gregory C. Gray and Dan Blasiole for critical preceding work, including collection and initial analysis of strains that we reanalyzed, and Beth Metzgar and Valerie de Crecy-Lagard for critically reading the manuscript.

Partial support was provided by the Epidemic Outbreak Surveillance Consortium[2] funded by the US Air Force Surgeon General Office, the Modernization Directorate, the Defense Threat Reduction Agency, and the Office of Naval Research.

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References

1. Rubin BA. Clinical picture and epidemiology of adenovirus infections. Acta Microbiol Hung. 1993;40:303–23.
2. Hierholzer JC, Stone YO, Broderson JR. Antigenic relationships among the 47 human adenoviruses determined in reference horse antisera. Arch Virol. 1991;121:179–97.
3. Wadell G. Molecular epidemiology of human adenoviruses. Curr Top Microbiol Immunol. 1984;110:191–220.
4. Rosen L. A hemagglutination-inhibition technique for typing adenoviruses. Am J Hyg. 1960;71:120–8.
5. Hierholzer JC. Further subgrouping of the human adenoviruses by differential hemagglutination. J Infect Dis. 1973;128:541–50.
6. Schmitz H, Wigand R, Heinrich W. Worldwide epidemiology of human adenovirus infections. Am J Epidemiol. 1983;117:455–66.
7. Brandt CD, Kim HW, Vargosko AJ, Jeffries BC, Arrobio JO, Rindge B, et al. Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. Am J Epidemiol. 1969;90:484–500.
8. Fox JP, Hall CE, Cooney MK. The Seattle Virus Watch. VII. Observations of adenovirus infections. Am J Epidemiol. 1977;105:362–86.
9. Takafuji ET, Gaydos JC, Allen RG, Top FH Jr. Simultaneous administration of live, enteric-coated adenovirus types 4, 7 and 21 vaccines: safety and immunogenicity. J Infect Dis. 1979;140:48–53.
10. Van der veen J. The role of adenoviruses in respiratory disease. Am Rev Respir Dis. 1963;88:167–80.
11. Gooch WM III, Mogabgab WJ. Simultaneous oral administration of live adenovirus types 4 and 7 vaccines: protection and lack of emergence of other types. Arch Environ Health. 1972;25:388–94.
12. Griffin JP, Greenberg BH. Live and inactivated adenovirus vaccines: clinical evaluation of efficacy in prevention of acute respiratory disease. Arch Intern Med. 1970;125:981–6.
13. Top FH Jr, Dudding BA, Russell PK, Buescher EL. Control of respiratory disease in recruits with types 4 and 7 adenovirus vaccines. Am J Epidemiol. 1971;94:142–6.
14. Gray GC, Goswami PR, Malasig MD, Hawksworth AW, Trump DH, Ryan MA, et al. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. For the Adenovirus Surveillance Group. Clin Infect Dis. 2000;31:663–70.
15. Gaydos CA, Gaydos JC. Adenovirus vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. Fourth ed. Philadelphia: W.B. Saunders; 2003, p. 863–85.
16. Blasiole DA, Metzgar D, Daum LT, Ryan MA, Wu J, Wills C, et al. Molecular analysis of adenovirus isolates from vaccinated and unvaccinated young adults. J Clin Microbiol. 2004;42:1686–93.
17. Kajon AE, Wadell G. Sequence analysis of the E3 region and fiber gene of human adenovirus genome type 7h. Virology. 1996;215:190–6.

[2]The members of the Epidemic Outbreak Surveillance Consortium are Peter F. Demitry, Theresa Lynn Difato, Robb K. Rowley, Clark Tibbetts, Eric H. Hanson, Rosana R. Holliday, Curtis White, David A. Stenger, Donald Seto, Elizabeth A. Walter, Jerry Diao, Brian K. Agan, Kevin Russell, David Metzgar, Gary J.Vora, Baochuan Lin, Dzung Thach, Jing Su, Chris Olsen, Dong Xia, John Gomez, John McGraw, Linda Canas, Margaret Jesse, Mi Ha Yuen, Robert Crawford, Sue A. Worthy, Sue Ditty, John McGraw, Michael Jenkins, Zheng Wang, Cheryl J. James, Kathy Ward, Kenya Grant, and Kindra Nix.

18. Lin B, Vora GJ, Thach D, Walter E, Metzgar D, Tibbetts C, et al. Use of oligonucleotide microarrays for rapid detection and serotyping of acute respiratory disease-associated adenoviruses. J Clin Microbiol. 2004;42:3232–9.
19. Vabret A, Gouarin S, Joannes M, Barranger C, Petitjean J, Corbet S, et al. Development of a PCR-and hybridization-based assay (PCR adenovirus consensus) for the detection and the species identification of adenoviruses in respiratory specimens. J Clin Virol. 2004;31:116–22.
20. Xu W, McDonough MC, Erdman DD. Species-specific identification of human adenoviruses by a multiplex PCR assay. J Clin Microbiol. 2000;38:4114–20.
21. Crawford-Miksza LK, Nang RN, Schnurr DP. Strain variation in adenovirus serotypes 4 and 7a causing acute respiratory disease. J Clin Microbiol. 1999;37:1107–12.
22. Malasig MD, Goswami PR, Crawford-Miksza LK, Schnurr DP, Gray GC. Simplified microneutralization test for serotyping adenovirus isolates. J Clin Microbiol. 2001;39:2984–6.
23. Xu W, Erdman DD. Type-specific identification of human adenovirus 3, 7, and 21 by a multiplex PCR assay. J Med Virol. 2001;64:537–42.
24. Pring-Akerblom P, Trijssenaar FE, Adrian T, Hoyer H. Multiplex polymerase chain reaction for subgenus-specific detection of human adenoviruses in clinical samples. J Med Virol. 1999;58:87–92.
25. Mei YF, Wadell G. Hemagglutination properties and nucleotide sequence analysis of the fiber gene of adenovirus genome types 11p and 11a. Virology. 1993;194:453–62.
26. Basler CF, Horwitz MS. Subgroup B adenovirus type 35 early region 3 mRNAs differ from those of the subgroup C adenoviruses. Virology. 1996;215:165–77.
27. Fife KH, Ashley R, Shields AF, Salter D, Meyers JD, Corey L. Comparison of neutralization and DNA restriction enzyme methods for typing clinical isolates of human adenovirus. J Clin Microbiol. 1985;22:95–100.
28. Adhikary AK, Inada T, Banik U, Numaga J, Okabe N. Identification of subgenus C adenoviruses by fiber-based multiplex PCR. J Clin Microbiol. 2004;42:670–3.
29. Hierholzer JC. Adenoviruses in the immunocompromised host. Clin Microbiol Rev. 1992;5:262–74.
30. Meinschad C, Winnacker EL. Recombination in adenovirus. I. Analysis of recombinant viruses under non-selective conditions. J Gen Virol. 1980;48:219–24.
31. Boursnell ME, Mautner V. Recombination in adenovirus: crossover sites in intertypic recombinants are located in regions of homology. Virology. 1981;112:198–209.
32. Sambrook J, Williams J, Sharp PA, Grodzicker T. Physical mapping of temperature-sensitive mutations of adenoviruses. J Mol Biol. 1975;97:369–90.
33. de Jong PJ, Valderrama G, Spigland I, Horwitz MS. Adenovirus isolates from urine of patients with acquired immunodeficiency syndrome. Lancet. 1983;1:1293–6.
34. Kojaoghlanian T, Flomenberg P, Horwitz MS. The impact of adenovirus infection on the immunocompromised host. Rev Med Virol. 2003;13:155–71.
35. Davison AJ, Benko M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol. 2003;84:2895–908.
36. Stone D, Furthmann A, Sandig V, Lieber A. The complete nucleotide sequence, genome organization, and origin of human adenovirus type 11. Virology. 2003;309:152–65.
37. Houng HH, Clavio S, Graham K, Kuschner R, Sun W, Russell KL, et al. Emergence of a new human adenovirus type 4 (Ad4) genotype: identification of a novel inverted terminal repeated (ITR) sequence from a majority of Ad4 isolates from US military recruits. J Clin Virol. 2006;35:381–7.
38. Erdman DD, Xu W, Gerber SI, Gray GC, Schnurr D, Kajon AE, et al. Molecular epidemiology of adenovirus type 7 in the United States, 1966–2000. Emerg Infect Dis. 2002;8:269–77.
39. Li Q, Wadell G. Genetic variability of hexon loops 1 and 2 between seven genome types of adenovirus serotype 7. Arch Virol. 1999;144:1739–49.
40. Sugarman BJ, Hutchins BM, McAllister DL, Lu F, Thomas BK. The complete nucleic acid sequence of the adenovirus type 5 reference material (ARM) genome. Bioprocessing Journal. 2003;2:27–32.
41. Mitchell S, O'Neill HJ, Ong GM, Christie S, Duprex P, Wyatt DE, et al. Clinical assessment of a generic DNA amplification assay for the identification of respiratory adenovirus infections. J Clin Virol. 2003;26:331–8.

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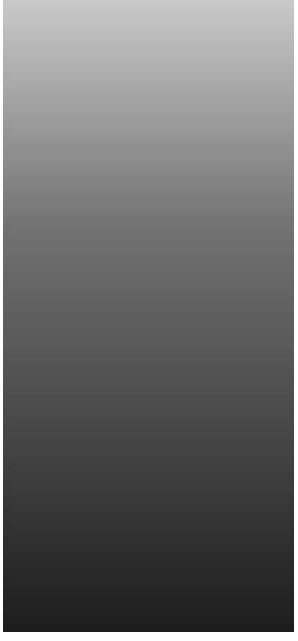

Norwalk Virus–specific Binding to Oyster Digestive Tissues

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The primary pathogens related to shellfishborne gastroenteritis outbreaks are noroviruses. These viruses show persistence in oysters, which suggests an active mechanism of virus concentration. We investigated whether Norwalk virus or viruslike particles bind specifically to oyster tissues after bioaccumulation or addition to tissue sections. Since noroviruses attach to carbohydrates of the histo-blood group family, tests using immunohistochemical analysis were performed to evaluate specific binding of virus or viruslike particles to oyster tissues through these ligands. Viral particles bind specifically to digestive ducts (midgut, main and secondary ducts, and tubules) by carbohydrate structures with a terminal N-acetylgalactosamine residue in an α linkage (same binding site used for recognition of human histo-blood group antigens). These data show that the oyster can selectively concentrate a human pathogen and that conventional depuration will not eliminate noroviruses from oyster tissue.

Twelve years ago, the question, "Should shellfish be purified before public consumption?" was asked in Lancet (*1*). Since then, new evidence of gastroenteritis outbreaks linked to shellfish consumption, even depurated shellfish, has been published, and raw or cooked oysters are the predominant bivalve mollusks involved (*2–5*). Regulations for *Escherichia coli* counts in shellfish-growing waters (United States) or shellfish meat (European Community) have failed to protect consumers because most shellfish-associated gastroenteritis outbreaks have a viral origin (*4*). Enteric viruses are different from enteric bacteria in terms of resistance to sewage treatment, persistence under unfavorable conditions such as occur in sea water, and transmission into the environment (*6–8*). Shellfish mollusks cultivated in coastal areas close to human activities can be contaminated by human sewage, which can spread >100 types of viruses (*9*). Viruses persist in shellfish for an extended period and can adversely affect public health; despite improvements, depuration does not eliminate viral particles (*2,10–14*).

Noroviruses are the most frequent cause of diarrhea outbreaks in all age groups (*8,15*). These viruses, which are commonly associated with foodborne and waterborne outbreaks, are resistant to sewage treatment and are present in high concentrations during the epidemic season (*3,7,15*). They are the primary pathogens associated with shellfishborne outbreaks worldwide (*3,4*). Oysters are rapidly contaminated, as shown by outbreaks linked to accidental input, and viruses then persist for up to several weeks (*2,13,16*). These data suggest that contamination by a passive process during mollusk filter feeding may be too simplistic an explanation. We tested whether oysters can actively capture noroviruses and determined the fate of prototype genogroup I Norwalk virus particles in bioaccumulation experiments. Since noroviruses can attach to carbohydrates of the ABH and Lewis histo-blood group family in humans, we also examined the possibility of a similar specific binding to oyster tissues through related carbohydrates (*17,18*).

Materials and Methods

Norwalk Virus Strain and Recombinant Viruslike Particles

Norwalk virus strain 8FIIa was purified from a stool sample of an infected volunteer and assayed by reverse transcription–polymerase chain reaction (RT-PCR) endpoint dilution (RT-PCR units) to determine the amount of viral RNA in the sample (*19*). Based on the equivalence of 1 RT-PCR unit to 30–40 genomic copies of RNA, the titer was estimated to be $\approx 3 \times 10^{10}$ particles/mL (*2*).

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Constructs containing open reading frames 2 and 3 were used to produce recombinant viruslike particles (VLPs) as described previously (*20*). Virus concentration, as calculated with the detergent compatible protein assay (Bio-Rad Laboratories, Hercules, CA, USA), was 2.11×10^{15} particles/mL. The number of particles in 1 μg of VLPs was calculated by multiplying the molecular mass of the major capsid protein VP1 (58 kDa) by the number of copies of the protein in a particle (180) and by a mass of 1 Da (1.66×10^{-24} g) (*21*).

Mutants in the P2 subdomain of VP1 were generated by Ala point substitution and used to produce recombinant VLPs His 329 (H329A), Asn 331 (N331A), and Trp 375 (W375A) (*22*). Concentrations of these 3 mutant VLPs were adjusted to 5.78×10^{14} particles/mL.

Oysters

All experiments were performed with *Crassostrea gigas* from a clean area (Class A, European Regulations), with no viral contamination, as tested after viral concentration, RNA extraction, and RT-PCR (*2*). Two batches of oysters collected in March and October 2004 were used to prepare tissue sections.

Bioaccumulation of Norwalk Virus and Recombinant VLPs

Norwalk virus (5×10^8 particles) or recombinant VLPs (10^{12} or 10^9 particles) were added to 500 mL clean sea water and homogenized for 5 min. Three oysters were added to the sea water and incubated at room temperature for 12 or 24 h. Sea water was continuously aerated to maintain adequate oxygen levels. A negative control was used under similar conditions but without the addition of Norwalk virus or recombinant VLPs.

Immunohistochemical Analysis

Oysters (uncontaminated or after bioaccumulation) were shucked, and the flesh was fixed in 10% formaldehyde for 48 hours. The digestive gland was then dissected, embedded in paraffin, and sliced into thin sections (5 μm). After preparation of tissue sections, sections from uncontaminated oysters were covered with 4×10^9 particles of recombinant wild-type VLPs, 3×10^9 particles of Norwalk virus, or 2×10^8 particles of mutant VLPs, incubated overnight at 4°C, and washed 3 times (5 min per wash) in phosphate-buffered saline (PBS) at room temperature (*23*). VLPs bound to oyster tissue or virus trapped after the bioaccumulation experiments was detected by using a rabbit polyclonal antibody to recombinant VLP as previously described (*23*). Negative controls included sections from uncontaminated samples not exposed to Norwalk virus or recombinant VLPs and virus-exposed sections without antibody. Immunoreactivity, which was detected by microscopic analysis, was graded as strong (intense brown-red staining), weak (pale brown-red staining), or negative (no staining).

Inhibition of Recombinant VLP Binding to Tissue Sections

Treatment with periodate was performed as previously described (*23*). VLPs were incubated overnight on slides, and the immunohistochemical detection was conducted. Saliva samples from 18 persons with secretor type O, 14 with secretor type A, 4 with secretor type B, and 5 nonsecretors were tested. Phenotyping was performed by enzyme-linked immunosorbent assay, and secretor phenotype was confirmed by genotyping as previously described (*17*). For the inhibition assay, VLPs were mixed with saliva samples diluted 1:100 for 90 min at room temperature, placed on shellfish tissues, and incubated overnight at 4°C. Positive (without treatment) and negative (without recombinant VLPs) samples were included in this assay.

Binding and Inhibition by Specific Lectins and Carbohydrate-specific Antibodies

Three biotinylated lectins, derived from *Helix pomatia* (HPA), *Dolichos biflorus* (DBA), and *Ulex europaeus* (UEA-1) (Biovalley SA, Marne la Vallée, France), were used for analysis of binding to tissues and inhibition of VLP binding. UEA-1 recognizes the H type 2 trisaccharide and shows some cross-reactivity with Le^y. HPA and DBA recognize α-linked N-acetylgalactosamine residues. For the binding assay, lectins were applied to shellfish tissues for 30 min at different concentrations (50, 20, 10, 5, and 1 μg/mL).

Monoclonal antibodies were used for tissue staining. The antibodies used were anti-A (3-3A), which recognizes all A epitopes; anti-A types 3/4 (III-2A3, III-2A18, and III-2A24); anti-A type 2 (III-2A5); anti-H/Le^b (LM-137); anti-H type 2 (19-OLE); anti-Le^y (12-4LE); and anti-H type 1. The primary antibodies were serially diluted, incubated with tissues overnight at 4°C, and subjected to standard immunohistochemical analysis (*23*). Negative (without lectin or primary antibody) and positive (known rat or human tissue sections) controls were also tested. For inhibition assays, antibodies or lectins were applied to shellfish tissues for 1 h at 37°C or 1 h at 4°C, respectively, at concentrations 10-fold higher than the lowest dilution that showed binding to the oyster tissues, incubated with VLPs, and analyzed as described above.

Results

Binding of Virus in Oysters after Bioaccumulation

Oysters were immersed in sea water seeded with Norwalk virus at a final concentration of 10^6 particles/mL

or recombinant VLPs at final concentrations of 2×10^9 and 2×10^6 particles/mL. After incubation for 12 h, virus was detected in digestive diverticula. Immunostaining showed particles in the lumen of tubules and ducts or in phagocytes between epithelial cells and in the surrounding connective tissue (Figure, panels A and B). No difference was observed for the 2 recombinant VLP concentrations used. After incubation for 24 h in sea water at final concentrations of 5×10^8 and 5×10^9 particles/mL for Norwalk virus and recombinant VLPs, respectively, similar results were obtained with particles binding to digestive ducts and isolated cells in connective tissue (data not shown).

Binding of Virus in Oyster Digestive Tissues

The binding of virus or recombinant VLPs was tested directly on oyster gut sections. Both recombinant VLPs and native virions bound to the midgut, the main and secondary ducts of the digestive diverticula, and tubules, although labeling of tubules was weaker (Figure, panels C, D, and E). Binding to connective tissue was not observed. Staining was not observed in negative controls.

Recombinant VLPs can attach to human digestive epithelial cells by recognizing carbohydrate structures. To determine if attachment to oyster digestive cells was also carbohydrate dependent, tissues sections were treated with 10 mmol/L sodium periodate before adding the VLPs. A reduction in VLP binding occurred, which suggested that, similar to the case with human tissue, binding to oyster tissue involves carbohydrates (Figure, panels E and F).

Inhibition of Virus Binding to Oyster Digestive Cells by Human Saliva

Since attachment of recombinant VLPs to human tissue sections involves carbohydrates of the ABH, secretor, and Lewis histo-blood group family, which are present on human salivary mucins, we evaluated the ability of saliva of different ABO and secretor phenotypes to block the binding of VLPs to shellfish tissues. Complete inhibition of binding was observed after pretreatment of the recombinant VLPs with type A secretor saliva, and binding was markedly reduced after incubation with type O secretor saliva. However, after incubation with type B secretor saliva or nonsecretor saliva, binding was as strong as that observed in positive controls (Table 1). All samples in subgroups of saliva showed similar patterns.

Binding of Mutant Recombinant VLPs

Our results suggested that attachment of VLPs to oyster tissue involves carbohydrate binding sites that overlap the site that attaches to human digestive cells. This site has been mapped to a restricted area of the viral capsid P2 domain. Mutations in key residues in this domain inactivate the binding activity to histo-blood group structures. To confirm that the binding to oyster tissue involved the same binding site, VLPs with point mutations were tested. Among 3 mutants, only mutant N331A binds to tissues similar to parental recombinant VLP (Figure, panel G). The 2 other mutants, H329A and W375A, did not bind to tissues (Figure, panel H).

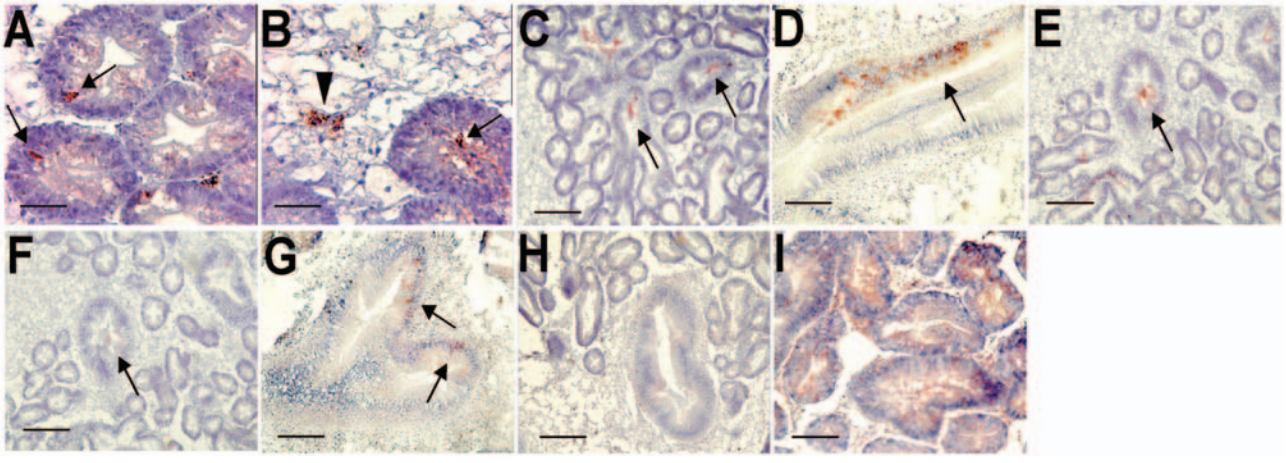

Figure. Immunohistochemical detection of Norwalk viruslike particles (VLPs) in oyster digestive tissue. A) VLPs in the digestive diverticulum 12 h after seeding sea water with 10^9 particles. The arrows show immunoreactivity detected in intraepithelial cells. B) VLPs in the digestive diverticulum 12 h after seeding sea water with 10^{12} particles. The arrowhead shows immunoreactivity in a phagocyte located in connective tissue, and the arrow shows immunoreactivity in the lumen of a digestive tubule. C) Attachment of recombinant VLPs to secondary ducts of the digestive diverticula after incubation on tissue sections. The arrows show immunoreactivity in the lumen of ducts. D) Attachment of Norwalk virus to a main digestive duct. The arrow shows immunoreactivity in epithelial duct cells. E and F) Attachment (arrows) of recombinant VLPs to digestive ducts without (E) or with (F) periodate treatment of serial tissue sections. G) Binding of VLPs (arrows) to a main digestive duct of VLPs from the H331A mutant capsid protein. H) Lack of binding of the H329A mutant. I) Binding of HPA lectin to the digestive diverticula. Scale bars: A and B, 10 μm; C, E, F, and H, 40 μm; D, G, and I, 20 μm.

Table 1. Inhibition of binding of Norwalk viruslike particles (VLPs) to oyster digestive tissues by saliva

Saliva	No. samples tested	VLP binding*
A	14	–
O	18	+
B	4	+++
Nonsecretor	5	+++
Control	5	+++

*After incubation with saliva, no (–), weak (+), or strong (+++) VLP binding to oyster digestive tissues was observed. For controls, VLPs were applied to shellfish tissues in phosphate-buffered saline/1% bovine serum albumin instead of a saliva sample diluted in the same buffer.

Inhibition of VLP Binding by Antibodies to Carbohydrate and Lectins

Antibodies to carbohydrates on cells in the human digestive tract can inhibit binding of recombinant VLPs to human tissue (*18*). Primary antibodies that recognize various determinants of the histo-blood group family were evaluated for binding to oyster digestive tract and inhibition of VLP binding. Antibodies that recognize all types of A determinants (3-3A) or those that are restricted to A type 3/4 determinants bound strongly to shellfish tissues (Table 2). Anti-H type 1 BG-4 labeled oyster digestive cells intracellularly and also labeled connective tissue. Antibody LM137, which recognizes all H determinants and Le^b tetrasaccharide, also labeled connective tissue and digestive epithelial cells intracellularly. However, staining had a more punctiform appearance. Despite recognition of oyster ligands, in inhibition assays none of these antibodies inhibited binding of VLPs to oyster tissues. Antibodies directed against H type 2 (19-OLE,), Le^y (12-4LE), or A type 2 epitopes (III-2A-5) showed little staining of shellfish tissues and when positive, staining did not correspond to cells to which VLPs bind. These results indicate that oyster tissues have carbohydrates that resemble human histo-blood group antigens, but structures recognized by VLPs were not identical to those recognized by these antibodies.

Lectins have been used to determine the distribution of carbohydrate residues in tissue. Since lectins have a broader reactivity than monoclonal antibodies, we used lectins to identify recombinant VLP-specific carbohydrate ligand in oyster tissue. UEA-1 bound to shellfish tissues, but only at high concentration (50 μg/mL), whereas 2 other lectins, DBA and HPA, bound at a lower concentration (1 μg/mL) (Table 3). Tissue and cellular distribution of staining overlapped with that of the recombinant VLPs (Figure, panel I). When these lectins were used in an inhibition assay, only HPA had an inhibitory effect on binding of recombinant VLPs to shellfish tissues. Complete inhibition was observed at a concentration of 25 μg/mL (Table 3). This inhibitory effect was reproducible and observed on tissues from different shellfish, indicating that attachment of recombinant VLPs to oysters involves carbohydrate structures with a terminal N-acetylgalactosamine residue in an α linkage.

Discussion

Virus-mediated disease can be transmitted when contaminated shellfish are eaten. Oysters are believed to act as filters or ionic traps, passively concentrating particles. A simple depuration process should be sufficient to rid oysters of virus, as observed for bacteria (*12*). However, long-term virus persistence in shellfish is a serious public health issue, and depuration or relaying is known to be inefficient (*10,12,14*). After bioaccumulation, only 7% of Norwalk virus is depurated, compared to a 95% reduction in bacterial levels (*13*). Virus is located mainly in pancreatic tissue (digestive diverticula), and various mechanisms have been suggested to explain differences between oyster species regarding virus accumulation, such as mechanical entrapment or ionic bonding (*10,13,24,25*).

Our data demonstrate specific binding of viral particles from a genogroup I norovirus to the oyster digestive tract and suggest a specific mechanism for concentration of virus particles. We tested recombinant VLPs of prototype genogroup I Norwalk virus. VLPs are stable in the marine environment and the disinfection processes (*14,26*). Bioaccumulation and tissue-binding experiments showed no difference between native Norwalk virus and VLPs, which confirmed that VLPs are good surrogates of infectious virions.

Different results were seen between bioaccumulation experiments and particle binding to tissues sections. After bioaccumulation, some viral particles were detected in phagocytes in either epithelium or connective tissue. This finding could reflect elimination of virus during digestion. The time required for food to pass through the entire shellfish intestinal tract varies from 90 to 150 min. We do not know whether immunoreactive material detected in phagocytes corresponds to particle degradation and digestion or if particles can escape digestion. Binding to main ducts may provide a mechanism for viral particles to avoid entering the digestive system and being degraded. Specific attachment of virus to oyster cells and capture by

Table 2. Binding of carbohydrate antibodies to oyster digestive tissues

Antibody	Binding*	Tissue
Anti-A all types	+++	Digestive cells
Anti-A types 2	+	
Anti-A type 3/4	+++	Digestive cells
Anti-H type 1	+++	Digestive cells (intracellularly) and connective tissue
Anti-H type 2	+	
Anti-H/Le^b	+++	Digestive cells (intracellularly) and connective tissue
Anti-Le^y	+	

*+++, strong; +, weak.

Table 3. Lectin binding to oyster digestive tissues and inhibitory effect on binding of Norwalk viruslike particles (VLPs)

Lectin*	Binding (μg/mL)†	VLP inhibition (μg/mL)‡
DBA	1	None
UEA-1	50	None
HPA	1	25

*Derived from *Dolichos biflorus* (DBA), *Ulex europaeus* (UEA-1), and *Helix pomatia* (HPA).
†Lowest concentration showing binding to digestive tissues.
‡Lowest dilution showing a complete inhibition of VLP binding to digestive tissues. None indicates no inhibition at 50 μg/mL.

phagocytes may explain why depuration in oysters is not an effective mechanism for eliminating virus.

Virus accumulation in oysters may depend on factors such as water temperature, mucus production, glycogen content of connective tissue, or gonadal development (*25*). In our study, no difference was seen in binding location between samples collected in March or October, although studies during other seasons are warranted.

Human susceptibility factors for norovirus infections that depend upon carbohydrates of the ABH, secretor, and Lewis histo-blood group family have been observed (*17*,*27*). We showed that recognition of oyster digestive epithelial cells by recombinant VLPs also involves carbohydrates. The ability of human saliva to inhibit attachment of VLPs to oyster tissue in a histo-blood group–dependent manner indicates involvement of the histo-blood group binding site and the viral P2 subdomain (*18*).

Similar to human histo-blood group structures, mutant VLPs showed that an alanine substitution at positions H329A and W375A prevented binding to oyster tissue. However, a mutant with a substitution at position N331A did not affect binding. This binding specificity identified the amino acids required for binding and further confirmed the similarity with the mechanism of recognition of human tissues (*22*). However, inhibition experiments using antibodies to carbohydrates showed that ligands on oyster tissues are not identical to those on human tissues. Since attachment of recombinant VLPs was blocked by lectin from *H. pomatia*, which recognizes α-linked N-acetylgalactosamine terminal residues of glycans, the oyster ligands are similar to those of histo-blood group A. The DBA lectin did not inhibit attachment of recombinant VLPs, which indicates that it does not bind oligosaccharide structures recognized by VLPs and HPA. Although DBA binds to N-acetylgalactosamine residues similar to HPA, the 2 lectins have different carbohydrate specificities that depend on the subjacent sugar residues (*28*). Thus, Norwalk virus binds to oyster tissues through an A-like carbohydrate structure recognized by HPA at a binding site also used for attachment to carbohydrate on human epithelial cells.

Genogroup I and II strains of norovirus show various binding patterns with different carbohydrate structures of the histo-blood group family, which suggests coevolution of this group of viruses and their host or carrier vector. The ability of Norwalk virus to bind to oysters tissues at the same binding site as that used to bind to human tissues suggests a possible coevolution mechanism involving the oyster as an intermediary vector. This mechanism would favor selection of some viruses, such as Norwalk virus, over other viruses that are unable to bind to the oyster and would not be transmittable by this intermediary host. Epidemiologic data suggest a predominance of genogroup I strains in oyster-related gastroenteritis outbreaks, whereas genogroup II strains are predominant in other food-related outbreaks (*2*,*3*,*5*,*8*,*15*,*16*). To clearly address this point, binding of other norovirus strains needs to be evaluated because VLP-carbohydrate binding patterns differ, and binding should also be evaluated with other shellfish species (*18*,*27*).

As knowledge increases in understanding the binding of these viruses in humans, we will likely further understand more about their behavior in shellfish. This knowledge may help identify species that could be less sensitive to contamination. Since the oyster can actively and specifically bind a human pathogen, this knowledge has practical consequences because conventional depuration cannot eliminate noroviruses from oyster tissues.

Acknowledgments

We thank Jezabel Rocher for technical assistance and R. Fraser, J. Bara, and Signet Pathology Systems for providing monoclonal antibodies.

This work was supported in part by the European Commission, QLRT-1999-0634, and the Institut National de la Santé et de la Recherche Médicale.

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References

1. Bosch A, Xavier Abad F, Gajardo R, Pinto RM. Should shellfish be purified before public consumption? Lancet. 1994;344:8928.
2. Le Guyader FS, Neil FH, Dubois E, Bon F, Loisy F, Kohli E, et al. A semi-quantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. Int J Food Microbiol. 2003;87:107–12.
3. 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.
4. Butt AA, Aldridge KE, Sanders CV. Infections related to the ingestion of seafood. Part I: viral and bacterial infections. Lancet Infect Dis. 2004;4:201–12.

5. Gallimore CL, Cheesbrough JS, Lamden K, Bingham C, Gray JJ. Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. Int J Food Microbiol. 2005;103:323–30.
6. Griffin DW, Donaldson KA, Paul JH, Rose JB. Pathogenic human viruses in coastal waters. Clin Microbiol Rev. 2003;16:129–43.
7. Duizer E, Bijkerk P, Rockx B, de Groot A, Twisk F, Koopmans M. Inactivation of caliciviruses. Appl Environ Microbiol. 2004;70:4538–43.
8. Lopman BA, Vennema H, Kohli E, Pothier P, Sanchez A, Negredo A, et al. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet. 2004;363:682–8.
9. Metcalf TG, Melnick JL, Estes MK. Environmental microbiology: from detection of virus in sewage and water by isolation to identification by molecular biology—a trip of over 50 years. Annu Rev Microbiol. 1995;49:461–87.
10. Metcalf TG. Viruses in shellfish growing waters. Environment International. 1982;7:21–7.
11. Heller D, Gill ON, Raynham E, Kirkland T, Zadick PM, Stanwell-Smith R. An outbreak of gastrointestinal illness associated with consumption of raw depurated oysters. BMJ. 1986;292:1726–7.
12. Richards GP. Microbial purification of shellfish: a review of depuration and relaying. J Food Prot. 1988;51:218–51.
13. Schwab KJ, Neill FH, Estes MK, Metcalf TG, Atmar RL. Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. J Food Prot. 1998;61:1674–80.
14. Loisy F, Atmar RL, Le Saux J-C, Cohen J, Caprais M-P, Pommepuy M, et al. Rotavirus virus like particles as surrogates to evaluate virus persistence in shellfish. Appl Environ Microbiol. 2005;71:6049–53.
15. Widdowson MA, Sulka A, Bulens SN, Beard RS, Chaves SS, Hammond R, et al. Norovirus and foodborne disease, United States, 1991–2000. Emerg Infect Dis. 2005;11:95–102.
16. Le Guyader FS, Neill FH, Estes MK, Monroe SS, Ando T, Atmar RL. Detection and analysis of a small round structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. Appl Environ Microbiol. 1996:62:4268–72.
17. Lindesmith L, Moe C, Marionneau S, Ruvoen N, Jiang X, Lindblad L, et al. Human susceptibility and resistance to Norwalk virus infection. Nat Med. 2003;9:548–53.
18. Tan M, Jiang X. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. Trends Microbiol. 2005;13:285–93.
19. Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. Norwalk virus infection of volunteers: new insights based on improved assays. J Infect Dis. 1994;170:34–43.
20. Bertolotti-Ciarlet A, Crawford SE, Hutson AM, Estes MK. The 3′ end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. J Virol. 2003;77:11603–15.
21. White LJ, Ball JM, Hardy ME, Tanaka TN, Kitamoto N, Estes MK. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. J Virol. 1996;70:6589–97.
22. Hutson AM, Chakravarty S, Atmar RL, Prasad BVV, Estes MK. Identifying the binding site on Norwalk virus for cell-surface carbohydrates. In: Abstracts of Digestive Disease Week and the 106th Annual Meeting of the American Gastroenterological Association; Chicago, IL; 2005 May 14–19; Abstract 393. Gastroenterology. 2005;128(Suppl 2):A-56.
23. Marionneau S, Ruvoen N, Le Moullac-Vaidye B, Clement M, Cailleau-Thomas A, Ruiz-Palacios G, et al. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology. 2002;122:1967–77.
24. Di Girolamo R, Liston J, Matches J. Ionic binding, the mechanism of viral uptake by shellfish mucus. Appl Environ Microbiol. 1977;33:19–25.
25. Burkhardt W, Calci KR. Selective accumulation may account for shellfish-associated viral illness. Appl Environ Microbiol. 2000;66:1375–78.
26. Caballero S, Abad FX, Loisy F, Le Guyader FS, Cohen R, Pinto RM, et al. Rotavirus virus-like particles as surrogates in environmental persistence and inactivation studies. Appl Environ Microbiol. 2004;70:3904–9.
27. Hutson AM, Atmar RL, Estes MK. Norovirus disease: changing epidemiology and host susceptibility factors. Trends Microbiol. 2004;12:279–87.
28. Piller V, Piller F, Cartron JP. Comparison of the carbohydrate-binding specificities of seven N-actyl-D-galactosamine-recognizing lectins. Eur J Biochem. 1990;31:461–6.

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Haemophilus influenzae Type b Reemergence after Combination Immunization

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An increase in *Haemophilus influenzae* type b (Hib) in British children has been linked to the widespread use of a diphtheria/tetanus/acellular pertussis combination vaccine (DTaP-Hib). We measured anti-polyribosyl-ribitol phosphate antibody concentration and avidity before and after a Hib booster in 176 children 2–4 years of age who had received 3 doses of DTP-Hib (either DT whole cell pertussis-Hib or DTaP-Hib) combination vaccine in infancy. We also measured pharyngeal carriage of Hib. Antibody concentrations before and avidity indices after vaccination were low (geometric mean concentration 0.46 μg/mL, 95% confidence interval [CI] 0.36–0.58; geometric mean avidity index 0.16, 95% CI 0.14–0.18) and inversely related to the number of previous doses of DTaP-Hib (p = 0.02 and p<0.001, respectively). Hib was found in 2.1% (95% CI 0.7%–6.0%) of study participants. Our data support an association between DTaP-Hib vaccine combinations and clinical Hib disease through an effect on antibody concentration and avidity.

After the introduction of conjugate *Haemophilus influenzae* type b (Hib) vaccines in October 1992, the incidence of invasive Hib disease in England and Wales dramatically declined. From 1990 to 1992, the annual incidence in children <5 years of age was 20.5–22.9 per 100,000 and by 1998 it had fallen to 0.65 per 100,000 (*1*). However, since 1999 the number of invasive Hib infections has risen, with an increase every year in the number of cases in children born from 1996 to 2001; by 2002 the disease incidence had reached 4.58 per 100,000 (*1*). This rise coincided with a temporary change in the type of Hib vaccine combinations given for primary immunization. An acellular pertussis combination vaccine (DTaP-Hib) was used from 1999 to 2002 because of a shortage of the whole cell pertussis combination (DTwP-Hib) vaccine. It also coincided with the introduction of routine immunization with meningococcal conjugate (MCC) vaccines in 1999.

Most, but not all (*2*), DTaP-Hib vaccines result in lower Hib antibody concentrations shortly after vaccination when compared with DTwP-Hib vaccines (*3*), but the persistence of this effect has not been studied and its clinical significance is controversial (*3*). However, a UK case control study has demonstrated an increased risk for invasive Hib disease in children who received ≥2 doses of DTaP-Hib, which suggests that this effect may be clinically relevant (*4*). Suppression of Hib antibody responses by other concomitantly administered vaccines has also been described. These include pneumococcal conjugate vaccines (*5*), inactivated polio vaccines (*6*), and MCC vaccines (*7*), even when given by separate injection. With respect to MCC vaccines, lower Hib responses were observed when the CRM-197 conjugate MCC vaccine was administered simultaneously as compared with the tetanus toxoid conjugate MCC vaccine (*7*).

In response to the rising incidence of Hib disease, a national booster campaign was initiated in 2003 in which an extra dose of Hib vaccine was offered for all children between 6 months and 4 years of age. We used this opportunity to assess whether the number of DTaP-Hib vaccines given during primary immunization was related to the serum concentration and avidity of Hib antibody both before and after the booster in children 2–4 years of age. We also aimed to assess the prevalence of pharyngeal Hib carriage in this age group.

Methods

The UK primary vaccine schedule consisted of 3 doses of Hib conjugate vaccine, DTP vaccine, oral polio vaccine, and MCC vaccine all administered at 2, 3, and 4 months of

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age. For their primary infant schedule, the participants in this study received either a DTwP-Hib (ACT-HIB DTP, Pasteur Merieux MSD Ltd, Maidenhead, UK) or DTaP-Hib (Infanrix-Hib, GlaxoSmithKline, Middlesex, UK). The Hib conjugate in both consisted of PRP conjugated to tetanus toxoid (PRP-T), and the acellular pertussis vaccine contained 3 pertussis components. The participants in the study may also have received 1 of 2 different meningococcal conjugate vaccines, a CRM197-based conjugate or a tetanus toxoid–based conjugate.

The names of children 2–4 years of age with full Hib vaccination (3 doses) were obtained from district computerized immunization records. After written informed parental consent was obtained, pharyngeal swabs and blood samples were obtained from the children, and doses of Hib conjugate vaccine were administered. Four to 6 weeks later, a second blood sample was obtained from each child. Dates and types of previous vaccinations were obtained from immunization records and the children's handheld record. We were particularly interested in the types of DTP-Hib and MCC vaccines the children had received. The study was approved by the Wandsworth Local Research ethics committee (reference 00.6.14).

We used an enzyme-linked immunosorbent assay (ELISA) method to measure immunoglobulin G antibodies to PRP. The minimum level of detection of the assay was 0.11 μg/mL, and values <0.11 μg/mL were recorded as 0.05 μg/mL for summary calculations. Anti-PRP concentrations were log transformed, and the geometric mean concentration (GMC) and 95% confidence intervals (CIs) were calculated. Avidity was determined by using a thiocyanate elution ELISA (*8*) and calculating the log-transformed data as the geometric mean avidity index. Throat swab specimens were obtained with a cotton-tipped swab, placed in transport media, and cultured on anti-serum agar. Strains identified as *H. influenzae* were analyzed by conventional slide agglutination and polymerase chain reaction (*9*).

We compared proportions by using Fisher exact test and GMCs between groups by using Mann-Whitney or Kruskal-Wallis tests. Linear regression was used to explore the effects of several variables on anti-PRP antibody concentration. The variables included in the model were age, number of DTaP-Hib vaccines, and type of MCC vaccines received, and for the post-booster anti-PRP concentration, the time between vaccination and blood sampling. For statistical analysis, we used SPSS version 12.0 (SPSS, Chicago, IL, USA) for Windows (Microsoft Corp., Redmond, WA, USA).

Results

We recruited 195 study participants from April 2003 to January 2004. Throat swabs were taken from 143 participants, and blood samples were taken from 176 participants. Their median age was 37.8 months (range 24–50 months), and 92% had received their third primary vaccine dose by 7 months of age.

We identified the type of DTP-Hib combination vaccine used in all 3 primary vaccinations in 163 (92.6%) participants and the number of doses and type of MCC vaccine in 159 (90.3%) participants. Participants were assigned to 1 of 4 groups based on the number of acellular pertussis vaccines (DTaP-Hib) received (0, 1, 2, or 3 doses). In 10 additional participants, 2 of the 3 vaccine combinations they had received were classified. Type of combination vaccine received varied according to participant age with younger children more likely to have received an acellular pertussis combination. The median age of participants who received 2 or 3 doses of DTaP-Hib was 32.4 months versus 40.9 months for those who received 2 or 3 doses of DTwP-Hib ($p = 0.001$).

Prebooster Anti-PRP Antibody Concentrations

The prebooster GMC for all participants was 0.46 μg/mL (95% CI 0.36–0.58) with 40 of 175 (22.9%) <0.15 μg/mL and 128 (73.1%) <1.0 μg/mL. For those who received all 3 doses as DTwP-Hib, the GMC was 0.61 μg/mL (95% CI 0.41–0.92); for those who received all 3 doses as DTaP-Hib, the GMC was 0.30 μg/mL (0.19–0.49). There was a significant trend in prebooster GMC according to the number of doses of acellular pertussis vaccine received, with increasing doses of DTaP-Hib associated with decreasing anti-PRP antibody concentrations ($p = 0.02$) (Figure 1). Receiving 2 or 3 doses of DTaP-Hib was associated with a higher proportion of children with a nonprotective concentration (<0.15 μg/mL) than receiving 2 or 3 doses of DTwP-Hib (36% vs. 14%, $p = 0.002$) (Figure 2). On linear regression analysis, only the type of DTP-Hib vaccine combination received ($p = 0.034$) was associated with prebooster antibody concentration.

With regard to MCC vaccines, 91% of children received the CRM197 containing conjugate vaccines, and 9% received the tetanus toxoid conjugate vaccine. No significant differences were found between anti-PRP antibody concentrations achieved according to type of MCC vaccine received (data not shown).

Postbooster Anti-PRP Antibody Concentrations

The postbooster GMC was 156.1 μg/mL (95% CI 133.5–182.4); 1 of 170 (0.6%) <1.0 μg/mL; 168 (99%) >10 μg/mL; median fold rise 439, range 0.9–9,200), obtained at a median of 31 days (range 26–64). The GMC was 153.1 μg/mL (113.5–207.0, n = 50) for those who had received all primary 3 doses as DTwP-Hib; 179.1 μg/mL (139.6–229.6, n = 38) for those who received 2 doses of DTwP-Hib and 1 dose of DTaP-Hib; 147.6 μg/mL (87.1–249.5, n = 27) for those who received 1 dose DTwP-

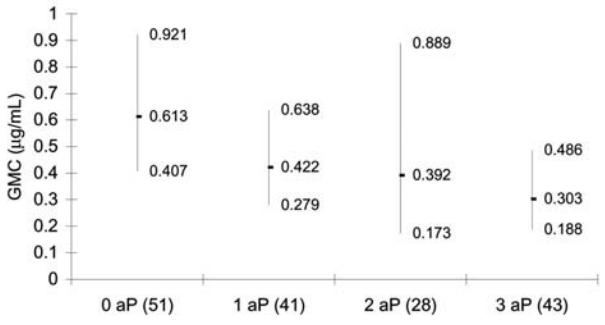

Figure 1. Geometric mean anti-polyribosyl-ribitol phosphate antibody concentration before booster (95% confidence intervals) in 2- to 4-year-old children, according to number of doses of acellular pertussis (aP) containing *Haemophilus influenzae* type b combination vaccines received in infancy. Number of participants is shown in parentheses. GMC, geometric mean concentration.

Hib and 2 doses DTaP-Hib; and 134.0 µg/mL (96.4–186.2, n = 42) for those who received all 3 doses as DTaP-Hib. None of the variables included in the model was associated with postbooster anti-PRP antibody concentration.

Anti-PRP Avidity

No significant differences in geometric mean avidity index were found before and after receiving the Hib booster vaccine (data not shown). A significant inverse trend to lower postbooster avidity levels was evident according to the number of doses of DTaP-Hib received ($p<0.001$) (Figure 3).

Pharyngeal Hib Carriage

Three of 143 participants (2.1%, 95% CI 0.7%–6.0%) were found to be carrying Hib on pharyngeal culture. One child had received all DTwP-Hib, and the other 2 had received all DTaP-Hib vaccines. The prebooster anti-PRP antibody concentrations in the 3 carriers were high: 63.9, 123.7, and 4.2 µg/mL. An additional 9 participants had prebooster anti-PRP antibody concentrations >5µg/mL (4 participants had concentrations >10 µg/mL), which suggests recent or current carriage of Hib or of a cross-reactive antigen.

Discussion

We have shown that Hib antibody concentrations in healthy UK children 2–4 years of age were low in 2003, with 23% of children unprotected based on a serologic correlate of 0.15 µg/mL and 73% of children unprotected based on a correlate of 1.0 µg/mL. This finding is consistent with national serologic data from 2000, which also showed that median anti-PRP antibody concentrations from children 2–4 years of age in 2000 were significantly lower than those from 1994 (*10*). The probable explanation is that children in this age group in 1994 had received 1 dose of Hib conjugate vaccine after 12 months of age as part of a catch-up program, while similarly aged children in 2000 and 2003 had received vaccine before 6 months of age as part of primary immunization. In the absence of vaccine boosting, anti-PRP antibody levels induced by vaccination in infancy wane over 2–3 years (*11*). However, we have shown a significant and lasting effect on anti-PRP antibody levels of the type of DTP-Hib combination vaccine used for primary vaccination. Participants who received all 3 primary doses as DTaP-Hib had antibody concentrations 2–4 years later that were approximately half those of participants who received all 3 primary doses as DTwP-Hib. This extends the findings of earlier studies in which Hib antibody concentrations were measured shortly after vaccination and found to be lower in recipients of DTaP-Hib vaccines (*3*).

Our data on avidity contrast with those of others who found that avidity appeared unaffected by receipt of acellular pertussis–containing Hib combination vaccines in the short term (to 1 year of age) (*12,13*). Our data suggest that DTaP can interfere with the normal antibody avidity maturation that occurs after priming with Hib vaccine. Since the avidity of serum antibody is likely to relate to the functional activity of the serum (*14*), the combination of decreased antibody concentration and decreased avidity suggests an increased susceptibility to Hib disease. This occurrence may provide the biological basis for the finding of an elevated risk of clinical vaccine failure in recipients of ≥2 doses of DTaP-Hib in a recent UK case control study (*4*).

Nearly all vaccinees responded well to the Hib booster dose, with 95% having >10-fold rises in antibody concentrations (median rise 439-fold) and 99% achieving concentrations >10 µg/mL. This result was independent of which combination vaccine they received in infancy. For most of

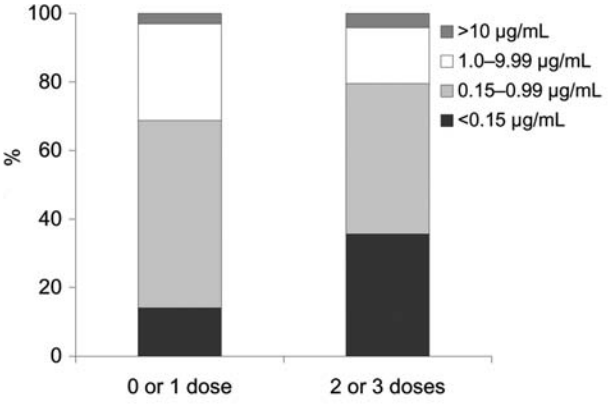

Figure 2. Anti-polyribosyl-ribitol phosphate antibody concentrations in 2- to 4-year-old children, according to number of doses of acellular pertussis containing *Haemophilus influenzae* type b combination vaccines received in infancy. Proportion achieving different concentrations is shown.

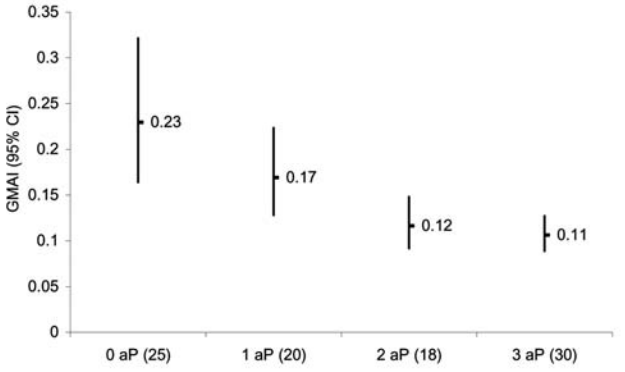

Figure 3. Geometric mean avidity index (GMAI) (95% confidence intervals [CI]) after booster in 2- to 4-year-old children, according to number of doses of acellular pertussis containing *Haemophilus influenzae* type b combination vaccines received in infancy. Number of participants is shown in parentheses.

the participants, the magnitude of their response is consistent with a memory response and implies that acellular pertussis–containing Hib combination vaccines do not impair immune memory. This finding was also demonstrated in earlier studies and formed the basis for the argument that suppression of Hib antibody concentrations by acellular pertussis–containing vaccines is not clinically relevant (*3*,*13*,*15*). Our data indicate, however, that the presence of memory, as judged by antibody response to a booster dose, is not necessarily a reliable surrogate of clinical protection. This conclusion is supported by the observation that many Hib-vaccinated children in whom clinical Hib disease later develops have a better convalescent-phase antibody response than unvaccinated children in whom Hib disease develops (*16*). In the absence of circulating antibody of sufficient quantity and quality, once Hib is encountered, the memory antibody response may be too slow to prevent invasion occurring.

The occurrence of cases of Hib disease implies ongoing transmission of Hib in the population, and our finding of a pharyngeal carriage rate of 2.1% implies that Hib was circulating in this susceptible age group. Significantly elevated antibody concentrations (>5 μg/mL) found in 9 additional participants suggests that this carriage rate will likely be a minimum estimate. Another UK study found no Hib carriage in similarly aged children in 1997 and 2002 (*17*). This difference may be explained by the population sampled or the consistent use of anti-serum agar in our study.

Reemergence of invasive Hib disease in a well-vaccinated population in Alaska in 1997 was attributed to ongoing carriage of Hib in the context of a low prevalence of Hib antibody because of a change of Hib conjugate vaccine (*18*). Similarly in the United Kingdom, the combination of low anti-PRP antibody concentration and quality, together with the presence of Hib carriage, likely explains the recent resurgence of invasive Hib disease. Low antibody concentrations in UK children 2–4 year of age is primarily due to the waning of vaccine-induced antibody after infant vaccination without a routine booster dose. This result may be a particular issue where accelerated schedules, such as the UK 2,3,4-month schedule, are employed because such schedules may be less immunogenic when compared to extended schedules (*19*). Our study indicates that this phenomenon has been compounded by the use of acellular pertussis Hib combination vaccines with lower Hib immunogenicity. As with the Alaskan experience, these results emphasize the importance of long-term surveillance of vaccine preventable diseases, particularly during changes to routine immunization schedules.

Acknowledgments

We thank John Robbins and Rachel Schneerson for providing antisera and comments on the manuscript, David Griffiths for providing antiserum agar plates, Robert Booy for providing samples from children and comments on the manuscript, and the children and parents for participating in the study.

Partial funding was received from GlaxoSmithKline. The funding body had no role in study design; collection, analysis, and interpretation of data; writing of the report; or the decision to submit the paper for publication.

P.T.H. and A.J.P. are investigators for clinical trials conducted on behalf of St. Georges, University of London, and Oxford University, respectively, sponsored by vaccine manufacturers including GlaxoSmithKline. P.T.H., A.J.P., J.U.R., and M.P.E.S. have received assistance from vaccine manufacturers to attend scientific meetings.

Dr Johnson is a consultant pediatrician with an interest in pediatric infectious diseases and immunization. He conducted this study during his pediatric training.

References

1. Ramsay ME, McVernon J, Andrews NJ, Heath PT, Slack MP. Estimating *Haemophilus influenzae* type b vaccine effectiveness in England and Wales by use of the screening method. J Infect Dis. 2003;188:481–5.
2. Mills E, Gold R, Thipphawong J, Barreto L, Guasparini R, Meekison W, et al. Safety and immunogenicity of a combined five-component pertussis-diphtheria-tetanus-inactivated poliomyelitis-*Haemophilus* b conjugate vaccine administered to infants at two, four and six months of age. Vaccine. 1998;16:576–85.
3. Eskola J, Ward J, Dagan R, Goldblatt D, Zepp F, Siegrist CA. Combined vaccination of *Haemophilus influenzae* type b conjugate and diphtheria-tetanus-pertussis containing acellular pertussis. Lancet. 1999;354:2063–8.
4. McVernon J, Andrews N, Slack MP, Ramsay ME. Risk of vaccine failure after *Haemophilus influenzae* type b (Hib) combination vaccines with acellular pertussis. Lancet. 2003;361:1521–3.

5. Buttery JP, Riddell A, McVernon J, Chantler T, Lane L, Bowen-Morris J, et al. Immunogenicity and safety of a combination pneumococcal-meningococcal vaccine in infants: a randomized controlled trial. JAMA. 2005;293:1751–8.
6. Rennels MB, Englund JA, Bernstein DI, Losonsky GA, Anderson EL, Pichichero ME, et al. Diminution of the anti-polyribosylribitol phosphate response to a combined diphtheria-tetanus-acellular pertussis/*Haemophilus influenzae* type b vaccine by concurrent inactivated poliovirus vaccination. Pediatr Infect Dis J. 2000;19:417–23.
7. Miller E, Southern J, Kitchin N, Cartwright K, Morris R, Burrage M, et al. Interaction between different meningococcal C conjugate vaccines and the Hib component of concomitantly administered diphtheria/tetanus/pertussis/Hib vaccines with either whole cell or acellular pertussis antigens. Proceedings of the 21st Annual Meeting of the European Society of Paediatric Infectious Diseases; 2003; Sicily. London: Triangle House.
8. Goldblatt D, Vaz AR, Miller E. Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. J Infect Dis. 1998;177:1112–5.
9. Heath PT, Bowen-Morris J, Griffiths D, Griffiths H, Crook DW, Moxon ER. Antibody persistence and *Haemophilus influenzae* type b carriage after infant immunisation with PRP-T. Arch Dis Child. 1997;77:488–92.
10. Trotter CL, McVernon J, Andrews NJ, Burrage M, Ramsay ME. Antibody to *Haemophilus influenzae* type b after routine and catch-up vaccination. Lancet. 2003;361:1523–4.
11. Heath PT, Booy R, Azzopardi HJ, Slack MP, Bowen-Morris J, Griffiths H, et al. Antibody concentration and clinical protection after Hib conjugate vaccination in the United Kingdom. JAMA. 2000;284:2334–40.
12. Zepp F, Schmitt HJ, Kaufhold A, Schuind A, Knuf M, Habermehl P, et al. Evidence for induction of polysaccharide specific B-cell-memory in the 1st year of life: plain *Haemophilus influenzae* type b-PRP (Hib) boosters children primed with a tetanus-conjugate Hib-DTPa-HBV combined vaccine. Eur J Pediatr. 1997;156:18–24.
13. Poolman J, Kaufhold A, De Grave D, Goldblatt D. Clinical relevance of lower Hib response in DTPa-based combination vaccines. Vaccine. 2001;19:2280–5.
14. Schlesinger Y, Granoff DM. Avidity and bactericidal activity of antibody elicited by different *Haemophilus influenzae* type b conjugate vaccines. The Vaccine Study Group. JAMA. 1992;267:1489–94.
15. Capiau C, Poolman J, Hoet B, Bogaerts H, Andre F. Development and clinical testing of multivalent vaccines based on a diphtheria-tetanus-acellular pertussis vaccine: difficulties encountered and lessons learned. Vaccine. 2003;21:2273–87.
16. McVernon J, Johnson PD, Pollard AJ, Slack MP, Moxon ER. Immunologic memory in *Haemophilus influenzae* type b conjugate vaccine failure. Arch Dis Child. 2003;88:379–83.
17. McVernon J, Howard AJ, Slack MP, Ramsay ME. Long-term impact of vaccination on *Haemophilus influenzae* type b (Hib) carriage in the United Kingdom. Epidemiol Infect. 2004;132:765–7.
18. Galil K, Singleton R, Levine OS, Fitzgerald MA, Bulkow L, Getty M, et al. Reemergence of invasive *Haemophilus influenzae* type b disease in a well-vaccinated population in remote Alaska. J Infect Dis. 1999;179:101–6.
19. Mallet E, Belohradsky BH, Lagos R, Gothefors L, Camier P, Carriere JP, et al. A liquid hexavalent combined vaccine against diphtheria, tetanus, pertussis, poliomyelitis, *Haemophilus influenzae* type b and hepatitis B: review of immunogenicity and safety. Vaccine. 2004;22:1343–57.

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Genetic Divergence of *Toxoplasma gondii* Strains Associated with Ocular Toxoplasmosis, Brazil

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Previous studies have shown a high prevalence of toxoplasmosis and the frequent occurrence of ocular disease in Brazil. To identify the genotypes of parasite strains associated with ocular disease, we compared 25 clinical and animal isolates of *Toxoplasma gondii* from Brazil to previously characterized clonal lineages from North America and Europe. Multilocus nested polymerase chain reaction analysis was combined with direct sequencing of a polymorphic intron to classify strains by phylogenetic methods. The genotypes of *T. gondii* strains isolated from Brazil were highly divergent when compared to the previously described clonal lineages. Several new predominant genotypes were identified from different regions of Brazil, including 2 small outbreaks attributable to foodborne or waterborne infection. These findings show that the genetic makeup of *T. gondii* is more complex than previously recognized and suggest that unique or divergent genotypes may contribute to different clinical outcomes of toxoplasmosis in different localities.

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of warm-blooded vertebrates and causes disease in agricultural animals and humans (*1*). *T. gondii* has a complex life cycle that includes an asexual cycle and sexual cycle; the asexual cycle occurs in a wide range of intermediate hosts, and the sexual cycle occurs exclusively in feline hosts, which shed infectious oocysts in their feces (*1*). *T. gondii* is mainly transmitted by ingesting cysts contained within tissues of a chronically infected host or by ingesting sporulated oocysts from fecally contaminated food or water (*2*). *T. gondii* is an influential foodborne pathogen in the United States (*3*) and a frequent cause of waterborne infection in parts of Brazil (*4*,*5*).

Despite having a sexual phase in its life cycle, the population structure of *T. gondii* is markedly clonal (*6*). Most strains analyzed from North America and Europe belong to 1 of 3 clonal lineages known as types I, II, and III (7–9). A small number (<5%) of isolates contain different combinations of the same alleles seen in the clonal types, which indicates that recombination occurs infrequently in the wild (*7*). Additionally, strains with more divergent genotypes have been isolated from locations such as French Guiana (*10*).

The serologic prevalence of *T. gondii* infection in Brazil includes 50%–80% of the adult population, with the highest values found in northern and southern states (*5*). Waterborne transmission has been implicated in high rates of *T. gondii* seropositivity in northern Rio de Janeiro State (*5*) and in a toxoplasmosis outbreak in Santa Isabel do Ivai in southern Paraná (*4*). High levels of ocular disease are associated with toxoplasmosis in Brazil (*11*). In the southern city of Erechim, Brazil, 184 (17.7%) of 1,042 adults were found to have retinal scars, thought to be caused by toxoplasmosis (*12*). Epidemiologic data indicate that many cases of ocular disease are acquired after birth rather than congenitally (*13*–*15*). Whether the increased prevalence and severity of ocular toxoplasmosis in Brazil are attributable to host or parasite genetic factors or differences in exposure rate is uncertain.

Polymerase chain reaction (PCR)–based typing at the *SAG2* locus has been used previously to suggest that type I strains predominate in Brazilian patients with ocular toxoplasmosis (*16*). While the *SAG2* marker provides accurate genotyping for most strains within the clonal lineages,

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it cannot detect recombinant strains or those with unusual genotypes (*17*). In fact, the exclusive use of any single locus may misrepresent the genotype of recombinant or unusual genotypes as having a simple genotype. This problem is partially alleviated by multilocus analysis, and random amplified polymorphic DNA (RAPD)–PCR analysis of Brazilian *T. gondii* strains with multiple markers showed that most strains contain both type I and III alleles at different loci (*18*). However, PCR-based markers, such as restriction fragment length polymorphism (RFLP) or RAPD, underestimate the true rate of nucleotide divergence and thus may not accurately classify *T. gondii* strains from new regions. For example, a high degree of polymorphism is detected at the *GRA6* locus by sequence analysis (9 allelic sequences among 30 strains), whereas PCR-RFLP analysis differentiates only 3 groups among these same strains (*19*).

We have recently described a sensitive method for multilocus genotyping consisting of nested PCR (nPCR) amplification of 4 different RFLP markers (*SAG2*, *GRA6*, *SAG3*, and *BTUB*). When combined with sequencing of the *UPRT*-1 intron, multilocus nPCR typing provides a robust means to classify strains as having clonal, recombinant, or novel genotypes (*17*). Multilocus nPCR analysis also can detect as few as 5 parasite genomes and thus is applicable to low-volume samples containing few parasites, as is typical of clinical specimens (*17*). In this study, we examined a group of Brazilian *T. gondii* strains from animal and human sources, including several outbreaks, to examine the population structure of *T. gondii* in Brazil.

Methods

Clinical Isolates

Patients were examined at Clinica Silveira, Erechim, Rio Grande do Sul State, by indirect ophthalmoscopy or biomicroscopy by using a slit-lamp microscope. Ocular disease was evaluated on the basis of parameters described previously (*11*). Patient consent was obtained at the time of sample acquisition. Recently acquired toxoplasmosis was confirmed by serologic tests that monitor immunoglobulin G (IgG) and IgM by enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park, IL, USA) (*11*). Ocular toxoplasmosis was diagnosed on the basis of recurrent episodes of necrotizing retinochoroiditis. Venous blood was collected before treatment in Vacutainer tubes containing heparin and cells, and serum was separated by centrifugation. The buffy-coat layer was removed, frozen at –20°C, and shipped to Washington University, Saint Louis, for analysis. Blood was also obtained from patients infected during 2 small outbreaks of toxoplasmosis in Santa Vitoria do Palmar, Rio Grande do Sul State, and Agronomica, Santa Catarina State, and processed in a similar manner. Buffy-coat samples were processed by using the DNAeasy tissue extraction kit (Qiagen Inc., Valencia, CA, USA) before PCR analysis.

Parasite Strains and Tissue Samples

Reference strains consisted of representative members of the 3 clonal lineages originally isolated from human or animal infections in North America and Europe. Reference strains for the clonal types included: 4 type I strains: ENT (ATCC 50850), RH (ATCC 50838), GT1, and VEL (ATCC 50852); 3 type II strains: Me49 (ATCC 50840), DEG (ATCC 50855), and PIH (ATCC 50857); and 3 type III strains: CTG (ATCC 50842), STRL, and VEG (ATCC 50861). In addition, 3 previously reported strains with more divergent genotypes were included: CAST (ATCC 50868), COUG, and MAS (ATCC 50870) (*20*). Parasite strains were grown in human fibroblast cells, harvested after natural egress from host cells and purified as above; cell lysates were prepared for PCR as described previously (*17*). Five Brazilian *T. gondii* strains isolated from Belo Horizonte, Minas Gerais, and one from São Paulo, as described previously (*18*), were also included.

Additional samples of porcine tissue collected from abattoirs in the Erechim region were included (R.N. Belfort, unpub. data). Six samples were analyzed, 2 negative controls and 4 samples that were positive by PCR (data not shown). Tissue samples were extracted with DNAzol, followed by an equal volume of chloroform. Polyacryl carrier (Molecular Research Center, Inc., Cincinnati, OH, USA) was added (5 μL) to the aqueous phase, and DNA was precipitated by adding an equal volume of ethanol and centrifugation at $5,000 \times g$ for 10 min.

Genotyping Isolates by PCR-RFLP

Multilocus nPCR analysis of 4 different loci was based on the markers 5′-*SAG2*, 3′-*SAG2*, *BTUB*, *GRA6*, and *SAG3* (*17*). Amplification was performed as described previously (*20*), and negative controls consisted of sterile, distilled water or proteinase K–treated cell lysate of noninfected host cells. The amplified products were digested with appropriate restriction enzymes for different loci, and the resulting fragments were analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide, and imaged by an Alpha Imager version 5.5 camera (Alpha Innotech Corp., San Leandro, CA, USA).

Restriction fragments were scored visually as present or absent, and a genetic distance matrix was calculated from the proportion of shared restriction sites by using the equation of Nei and Li (*21*). The neighbor-joining method was used to analyze the distance matrix, and dendrograms were generated by using the phylogenetic analysis program PAUP* version 4.0b (*22*). Bootstrap analysis was conducted for 1,000 replicates to obtain confidence

estimates for the taxonomic groupings. The conditions were set to distance, neighbor-joining, with mean character differences, and dendrograms were constructed by using the 50% majority rule.

UPRT-1 Intron Sequences Analysis

Sequence divergence among strains of *T. gondii* was determined at the uracil phosphoribosyl transferase (*UPRT*) intron 1 sequence (GenBank accession no. AY143141), as described previously (*17*). Following the previously described nPCR amplification of the *UPRT*-1 intron, a third set of internal primers was used for sequencing: UPRT-1seqF 5′-CTCGTCCTCGTTTTCCTT-3′ and UPRT-1seqR 5′-TGAAAGGAAGCACGTAAAGT-3′. Sequencing was conducted on 3 independent PCR-amplified templates by using BigDye cycle sequencing (Applied Biosystems, Foster City, CA, USA) (conducted by SeqWright DNA Technology Services, Houston, TX, USA). ClustalX/W (*23*) was used to align the sequences for comparison with default settings. After removal of primer sequences, the UPRT-1 intron sequence used for comparison was 467 bp in length. Unrooted phylogenetic comparisons were conducted with distance and parsimony methods by using PAUP*4.0b (*22*). The conditions were set to distance (mean character difference, minimal evolution, negative branches = 0), and 1,000 bootstrap replicates were performed by using the BioNeighbor-Joining algorithm. Alternatively, parsimony analysis was conducted by heuristic stepwise searching, with bootstrapping for >1,000 replicates. Consensus trees were drawn with an arbitrary root according to the bootstrap 50% majority rule.

Results

Brazil has a high prevalence of ocular toxoplasmosis, and many of these cases are recurrent and serious in nature (*11*,*12*). This situation prompted us to consider whether sampling patient blood might allow diagnosis of recent (acute) or recurrent infection by direct PCR amplification. Blood was collected from 77 patients seen at the Clinica Silveira, Erechim, Brazil, from 2003 to 2005, and the buffy-coat that contained leukocytes was separated by centrifugation and used for analysis. Nested PCR analysis of these samples by using the *SAG3* gene showed that 11 of 77 were positive, including 6 patients with acute disease and 5 patients with recurrent disease (Table) (locations of the patients with positive samples are shown in Figure 1). We also analyzed several sets of samples from 2 small outbreaks of acute toxoplasmosis. The first in Santa Vitoria do Palmar consisted of 10 persons from a single family that shared a meal of home-cured sausage that contained pork. Symptoms in infected persons included lymphadenitis, myalgia, fever, headache, and sweating. One of these patients, a 53-year-old woman, had severe retinochoroiditis. Only a single sample from these 10 persons was positive by *SAG3* nPCR analysis of buffy-coat cells. A second outbreak consisted of 8 infected persons from Agronomica, a town of ≈4,000 residents located 200 km from Florianopolis. These 8 persons shared the same source of nontreated water in a common neighborhood, and their illness was likely caused by waterborne infection. Three of these persons had positive results by *SAG3* nPCR.

To determine the genotype of *T. gondii* strains present in clinical samples from Brazil, multilocus nPCR was conducted by using 4 independent markers, *SAG2*, *BTUB*, *GRA6*, and *SAG3*, as described previously (*17*). The 15 clinical samples found to be positive for *SAG3* were genotyped for most of these markers, although in some cases, insufficient material was available to type all markers (Table). We compared these isolates to strains previously characterized from Brazil and to the clonal lineages common in North America and Europe. In total, 38 strains were subjected to multilocus nPCR analysis, and after restriction digestion and gel electrophoresis of the products, the strains were classified on the basis of the alleles present relative to the reference strains (Table, Figure 2) (*17*). Three of the ocular toxoplasmosis samples carried alleles characteristic of type I strains at 3 or more independent markers, and 2 Brazilian chicken strains possessed alleles typical of type III strains at all loci. All of the remaining Brazilian samples had genotypes consisting of different combinations of alleles seen in the clonal types. Nine clinical samples, including samples from Agronomica from 2 outbreaks, possessed the same profile that consisted of alleles typical of type I and type II lineages. The nPCR assay used here can detect both alleles equally well for all the makers studied, yet in no case were 2 alleles detected at a single locus within a single strain (data not shown). Consequently, the genotypes observed in Brazilian isolates cannot be explained by "mixtures" of >1 strain in a given patient or sample.

The percentage of nucleotide divergence between strains was estimated from the proportion of shared restriction sites at each locus, and a distance matrix was used to construct a dendrogram by using neighbor-joining analysis (Figure 3). All the strains belonging to type II were clustered together with a high degree of confidence. All type III strains and 2 chicken strains from Brazil were grouped together with a similarly high confidence. The 4 type I reference strains and 3 human Brazilian clinical isolates (ER8, ER11, and AG2) were clustered together. However, most Brazilian *T. gondii* strains were clustered into 2 new groups that were intermediate between types I and III. These results suggest the presence of at least 2 additional haplotypes that are prevalent in Brazil and which differ from North America and European lineages. MAS, which

Table. Genotypes of *Toxoplasma gondii* in human ocular toxoplasmosis samples from Brazil*

Strain name	Type of sample	Source	Locus						Reference¶	Location
			5′-SAG2†	3′-SAG2‡	BTUB§	SAG3	GRA6	Genotype		
ENT	C#	Human	1**	1	1,1	1	1	I	ENT	F
RH	C	Human	1	1	1,1	1	1	I	RH	USA-OH
GT1	C	Goat	1	1	1,1	1	1	I	GT1	USA-MD
VEL	C	Human	1	1	1,1	1	1	I	VEL	USA-CA
Me49	C	Sheep	1	2	2,2	2	2	II	Me49	USA-CA
DEG	C	Human	1	2	2,2	2	2	II	DEG	F
PIH	C	Human	1	2	2,2	2	2	II	PIH	USA-CA
CTG	C	Cat	2	1	2,1	3	3	III	CTG	USA-NIH
STRL	C	Human	2	1	2,1	3	3	III	STRL	USA-CA
VEG	C	Human	2	1	2,1	3	3	III	VEG	USA-CA
CAST	C	Human	1	1	1,1	1	1	I	CAST	USA-CA
COUG	C	Cougar	1	2	2,2	3	2	I/II/III	COUG	CAN-BC
MAS	C	Human	1	1	2,1	3	3	I/III	MAS	F
PBR	C	Dog	1	1	1,1	3	3	I/III	MG1	SP
D3	C	Dog	1	1	2,1	3	3	I/III	MG2	MG
CH1	C	Chicken	2	1	2,1	3	3	III	MG3	MG
CH2	C	Chicken	2	1	2,1	3	3	III	MG4	MG
EFP	C	Human	1	1	2,1	3	3	I/III	MG5	MG
SAF	C	Human	1	1	2,1	3	3	I/III	MG6	MG
6T	A††	Porcine	1	1	2,1	3	3	I/III	P1	EC
7T	A	Porcine	1	1	2,1	3	3	I/III	P2	EC
8T	A	Porcine	1	1	2,1	3	2	I/II/III	P3	EC
9T	A	Porcine	1	1	2,1	3	3	I/III	P4	EC
2147	CI‡‡	Recurrent ocular	1	1	1,1	2	1	I/II	ER1	ER
2324	CI	Acute ocular	1	1	1,1	2	1	I/II	ER2	ER
2296	CI	Acute ocular	1	1	1,1	2	1	I/II	ER3	ER
2323	CI	Acute ocular	1	1	1,1	2	1	I/II	ER4	EC
2434	CI	Recurrent ocular	1	1	1,1	2	1	I/II	ER5	ER
2325	CI	Acute ocular	1	1	1,1	3	1	I/III	ER6	ER
2566	CI	Recurrent ocular	1	2	1,1	3	–§§	I/II/III	ER7	ER
2583	CI	Acute ocular	1	1	1,1	1	–	I	ER8	ER
2612	CI	Recurrent ocular	1	–	1,1	2	–	I/II	ER9	ER
2670	CI	Recurrent ocular	1	–	1,1	2	–	I/II	ER10	EC
2728	CI	Acute ocular	1	1	1,1	1	–	I	ER11	EC
2694	CI	Outbreak	1	2	1,1	3	–	I/II/III	SV	SV
2712	CI	Outbreak	1	–	1,1	2	–	I/II	AG1	AG
2717	CI	Outbreak	1	–	1,1	1	–	I	AG2	AG
2719	CI	Outbreak	1	1	1,1	2	1	I/II	AG3	AG

*C, culture; A, animal; CI, clinical; F, France; CA, California; NIH, National Institutes of Health; CAN-BC, British Columbia, Canada; SP, São Paulo; MG, Belo Horizonte, Minas Gerais; EC, Erechim City; ER, Erechim region; SV, Santa Vitttoria do Palmar; AG, Agronomica.
†Genotypes I and II are the same.
‡Genotypes I and III are the same.
§Alleles represent *Bsi*EI and *Taq*I, respectively.
¶As referred in figures.
#Culture strains used as reference.
**Alleles defined by pattern in type I strain = 1, second allele = 2; allele 3 is defined by the presence of a second biallelic polymorphism.
††Meat tissue samples, primary source.
‡‡Human ocular toxoplasmosis.
§§Refers to negative amplification product.

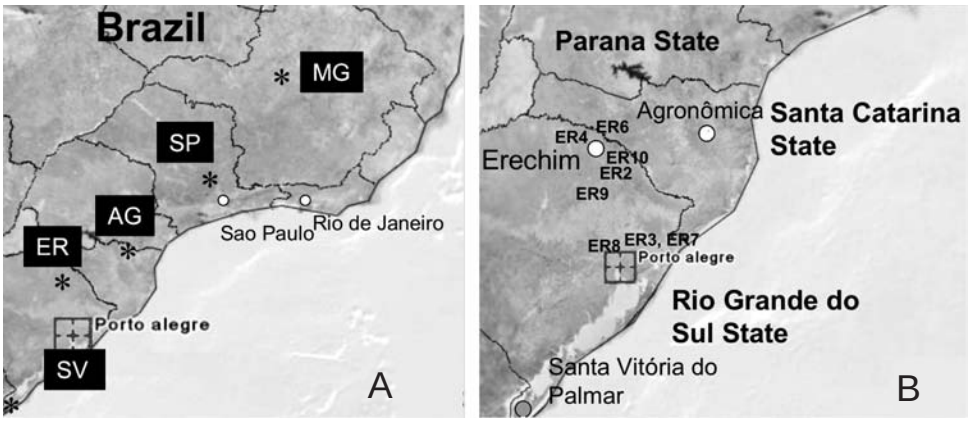

Figure 1. Location of samples obtained from Brazil. A) Samples were collected from Belo Horizonte, Minas Gerais (MG), Erechim City (ER), São Paulo (SP), Agronomica (AG), and Santa Vitttoria do Palmar (SV) (abbreviations as found in the Table). B) Clinical samples were collected from Erechim, the surrounding region (numbered as in the Table), and from 2 outbreaks in AG and SV.

was isolated from a patient with a congenital case of toxoplasmosis in France, clustered with 1 of these Brazilian haplotypes.

To more accurately assess genetic divergence, we characterized the strains by *UPRT*-1 intron sequencing, a method that is highly sensitive for detecting divergent strains (*17*). *UPRT*-1 intron sequences from 35 strains (1 clinical sample was not available in sufficient quantity for analysis, and 2 samples gave unsatisfactory sequence quality) were aligned by using Clustal X (online Appendix Figure, available at http://www.cdc.gov/ncidod/EID/vol12no06/06-0025-appG.htm), and the relative divergence of different Brazilian strains was determined by phylogenetic comparison. The results of parsimony and distance analysis were similar and the neighbor-joining distance analysis is shown in Figure 4. Because of the strongly biallelic pattern of *T. gondii*, types II and III are identical at the *UPRT* locus, while type I possess a unique haplotype distinguished by 6 single nucleotide polymorphisms (*17*,*20*). Most Brazilian *T. gondii* strains (13 of 22) shared a new allele that was distinguished by 6 additional polymorphisms not seen in the clonal lineages (online Appendix Figure). This new Brazilian allele was also shared by the previously characterized divergent strain MAS (Figure 4). Additionally, 3 outbreak strains (AG1, AG2, AG3) from Agronomica and 1 strain each from chickens (MG4) and pigs (P3) were found in this group that otherwise contained a majority of ocular toxoplasmosis isolates from the Erechim region. Other strains from Brazil contained equally divergent but unique alleles that in some cases formed smaller groups (i.e., P1, P2, P4 and MG1, ER4) (Figure 4). Only a single Brazilian strain (ER8) contained a haplotype characteristic of 1 of the clonal lineages, and this strain was identical to the type I lineage in both the PCR-RFLP and *UPRT*-1 intron trees.

Discussion

T. gondii is highly prevalent in Brazil, where human infection is associated with an unusually high occurrence of ocular disease in some locations. We examined the genotype of *T. gondii* strains collected from a variety of sources in southern Brazil. Included in this study were a group of patients seen at an eye clinic in Erechim, a region known for high levels of ocular toxoplasmosis (*11*). We also examined strains from several small outbreaks from nearby regions and compared these strains to animal isolates from Erechim and the more central region of Minas Gerais. Multilocus PCR-RFLP and sequenced-based analysis showed that they differ substantially from the previously described clonal lineages and instead define several new haplotypes that appear to be predominant in Brazil. The abundance of genotypes that do not fit the conventional classification shows the global pattern of *T. gondii* population structure to be more complex than previously thought. These findings have implications for the transmission of *T. gondii* as a waterborne and foodborne human pathogen and for studies on the role of genetic composition in virulence, pathogenesis, and life cycle dynamics.

Most human infections of *T. gondii* are not clinically severe and progress rapidly to a chronic state that is characterized by semidormant tissue cysts (*2*,*24*). During the chronic infection, parasites are generally not found in circulation, and obtaining parasites without performing invasive procedures such as tissue biopsy is relatively difficult. Previous reports have suggested that parasites may be found circulating in blood during reactivation of toxoplasmosis in AIDS patients (*25*,*26*). Our studies show that by using highly sensitive and specific nPCR, small numbers of parasites may be detected in circulating blood from some patients with either acute-onset or recurrent ocular toxoplasmosis. False-positive PCR amplification did not appear to be a substantial problem, as shown by consistently negative results for water and host-cell-only samples and the fact that the genotypes of clinical strains did not resemble common laboratory strains that would be the likely source of any contamination.

Genotyping *T. gondii* strains found in clinical and animal samples from Brazil showed that all strains except 1

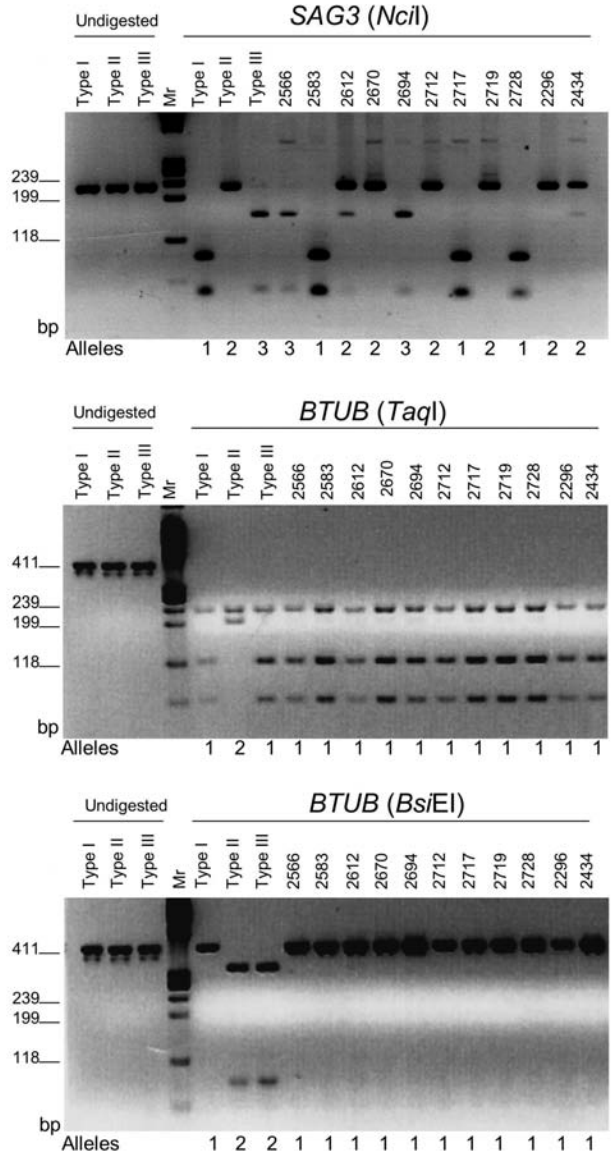

Figure 2. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analyses of clinical isolates from Brazil compared to analyses of clonal strains. Shown are the PCR markers *SAG3* and *BTUB*, with their respective restriction digests. Alleles are designated below each figure panel and match those given in the Table. Agarose gel electrophoresis of undigested and restriction digested products for type strains (type I RH, type II Me49, type III CTG). Products were resolved on 3% agarose gels strained with ethidium bromide. Mr refers to size markers from ϕX174 digested with *Hae*III.

(ER8) had different genotypes from clonal lineages that predominate in North America and Europe. When analyzed by multilocus PCR-RFLP, these new South American genotypes initially appeared to be composed of different combinations of alleles seen in the clonal types, similar to findings of a previous report from Brazil (*18*). This pattern could indicate that Brazilian strains of *T. gondii* undergo more frequent sexual recombination, resulting in mixed genotypes. However, the true extent of sequence divergence is not captured by multilocus RFLP analysis. We have previously shown that direct sequencing of introns from housekeeping genes provides a more accurate picture of sequence divergence (*17*,*20*). Introns are also likely to be selectively neutral and therefore well suited for phylogenetic comparisons (*27*). In the present study, when the *UPRT*-1 intron sequence was compared, all strains from Brazil except 1 (ER8) had multiple additional polymorphisms not seen in the clonal lineages. This locus indicates a low genetic diversity in *T. gondii* strains in Brazil, although they include genotype(s) uncommon in North America and Europe. Both the RFLP and intron analysis indicate several predominant haplotypes in Brazil, along with less common unique genotypes. Further studies will be necessary to define the population structure of *T. gondii* in Brazil and other South American locations.

The high seropositivity to *T. gondii* (*11*,*28*,*29*), combined with unusually high levels of ocular disease in some regions, shows that toxoplasmosis is a notable health problem in Brazil. Previous studies have shown a high prevalence of *T. gondii* in food animals such as pigs (*30*) and chickens (*31*) and in companion animals such as dogs (*32*) and cats (*33*). Although companion animals are not typically a source of human infection, the high prevalence in these species indicates a high level of transmission in Brazil. A recent survey of pig samples obtained from

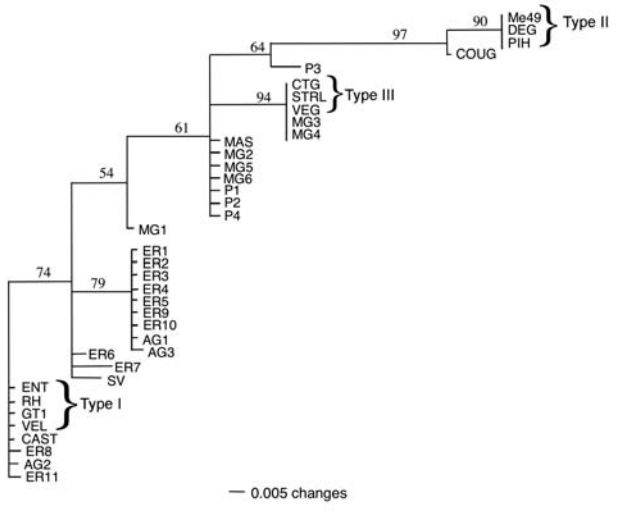

Figure 3. Neighbor-joining phylogram of 38 *Toxoplasma gondii* strains derived from polymerase chain reaction–restriction fragment length polymorphism typing at loci (*SAG2, SAG3, GRA6,* and *BTUB*). Distances were calculated according to Nei and Li (*21*) and the distance matrix analyzed using the phylogenetic analysis program PAUP*4.0b to generate an unrooted phylogram (*22*). The numbers on the branches indicate the bootstrap values (1,000 replicates). Strain designations are shown in the Table.

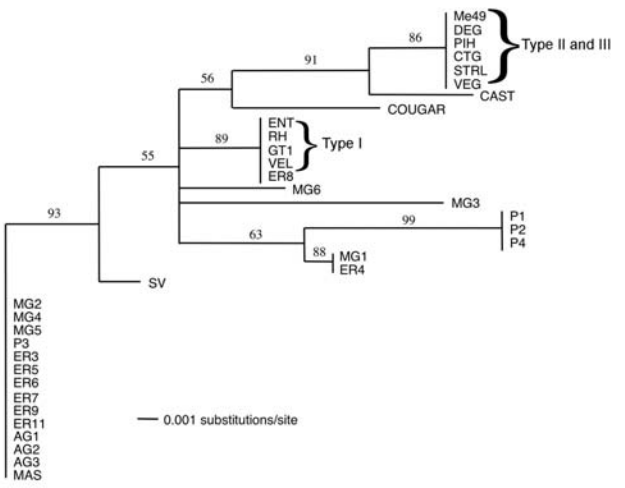

Figure 4. A phylogram of 35 *Toxoplasma gondii* strains was constructed from a Clustal alignment of UPRT-1 intron sequences using the phylogenetic analysis program PAUP*4.0b (*22*). The BioNeighbor-Joining algorithm was used to determine the divergence distance among different strains and generate an unrooted phylogram. Consensus trees were bootstrapped for 1,000 replicates and drawn with an arbitrary root according to the 50% majority rule. Strain designations are shown in the Table. A complete listing of intron sequences is found in online Appendix Figure (available at http://www.cdc.gov/ncidod/EID/vol12no06/06-0025-appG.htm).

abattoirs in the Erechim region indicated a high prevalence of *T. gondii* (35%–66% positive by PCR) (R.N. Belfort, unpub. data). Previous studies have shown a high level of recurrent ocular disease from this region, where 17.7% adults were found to have retinal scars, likely due to toxoplasmosis (*11*,*12*). In addition, drinking unfiltered water has been associated with an increased risk of *T. gondii* seropositivity in north Rio de Janeiro State, Brazil (*5*).

Collectively, these epidemiologic features suggest that infection with *T. gondii* in Brazil is more likely to lead to serious ocular disease, even in otherwise healthy persons. The extent to which host genetics, immune status, and exposure rate contribute to this pattern is unknown. However, an obvious difference is the markedly different genetic makeup of Brazilian strains of *T. gondii*. Previous studies of recurrent ocular toxoplasmosis in patients in the United States have also shown an elevated frequency of unusual genotypes (*34*). Although small animal models have been used for evaluating virulence traits of *T. gondii* strains (*6*), comparisons have not yet been made between North American and South American strains in terms of their potential to cause ocular disease.

We have previously advocated using SAG2 for genotyping *T. gondii* strains, since it is capable of distinguishing all 3 clonal genotypes at a single locus (*35*). This approach works well in North America and Europe, where the 3 major lineages predominate because of extreme linkage disequilibrium (*7*). Our current findings indicate that most strains from Brazil do not fit the clonal pattern seen in North America. Additionally, *T. gondii* strains isolated from French Guiana are also genetically distinct from the clonal lineages seen in North America (*10*). Consequently, studies that rely solely on *SAG2* typing will necessarily underrepresent the true genetic divergence in many regions. *SAG2* typing has been used for genotyping *T. gondii* isolates from various animals in Brazil (*30*–*33*,*36*), other parts of South America (*37*,*38*), and Africa (*39*,*40*). Researchers also recently suggested that strains associated with an outbreak of waterborne toxoplasmosis in Paraná, Brazil, were type I strains, based solely on genotyping with the SAG2 marker (*4*). However, analyses based solely on SAG2 almost certainly underestimate the genetic diversity of *T. gondii* in these regions. Further strain comparisons based on a wider set of sequence-based markers will be necessary to define the global population structure of *T. gondii* and to resolve the relationships between major strain types seen in different regions. Establishing the population structure of *T. gondii* is highly relevant to transmission dynamics because the suggestion has been made that recently derived clonal lineages arose through a process of recombination that led to enhanced asexual oral transmission (*20*). Whether other, more divergent strains also express this trait and to what extent their genetic makeup contributes to transmission are highly relevant to understanding the pathogenesis of toxoplasmosis.

Acknowledgments

We thank J.P. Dubey, Tovi Lehmann, Chunlei Su, Ben Rosenthal, Michael Grigg, Jim Ajioka, and Blima Fux for helpful comments and discussions and Julie Suetterlin for expert technical assistance.

This study was supported by a grant from the National Institutes of Health (AI059176).

Dr Khan is a postdoctoral research associate at Washington University. His research interests include molecular epidemiology and microbial pathogenesis.

References

1. Dubey JP, Beattie CP. Toxoplasmosis of animals and man. Boca Raton (FL): CRC Press; 1988. p. 220.
2. Frenkel JK. Toxoplasmosis in human beings. J Am Vet Med Assoc.. 1990;196:240–8.
3. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Dis. 1999;5:607–25.
4. Moura L, Bahia-Oliveira LMG, Wada MA, Jones JL, Tuboi SH, Carmo EH, et al. Waterborne toxoplasmosis, Brazil, from field to gene. Emerg Infect Dis. 2006;12:326–9.

5. Bahia-Oliveira LM, Jones JL, Azevedo-Silva J, Alves CC, Orefice F, Addiss DG. Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. Emerg Infect Dis. 2003;9:55–62.
6. Sibley LD, Boothroyd JC. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature. 1992;359:82–5.
7. Howe DK, Sibley LD. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis. 1995;172:1561–6.
8. Dardé ML, Bouteille B, Pestre-Alexandre M. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. J Parasitol. 1992;78:786–94.
9. Ajzenberg D, Cogne N, Paris L, Bessieres MH, Thulliez P, Fillisetti D, et al. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis and correlation with clinical findings. J Infect Dis. 2002;186:684–9.
10. Ajzenberg D, Bañuls AL, Su C, Dumètre A, Demar M, Carme B, et al. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. Int J Parasitol. 2004;34:1185–96.
11. Silveira C, Belfort R Jr, Muccioli C, Abreu MT, Martins MC, Victora C, et al. A follow-up study of *Toxoplasma gondii* infection in southern Brazil. Am J Ophthalmol. 2001;131:351–4.
12. Glasner PD, Silveira C, Kruszon-Moran D, Martins MC, Burnier M, Silveira S, et al. An unusually high prevalence of ocular toxoplasmosis in southern Brazil. Am J Ophthalmol. 1992;114:136–44.
13. Gilbert RE, Stanford MR. Is ocular toxoplasmosis caused by prenatal or postnatal infection? Br J Ophthalmol. 2000;84:224–6.
14. Holland GN. Reconsidering the pathogenesis of ocular toxoplasmosis. Am J Ophthalmol. 1999;128:502–5.
15. Holland GN. Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. Am J Ophthalmol. 2003;136:973–88.
16. Vallochi AL, Muccioli C, Martins MC, Silveira C, Belfort R, Jr, Rizzo LV. The genotype of *Toxoplasma gondii* strains causing ocular toxoplasmosis in humans in Brazil. Am J Ophthalmol. 2005;139:350–1.
17. Khan A, Su C, German M, Storch GA, Clifford D, Sibley LD. Genotyping of *Toxoplasma gondii* strains from immunocompromised patients reveals high prevalence of type I strains. J Clin Microbiol. 2005;43:5881–7.
18. Ferreira AM, Vitor RW, Carneiro ACAV, Brandão GP, Melo MN. Genetic variability of Brazilian *Toxoplasma gondii* strains detected by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and simple sequence repeat anchored-PCR (SSR-PCR). Infect Genet Evol. 2004;4:131–42.
19. Fazaeli A, Carter PE, Dardé ML, Pennington TH. Molecular typing of *Toxoplasma gondii* strains by GRA6 gene sequence analysis. Int J Parasitol. 2000;30:637–42.
20. Su C, Evans D, Cole RH, Kissinger JC, Ajioka JW, Sibley LD. Recent expansion of *Toxoplasma* through enhanced oral transmission. Science. 2003;299:414–6.
21. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci U S A. 1979;76:5269–73.
22. Swofford DL. Phylogenetic analysis using parsimony (* and other methods). Vol. 4.0b10. Sunderland (MA): Sinauer Associates; 2002.
23. Higgins DG, Thompson JD, Gibson TJ. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 1996;266:382–402.
24. Frenkel JK. Pathophysiology of toxoplasmosis. Parasitol Today. 1988;4:273–8.
25. Dupouy-Camet J, de Souza SL, Maslo C, Paugam A, Saimot AG, Benarous R, et al. Detection of *Toxoplasma gondii* in venous blood from AIDS patients by polymerase chain reaction. J Clin Microbiol. 1993;31:1866–9.
26. Tirard V, Niel G, Rosenheim M, Katlama C, Ciceron L, Ogunkolade W, et al. Diagnosis of toxoplasmosis in patients with AIDS by isolation of the parasite from the blood. N Engl J Med. 1991;324:634.
27. Li WH, Graur D, Fundamentals of molecular evolution. Sunderland (MA): Sinauer Associates; 1991.
28. Neto EC, Anele E, Rubim R, Brites A, Schulte J, Becker D, et al. High prevalence of congenital toxoplasmosis in Brazil estimated in a 3-year prospective neonatal screening study. Int J Epidemiol. 2000;29:941–7.
29. Petersen E, Pollak A, Reiter-Owona I. Recent trends in research on congenital toxoplasmosis. Int J Parasitol. 2001;31:115–44.
30. Santos CBA, Carvalho ACFB, Ragozo AMA, Soares RM, Amaku M, Yai LEO, et al. First isolation and molecular characterization of *Toxoplasma gondii* from finishing pigs from São Paulo State, Brazil. Vet Parasitol. 2005;131:207–11.
31. Dubey JP, Graham DH, Blackston CR, Lehmann T, Gennari SM, Ragozo AMA, et al. Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: unexpected findings. Int J Parasitol. 2002;32:99–105.
32. Silva AV, Pezerico SB, Lima VY, d'Arc Moretti L, Pinheiro JP, Tanaka EM, et al. Genotyping of *Toxoplasma gondii* strains isolated from dogs with neurological signs. Vet Parasitol. 2005;127:23–7.
33. Dubey JP, Navarro IT, Sreekumar C, Dahl E, Freire RL, Kawabata HH, et al. *Toxoplasma gondii* infections in cats from Paraná, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. J Parasitol. 2004;90:721–6.
34. Grigg ME, Ganatra J, Boothroyd JC, Margolis TP. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J Infect Dis. 2001;184:633–9.
35. Howe DK, Honoré S, Derouin F, Sibley LD. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J Clin Microbiol. 1997;35:1411–4.
36. Dubey JP, Graham DH, da Silva DS, Lehmann T, Bahia-Oliveira LM. *Toxoplasma gondii* isolates of free-ranging chickens from Rio de Janeiro, Brazil: mouse mortality, genotype, and oocyst shedding by cats. J Parasitol. 2003;89:851–3.
37. Dubey JP, Venturini MC, Venturini L, Piscopo M, Graham DH, Dahl E, et al. Isolation and genotyping of *Toxoplasma gondii* from free-ranging chickens from Argentina. J Parasitol. 2003;89:1063–4.
38. Dubey JP, Gomez-Marin JE, Bedoya A, Lora F, Vianna MC, Hill D, et al. Genetic and biologic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Colombia, South America. Vet Parasitol. 2005;134:67–72.
39. Dubey JP, Karhemere S, Dahl E, Sreekumar C, Diabate A, Dabire KR, et al. First biological and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). J Parasitol. 2005;91:69–72.
40. Dubey JP, Graham DH, Dahl E, Hilali M, El-Ghaysh A, Streekumar C, et al. Isolation and molecular characterization of *Toxoplasma gondii* from chickens and ducks from Egypt. Vet Parasitol. 2003;114:89–95.

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Social Behavior and Meningococcal Carriage in British Teenagers

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Understanding predisposing factors for meningococcal carriage may identify targets for public health interventions. Before mass vaccination with meningococcal group C conjugate vaccine began in autumn 1999, we took pharyngeal swabs from ≈14,000 UK teenagers and collected information on potential risk factors. *Neisseria meningitidis* was cultured from 2,319 (16.7%) of 13,919 swabs. In multivariable analysis, attendance at pubs/clubs, intimate kissing, and cigarette smoking were each independently and strongly associated with increased risk for meningococcal carriage (p<0.001). Carriage in those with none of these risk factors was 7.8%, compared to 32.8% in those with all 3. Passive smoking was also linked to higher risk for carriage, but age, sex, social deprivation, home crowding, or school characteristics had little or no effect. Social behavior, rather than age or sex, can explain the higher frequency of meningococcal carriage among teenagers. A ban on smoking in public places may reduce risk for transmission.

Pharyngeal carriage of *Neisseria meningitidis*, however brief, is a prerequisite for invasive meningococcal disease. Highest age-specific disease attack rates are seen in young infants. Another peak of disease that is accompanied by higher frequency of pharyngeal carriage is seen in teenagers (*1*). *N. meningitidis* may be cultured from the pharynx in as many as 1 in 4 teenagers (*2*). Male sex (*2–5*), cigarette smoking (*6–8*), passive exposure to smoke (*8*), bar patronage, discotheque visits (*9*), antimicrobial drug use (*9,10*), kissing (*5*), and overcrowding (*11*) have been associated with carriage, and many of these factors are also risk factors for meningococcal disease (*12–15*). Outbreaks of meningococcal disease are well documented in educational institutions (*16*), but no data exist on institutional factors that might contribute to carriage and transmission of meningococci. Social deprivation is associated with meningococcal disease (*17*), but whether it is associated with carriage is unknown. Greater knowledge of risk factors for meningococcal carriage may help to identify useful public health interventions.

In 1999, meningococcal group C conjugate vaccine (MenC) was offered to all persons ≤18 years of age in the United Kingdom (*18*). We identified risk factors for carriage among 14,000 teenagers as an integral part of a large, 3-year, multicenter study to determine the effect of this mass vaccination program on the carriage of meningococci. A reduction in serogroup C carriage after this intervention has already been reported (*19*). The study size gave us high statistical power to investigate the independent effects of risk factors at both individual and school levels.

Methods

Study Population

Students from 15 to 19 years of age who were attending school or college full- or part-time (but not at university) were recruited from centers in 8 geographic regions

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throughout the United Kingdom (Table 1) as previously described (*19*). The study was approved by the Trent MultiCentre Research Ethics Committee. Culture-positive data were not available for the London center in 1999; consequently, this center was not included in the analysis for this study.

The local consultant in communicable disease control (public health) asked schools and colleges in their health authority area to participate in the study, with the aim of selecting a sample of schools that broadly represented the social diversity of that population. Each center trained staff to take pharyngeal swabs according to a standard protocol. The swabbing teams visited schools and colleges from October to December for 3 successive years. In year 1 of the study (1999), swabbing took place immediately before MenC vaccination. All students 15–19 years of age in the last 2 school years before university were eligible for the study. After obtaining signed informed consent from the student (or parent/guardian), swabbing teams took a pharyngeal swab, and the student completed a short questionnaire assessing risk factors for carriage. Participants were questioned about age, sex, home postal code, school year, number of persons and rooms in household (to derive persons/room), sharing of bedroom, previous vaccination with meningococcal polysaccharide vaccine, current and recent antimicrobial drug use, active smoking, passive smoking at home, number of days in the last week they had visited a pub or club, and number of people they had intimately kissed in the last week.

Using data from the 1991 census, the postal code of home residence was used to link each person to an electoral ward and its Carstairs deprivation score (*20*) (http://www.mimas.ac.uk). A higher score reflects a greater level of social deprivation.

The following information was requested about schools and colleges: type of establishment, selective or nonselective entry, independent or state funded, single sex or coeducational, day pupils with or without boarders, and school size (small [<200 pupils], medium [200–499 pupils], or large [>500 pupils]).

Here we present the results from the first year of the study; swabs were collected in November and December 1999. The results represent meningococcal carriage just before MenC immunization.

Laboratory Methods

Swabs were plated onto selective medium either directly or within 6 hours and incubated in CO_2 at 37°C. Colonies resembling meningococci were identified by conventional tests, and oxidase-positive, gram-negative diplococci were frozen and stored in duplicate as putative meningococci at –70°C. Plates negative after 24 h were reincubated and examined again after 48 h. All isolates from England and Wales were sent to the Meningococcal Reference Laboratory, Manchester, for typing and subtyping. Scottish isolates were sent to the Scottish Pneumococcal and Meningococcal Reference Laboratory, Stobhill, Glasgow. A sample was considered positive if *N. meningitidi*s was confirmed by the reference laboratory. The duplicate isolate was examined if a viable *Neisseria* sp. was not obtained from the initial isolate.

Statistical Methods

Data from questionnaires were entered twice and validated by using Epi Info version 6.0 (*21*). Data inconsistencies were found and corrected when possible, and efforts were made to clarify incomplete dates of birth. Individual-level risk factors for meningococcal carriage were initially analyzed in single-variable models by using logistic regression in the package Stata 8.0 (StataCorp, College Station, TX, USA). School-level risk factors were initially analyzed individually within a multilevel model with students at level 1 and schools at level 2. All risk factors with $p \leq 0.1$ were then included in a multilevel logistic regression model for a multivariable analysis; again, individual students were at level 1, and schools were at level 2 of the model with explanatory variables at both levels. Center was regarded as having a fixed effect at the school level. A further analysis regarding center as a third level was undertaken but gave similar results, and the 2-level model is

Table 1. Sample characteristics by study center, UK Meningococcal Carriage Study, 1999

	Swabs		No. swabs by student's sex		No. swabs by school year			No. schools visited by type				
Center	n	No. analyzed	M	F	12	13	Other	Comprehensive	Independent/ grammar	Sixth form college	Further education college	Plating method
Bangor	972	971	439	532	529	344	94	3	0	0	3	Direct
Cardiff	1,718	1,712	829	883	916	692	102	7	0	1	1	Direct
Glasgow	2,896	2,896	1,317	1,499	1,823	1,073	0	20	2	0	0	Indirect
Nottingham	1,685	1,654	848	806	659	475	489	1	3	5	0	Direct
Oxford	2,398	2,391	1,175	1,216	1,239	822	309	7	0	0	4	Direct
Plymouth	1,394	1,389	688	701	585	437	366	9	4	0	1	Indirect
Stockport	3,011	2,906	1,598	1,408	1,514	1,023	320	0	0	3	0	Indirect
Total	14,074	13,919	6,874	7,045	7,265	4,866	1,680	47	9	9	9	

presented (the 3-level model is available on request). Each variable included was tested for significance by using the Wald test. Interactions between significant variables were also investigated. The multilevel analysis was carried out in MLwiN (*22*), and the gllamm command (*23*) in Stata 8.0 was used.

Results

A total of 14,057 swab samples were obtained. Persons were excluded if they were <15 years or >19 years (n = 101) of age, if their age was not known and their attendance in the last 2 school years (n = 16) could not be confirmed, or if their questionnaire was missing (n = 21). A total of 13,919 (99.0%) questionnaires remained for analysis (Table 1). The analysis included 6,874 male students and 7,045 female students from 74 schools or colleges. The overall frequency of carriage was 2,319 (16.7%) of 13,919 students.

Single Variable Analysis

Meningococcal carriage increased with age (Table 2, Figure 1). Some social and behavioral factors (level 1 factors) had a strong positive association with meningococcal carriage, namely, cigarette smoking, exposure to passive smoke at home, intimate kissing of ≥1 persons, and attendance at pubs or clubs in the previous week (Table 2). Weak evidence was found of an association with Carstairs score and number of persons per room. Current or recent antimicrobial drug use was negatively associated with meningococcal carriage. School year was associated with carriage; however, this variable was highly correlated with age and was not considered in the multivariable model. No association was found between carriage and sex, sharing a bedroom, and previous meningococcal polysaccharide vaccination. School-level analysis (Table 3) showed variation in frequency of carriage between centers (7.7%–23.7%, p<0.001). Associations with school type and school size were also highly significant. The presence of boarders, the source of funding, and the gender mix showed no significant association.

Multivariable Analysis

In the multivariable analysis, strong associations were found with cigarette smoking, intimate kissing, pub or club patronage, and antimicrobial drug use (Table 4). The association with pub or club attendance showed a clear dose-response relationship. The association with passive smoking was not as strong but remained significant. The rise in carriage by age was much reduced after controlling for these other factors (Figure 1, Table 4). The 15-year-olds had the highest adjusted carriage, but the numbers in this age group were relatively small, and the significant trend in age is attributable to the rise in carriage from 16 years to 18 or 19 years. Associations with Carstairs score and persons per room were no longer significant. Of the level 2 factors, no school characteristics were linked to carriage, and only the association with center remained significant.

The analysis of interactions showed evidence of small, but significant interactions between smoking and kissing (p = 0.005) and also between smoking and pub or club attendance (p = 0.003). Investigation of the relationship between these 3 variables showed that crude carriage rates varied from 7.8% to 32.8% (Figure 2). The interaction effect appears to be due to relatively high carriage (20.5%) in teenagers whose only risk factor among these 3 is smoking. This analysis was repeated by calculating odds ratios from the multivariable analysis for all combinations of the 3 variables, and the pattern was similar to that seen with the crude carriage rates.

Discussion

To our knowledge, this is the largest study, several times larger than other published studies (*2–10*), that examines risk factors for meningococcal carriage. Humans are the only natural hosts for meningococcus, and carriage in the nasopharynx, however brief, is both a prerequisite of invasive disease and essential for transmission. Our study strongly suggests that behavior, not age, is largely responsible for the increase in meningococcal carriage seen in teenagers. Active and passive smoking, intimate kissing, and attending pubs and clubs were all strongly and independently linked to the risk for meningococcal carriage. The size of this study allowed us to quantify these contributions and identify a "dose-dependent" increase in risk for attendance at pubs and clubs. The presence of all 3 risk factors increased the risk of carriage 4-fold, compared to the baseline risk in teenagers with none of these factors. The results of the risk factor analysis in the succeeding study years (2000 and 2001) were similar to those presented here. The same factors were significant, and a dose-response relationship to smoking was found in 2000.

Active and passive smoking have both been linked to risk for meningococcal carriage (*6*). Both are shown as independent risk factors in this study, and the increasing risk with the number of days that persons visited pubs or clubs may well be due to passive smoking. Other possible risk factors associated with pub and club attendance include alcohol consumption (*24*) and overcrowding (*11*). Loud music may indirectly increase risk for transmission as persons raise their voices and move closer to each other to be heard. Although salivary contact itself is probably not a risk factor (*25*), frequency of intimate kissing would be expected to increase risk for transmission through close contact with respiratory droplets from the nasopharynx (*14*).

Table 2. Single-variable analysis of risk factors for meningococcal carriage in British teenagers at an individual level*

Variable	No. swab samples	Total positive (%)	OR (95% CI)	p value
Sex				
Male	6,874	1,156 (16.8)	1.00, reference	
Female	7,045	1,163 (16.5)	0.98 (0.89–1.07)	0.625
Age (y)				
15	959	108 (11.3)	1.00, reference	
16	5,856	839 (14.3)	1.32 (1.06–1.63)	
17	5,575	1,027 (18.4)	1.78 (1.44–2.20)	
18, 19	1,511	342 (22.6)	2.31 (1.82–2.91)	<0.001
School year				
12	7,265	1,096 (15.1)	1.00, reference	
13	4,866	883 (18.1)	1.25 (1.13–1.38)	
Other	1,860	310 (18.5)	1.27 (1.11–1.46)	<0.001
Cigarettes smoked/day				
None	10,732	1,496 (13.9)	1.00, reference	
1–5	1,343	335 (24.9)	2.05 (1.79–2.35)	
6–10	1,016	277 (27.3)	2.31 (1.99–2.68)	
11–20	531	153 (28.8)	2.50 (2.05–3.04)	
>21	46	7 (15.2)	1.11 (0.49–2.48)	<0.001
Other smokers at home				
No	8,457	1,271 (15.0)	1.00, reference	
Yes	5,064	974 (19.2)	1.35 (1.23–1.48)	<0.001
No. persons kissed in last week				
0	7,564	935 (12.4)	1.00, reference	
1	4,910	1,049 (21.4)	1.93 (1.75–2.12)	
2	662	142 (21.5)	1.94 (1.59–2.36)	
3	233	68 (29.2)	2.92 (2.18–3.91)	
4–5	328	79 (24.1)	2.25 (1.73–2.92)	<0.001
No. nights attended pub or club in last week				
0	5,164	523 (10.1)	1.00, reference	
1	3,805	648 (17.0)	1.82 (1.6–2.06)	
2	2,301	482 (20.9)	2.35 (2.05–2.69)	
3	1,207	285 (23.6)	2.74 (2.34–3.22)	
4	562	150 (26.7)	3.23 (2.63–3.98)	
5–7	572	175 (30.6)	3.91 (3.20–4.78)	<0.001
No. persons sharing bedroom				
1	11,900	1,963 (16.5)	1.00, reference	
2	1,662	284 (17.1)	1.04 (0.91–1.20)	
>3	145	22 (15.2)	0.91 (0.57–1.43)	0.751
No. persons/room†				
0–1	13,197	2,196 (16.6)	1.00, reference	
>1–1.5	457	79 (17.3)	1.05 (0.82–1.34)	
>1.5	122	11 (9.0)	0.50 (0.27–0.92)	0.073
Recent antimicrobial drug use				
None	11,749	2,021 (17.2)	1.00, reference	
Current	682	64 (9.4)	0.50 (0.38–0.65)	
Stopped last week	303	48 (15.8)	0.91 (0.66–1.24)	
Stopped last month	733	99 (13.5)	0.75 (0.60–0.93)	<0.001
Prior polysaccharide vaccine				
No	12,493	2,078 (16.6)	1.00, reference	
Yes	1,042	170 (16.3)	0.98 (0.82–1.16)	0.791
Carstairs score, per unit			1.02 (1.00–1.03)	0.022
Month of swabbing				
Nov	7,050	1,192 (16.9)	1.00, reference	
Dec	6,869	1,127 (16.4)	0.96 (0.88–1.05)	0.428

*OR, odds ratio; CI, confidence interval.
†Derived data.

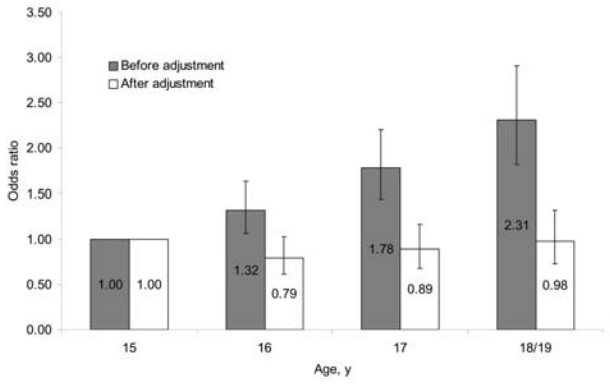

Figure 1. Relationship between age and meningococcal carriage in British teenagers 15–19 years of age before and after adjustment for other factors. Error bars indicate 95% confidence intervals.

The overall prevalence of carriage was close to expected levels for a European population of this age group, mainly 16- to 17-year-olds (*2*,*3*). Although increasing age showed a strong relationship with increasing prevalence of carriage in the univariable analysis, this increase was much reduced after adjustment for other factors. This observation is striking since other studies have suggested an increased risk of meningococcal carriage with age (*2*–*5*). We observed no association of sex with carriage, in contrast to results of other studies (*2*–*5*). This finding strongly suggests that behavior, rather than age or sex, is the driving force behind the increased risk of meningococcal carriage in teenagers. This study involved ≈14,000 persons, had more power than those previously undertaken, and was restricted to older teenagers.

This study is the first to examine a link between meningococcal carriage and social deprivation in the United Kingdom. We found no evidence of an association. The methods used had some limitations, since we were only able to link by postal code to a ward and not to individual households. These findings contrast with the increased risk for disease in young children found in lower socioeconomic groups by using similar methods (*26*–*28*). However, this association has only been reported in young children and may not apply to the teenage population. Some previous studies have reported an association between crowding and meningococcal carriage (*11*,*29*) and meningococcal disease (*13*,*30*). This study showed no evidence that increasing levels of crowding in the home, as measured by the number of rooms or persons per household and number of persons per room, was associated with increasing levels of meningococcal carriage. Crowding in the home may also be less relevant to teenagers than to young children because teenagers spend less time at home.

Table 3. Single variable analysis of risk factors for meningococcal carriage in British teenagers at school level*

Variable	No. schools	% positive†	OR (95% CI)	p value
School type				
Comprehensive	47	13.1	1.00, reference	
Independent/grammar	9	12.0	0.90 (0.60–1.35)	
Sixth form college	9	19.6	1.71 (1.18–2.48)	
Further education college	9	19.1	1.66 (1.14–2.42)	0.002
Funding				
State	68	14.6	1.00, reference	
Independent	6	13.8	0.94 (0.57–1.56)	0.808
Sex		10.5		
Single sex	5	14.8	1.00, reference	
Coeducational	69	14.7	1.52 (0.87–2.64)	0.139
School size				
Small (<200 pupils)	11	13.5	1.00, reference	
Medium (200–499)	47	12.8	0.98 (0.68–1.41)	
Large (≥500)	16	20.2	1.78 (1.18–2.69)	<0.001
Boarding				
No	71	14.6	1.00, reference	
Yes	3	13.1	0.79 (0.39–1.61)	0.512
Center				
Cardiff	9	14.2	1.00, reference	
Glasgow	22	11.7	0.75 (0.54–1.03)	
Bangor	6	19.4	1.47 (0.99–2.20)	
Nottingham	9	18.0	1.36 (0.94–1.95)	
Oxford	11	20.8	1.63 (1.16–2.29)	
Plymouth	14	7.9	0.52 (0.36–0.75)	
Stockport	3	23.5	1.92 (1.22–3.03)	<0.001

*OR, odds ratio; CI, confidence interval.
†This is calculated as the average of the percentages positive across the schools.

Table 4. Multivariable analysis of independent risk factors for meningococcal carriage in British teenagers, based on 12,437 samples with complete information*

Variable	OR (95% CI)	p value
Age (y)		
15	1.00, reference	
16	0.79 (0.61–1.03)	
17	0.89 (0.68–1.16)	
18, 19	0.98 (0.73–1.32)	0.025
No. cigarettes smoked/day		
None	1.00, reference	
1–5	1.55 (1.33–1.81)	
6–10	1.69 (1.43–2.00)	
11–20	1.62 (1.29–2.03)	
>21	0.95 (0.41–2.23)	<0.001
Other smokers at home		
No	1.00, reference	
Yes	1.17 (1.05–1.30)	0.004
No. persons kissed in last week		
0	1.00, reference	
1	1.49 (1.34–1.66)	
2	1.25 (1.00–1.57)	
3	2.00 (1.44–2.78)	
4–5	1.41 (1.05–1.91)	<0.001
Nights attended pub or club in last week		
0	1.00, reference	
1	1.52 (1.33–1.75)	
2	1.68 (1.44–1.96)	
3	1.84 (1.52–2.21)	
4	1.90 (1.50–2.42)	
5–7	2.27 (1.79–2.87)	<0.001
No. persons/room		
0–1	1.00, reference	
>1–1.5	1.01 (0.76–1.34)	
>1.5	0.57 (0.29–1.12)	0.267
Recent antimicrobial drug		
None	1.00, reference	
Current	0.51 (0.38–0.67)	
Stopped last week	0.81 (0.57–1.13)	
Stopped last month	0.66 (0.52–0.83)	<0.001
Carstairs score, per unit	1.00 (0.98–1.02)	0.909
School type		
Comprehensive	1.00, reference	
Independent/grammar	1.04 (0.67–1.60)	
Sixth college	0.67 (0.32–1.40)	
College	0.82 (0.49–1.37)	0.681
School size		
Small (<200 pupils)	1.00, reference	
Medium (200–499)	0.92 (0.65–1.30)	
Large (≥500)	1.18 (0.6–2.06)	0.617
Center		
Cardiff	1.00, reference	
Glasgow	0.89 (0.62–1.28)	
Bangor	1.33 (0.83–2.14)	
Nottingham	1.45 (0.86–2.44)	
Oxford	1.46 (0.99–2.16)	
Plymouth	0.48 (0.31–0.74)	
Stockport	1.99 (1.01–3.90)	<0.001

*OR, odds ratio; CI, confidence interval.

Of the study participants, 16% reported previous meningococcal vaccination. The only meningococcal vaccine available before this study was the plain polysaccharide vaccine. The lack of impact of this vaccine on carriage is not surprising. Any effect of polysaccharide vaccination on carriage is probably short term, and the most commonly used polysaccharide vaccine is directed against serogroups A and C. Very few carriers of serogroup A and C strains were found in this study. A protective effect from recent antimicrobial drug use was expected because many antimicrobial drugs temporarily suppress or eradicate meningococcal carriage (*31*).

The design and size of this study allowed us to examine school characteristics as possible risk factors for meningococcal carriage. Although outbreaks often occur in educational institutions, no previous data existed on institutional factors that might contribute to carriage and transmission of meningococci. We did not identify any school characteristics that had an independent effect on carriage. Differences between centers remained significant even after adjustment for other factors. These may have been true differences between centers or the result of differences in the methods of swabbing, plating, and laboratory procedures. For example, in 1 study, direct plating resulted in a doubling of the detectable frequency of carriage, compared to results of indirect plating (*32*).

In conclusion, this study suggests that the rise in meningococcal carriage in teenagers is driven by changes in social behavior. Since carriage is a prerequisite for invasive disease (*33*), this rise in carriage is likely to explain the well-documented peak in meningococcal disease attack rates in teenagers (*1,34–36*). Explaining the risks of smoking, intimate kissing, and pub and club attendance may be a useful public health intervention, particularly in an outbreak situation. In the United Kingdom, a ban on

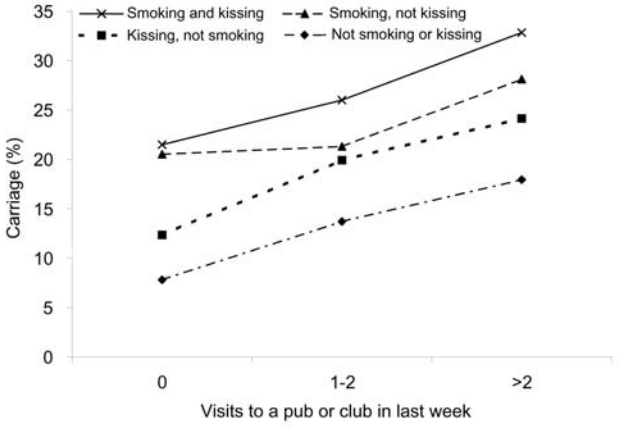

Figure 2. The combined effect of varying attendance at pubs and clubs, cigarette smoking, and intimate kissing on the risk for meningococcal carriage in British teenagers.

smoking in public places will be introduced in 2007 (*37*). Potential health benefits from such a measure may include a reduction in the risk of meningococcal meningitis and septicemia.

Acknowledgments

We thank the head teachers, staff, and pupils at the participating schools and colleges for their support and the public health and microbiology staff at the study centers for their essential contribution to the success of this study.

This work was funded by Project Grants from the Wellcome Trust nos. 059987 and 062057 (sampling in England and Wales), the Chief Scientist Office of the Scottish Executive Health Department (sampling in Scotland), and the Meningitis Trust (risk factor survey). M.C.J.M. was a Wellcome Trust Senior Research Fellow in Basic Biomedical Sciences. The sponsors of this study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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References

1. Coen PG, Cartwright K, Stuart J. Mathematical modelling of infection and disease due to *Neisseria meningitidis* and *Neisseria lactamica*. Int J Epidemiol. 2000;29:180–8.
2. Cartwright KAV, Stuart JM, Jones DM, Noah ND. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol Infect. 1987;99:591–601.
3. Caugant DA, Hoiby EA, Magnus P, Scheel O, Hoel T, Bjune G, et al. Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. J Clin Microbiol. 1994;32:323–30.
4. Gilmore A, Jones G, Barker RM, Soltanpoor N, Stuart J. Meningococcal disease at the University of Southampton: outbreak investigation. Epidemiol Infect. 1999;123:185–92.
5. Neal KR, Nguyen-Van-Tam J, Jeffrey N, Slack RCB, Madeley RJ, Ait-Tahar K, et al. Changing carriage rate of *Neisseria meningitidis* among university students during the first week of term: cross sectional study. BMJ. 2000;320:846–9.
6. Stuart JM, Cartwright KAV, Robinson PM, Noah ND. Effect of smoking on meningococcal carriage. Lancet. 1989;2:723–6.
7. Kremastinou J, Blackwell C, Tzanakaki G, Kallergi C, Elton R, Weir D. Parental smoking and carriage of *Neisseria meningitidis* among Greek schoolchildren. Scand J Infect Dis. 1994;26:719–23.
8. Riordan T, Cartwright K, Andrews N, Stuart J, Burris A, Fox A, et al. Acquisition and carriage of meningococci in marine commando recruits. Epidemiol Infect. 1998;121:495–505.
9. Conyn-van Spaendonck MAE, Reintjes R, Spanjaard L, Van Kregten E, Kraayeveld AG, Jacobs PHA. Meningococcal carriage in relation to an outbreak of invasive disease due to *Neisseria meningitidis* serogroup C in the Netherlands. J Infect. 1999;39:42–8.
10. Dominguez A, Cardenosa N, Izquierdo C, Sanchez F, Margall N, Vazguez JA, et al. Prevalence of *Neisseria meningitidis* carriers in the school population of Catalonia, Spain. Epidemiol Infect. 2001;127:425–33.
11. Glover JA. Observations of the meningococcus carrier rate and their application to the prevention of cerebro-spinal fever. Special Report series of the Medical Research Council (London). 1920;50:133–65.
12. Fischer M, Hedberg K, Cardosi P, Plikaytis BD, Hoesly FC, Steingart KR, et al. Tobacco smoke as a risk factor for meningococcal disease. Pediatr Infect Dis J. 1997;16:979–83.
13. Baker M, McNicholas A, Garrett N, Jones N, Steward J, Kobertsein V, et al. Household crowding a major risk factor for epidemic meningococcal disease in Auckland children. Pediatr Infect Dis J. 2000;19:983–90.
14. Stanwell-Smith RE, Stuart JM, Hughes AO, Robinson P, Griffin MB, Cartwright KAV. Smoking, the environment and meningococcal disease: a case control study. Epidemiol Infect. 1994;112:315–28.
15. Cookson ST, Corrales JL, Lotero JO, Regueira M, Binsztein N, Reeves MW, et al. Disco fever: epidemic meningococcal disease in northeastern Argentina associated with disco patronage. J Infect Dis. 1998;178:266–9.
16. Davison KL, Andrews N, White JM, Ramsay ME, Crowcroft NS, Rushdy AA, et al. Clusters of meningococcal disease in school and preschool settings in England and Wales: what is the risk? Arch Dis Child. 2004;89:256–60.
17. Jones IR, Urwin G, Feldman RA, Banatvala N. Social deprivation and bacterial meningitis in north east Thames region: three year study using small area statistics. BMJ. 1997;314:794–5.
18. Public Health Laboratory Service. Vaccination programme for group C meningococcal infection is launched. CDR Weekly. 1999;9:261–4.
19. Maiden MCJ, Stuart JM (for the UK Meningococcal Carriage Group). Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. Lancet. 2002;359:1829–30.
20. Carstairs V, Morris R. Deprivation, mortality and resource allocation. Community Medicine. 1989;11:364–72.
21. Centers for Disease Control and Prevention. Epi Info, version 6.04a, a word processing, database and statistics program for public health on IBM-compatible microcomputers. Atlanta (GA): the Centers; 1996.
22. A user's guide to M1wiN version 2.0. London (UK): London Institute of Education; 1999.
23. Rabe-Hesketh S, Skrondal A, Pickels A. A reliable estimation of generalised linear mixed models using adaptive quadrature. Stat J. 2002;2:1–21.
24. Imrey PB, Jackson LA, Ludwinski PH, England ACI, Fella GA, Fox BC, et al. Meningococcal carriage, alcohol consumption, and campus bar patronage in a serogroup C meningococcal disease outbreak. J Clin Microbiol. 1995;33:3133–7.
25. Orr HJ, Gray SJ, Macdonald M, Stuart JM. Saliva and meningococcal transmission. Emerg Infect Dis. 2003;9:1314–5.
26. Fone DL, Harries JM, Lester N, Nehaul L. Meningococcal disease and social deprivation: a small area geographical study in Gwent, UK. Epidemiol Infect. 2003;130:53–8.
27. Stuart JM, Middleton N, Gunnell DJ. Socioeconomic inequality and meningococcal disease. Commun Dis Public Health. 2002;5:327–8.
28. Heyderman R, Ben-Shlomo Y, Brennan CA, Somerset M. The incidence and mortality for meningococcal disease associated with area deprivation: an ecological study of hospital episode statistics. Arch Dis Child. 2004;89:1064–8.
29. Kaiser AB, Hennekens CH, Saslaw MS, Hayes PS, Bennett JV. Seroepidemiology and chemoprophylaxis of disease due to sulfonamide-resistant *Neisseria meningitidis* in a civilian population. J Infect Dis. 1974;130:217–24.

30. Deutch S, Labouriau R, Schonheyder HC, Ostergaard L, Norgard B, Sorensen HT. Crowding as a risk factor of meningococcal disease in Danish preschool children: a nationwide population based case-control study. Scand J Infect Dis. 2004;36:20–3.
31. Broome CV. The carrier state: *Neisseria meningitidis*. J Antimicrob Chemother. 1986;18(Suppl A):25–34.
32. Cunningham R, Matthews R, Lewendon G, Harrison S, Stuart J. Improved rate of isolation of *Neisseria meningitidis* by direct plating of pharyngeal swabs. J Clin Microbiol. 2001;39:4575–6.
33. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity. J Exp Med. 1969;129:1327–48.
34. Connolly M, Noah N. Is group C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993–6. Epidemiol Infect. 1999;122:41–9.
35. Ramsay M, Kaczmarski EB, Rush M, Mallard R, Farrington P, White J. Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. Commun Dis Rep CDR Rev. 1997;7:R49–54.
36. Abbott JD, Jones DM, Painter MJ, Young SEJ. The epidemiology of meningococcal infections in England and Wales, 1912–1983. J Infect. 1985;11:241–57.
37. British Broadcasting Corporation News Online [cited 2006 Feb 15]. Available from http://news.bbc.co.uk/1/hi/uk_politics/4709258.stm

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Coccidioidomycosis as a Common Cause of Community-acquired Pneumonia

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The early manifestations of coccidioidomycosis (valley fever) are similar to those of other causes of community-acquired pneumonia (CAP). Without specific etiologic testing, the true frequency of valley fever may be underestimated by public health statistics. Therefore, we conducted a prospective observational study of adults with recent onset of a lower respiratory tract syndrome. Valley fever was serologically confirmed in 16 (29%) of 55 persons (95% confidence interval 16%–44%). Antimicrobial medications were used in 81% of persons with valley fever. Symptomatic differences at the time of enrollment had insufficient predictive value for valley fever to guide clinicians without specific laboratory tests. Thus, valley fever is a common cause of CAP after exposure in a disease-endemic region. If CAP develops in persons who travel or reside in *Coccidioides*-endemic regions, diagnostic evaluation should routinely include laboratory evaluation for this organism.

Coccidioidomycosis (valley fever) is an infection caused by *Coccidioides immitis* or *Coccidioides posadasii*, which are fungi endemic to parts of Arizona, California, Utah, New Mexico, Texas, Mexico, and elsewhere in Central and South America (*1*). The most common clinical syndrome resulting from infection is community-acquired pneumonia (CAP), which is characterized by systemic illness, lower respiratory tract symptoms, and various immunologic manifestations such as rashes and skeletal discomfort (*2–5*). This syndrome, which occurs 1–3 weeks after inhalation of a fungal spore, may be present for protracted periods. Although infection occasionally spreads hematogenously to other parts of the body, most infections eventually resolve without complications or specific antifungal therapy.

Enormous disparities exist between the predicted number of illnesses caused by coccidioidal infection and the actual number of infections reported to state departments of public health. For example, rates of skin test conversion (*6*) and the size of the susceptible population in southern Arizona indicate that illness should develop in ≈30,000 persons per year. Similar frequencies are obtained from other indirect analyses (*7*). If these estimates were accurate, coccidioidomycosis would be a common cause of CAP in this disease-endemic region. In contrast, cases reported to the Arizona Department of Health Services from 1998 to 2001 averaged <2,000 annually (*8*) or 15-fold fewer than estimated. The figure that best reflects the actual frequency of illness has profound implications for diagnostic evaluation and therapeutic management in patients with exposure to *Coccidioides* species in a disease-endemic region.

In this report, we present results of evaluations of patients seeking care for symptoms of CAP with respect to the incidence of coccidioidomycosis. Because CAP is often managed by clinicians as part of general medical practices, we chose that setting for our studies. Our findings provide the first prospective evidence that coccidioidomycosis is a common cause of CAP in the study area. In addition to the relevance of these results for residents within *Coccidioides*-endemic regions, a similar risk for coccidioidal infection should be expected for others with CAP and a recent travel history to *Coccidioides*-endemic areas.

Methods

Study Sample

Patients were recruited from 3 primary care sites in Tucson, Arizona: the Urgent Care Center of University

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Medical Center and 2 of the medical offices of Arizona Community Physicians. Recruitment was conducted during 2 time periods: from December 1, 2003, through February 21, 2004, and from May 1, 2004, through August 14, 2004. Although we sought to enroll as many eligible persons as possible during these periods, study personnel were not always available to do so.

To be eligible for enrollment, patients had to exhibit a lower respiratory syndrome of <1 month's duration that included ≥1 of the following: pleuritic chest pain, dyspnea on exertion, having an evaluation by a chest radiograph, multiple visits for the same respiratory problem, or administration of an antibacterial drug for presumed CAP. Patients were excluded from enrollment if they had a previously diagnosed, laboratory-confirmed coccidioidal infection, another laboratory-confirmed diagnosis for inclusion-defining illness, were <18 years of age, or had not had previous exposure >1 week in a disease-endemic area. Fewer than 5% of the patients offered enrollment refused to participate in the study.

Study Protocol

This was an observational study, and medical management of each patient's condition remained entirely with the responsible clinician. After informed consent was obtained, persons were interviewed and their clinical records were reviewed to collect information regarding demographics, comorbid conditions, time ranges of exposure in a disease-endemic region, and recent antimicrobial therapy for current respiratory illness. Persons were asked to complete the Medical Outcomes Study 36-Item Short Form Health Survey (SF-36) (*9*), the Iowa Fatigue Scale (*10*), and a respiratory infection severity scale (*11*). Results from chest radiographs and complete blood counts, where obtained as part of routine medical care, were also recorded. A second visit was scheduled for all persons.

Serum samples were obtained at both visits. They were stored at –70°C until tested at the completion of the study. Persons identified as having a coccidioidal infection were also contacted during or within the next 6 months to determine the status of their illness.

Serologic Analysis

Anti-coccidioidal antibodies were measured in the laboratory of 1 of the authors (J.N.G.) by several conventional methods. The double immunodiffusion technique was used to measure tube precipitin–type and complement fixing–type anti-coccidioidal antibodies (*12*,*13*). Serum samples qualitatively positive for complement fixing–type anti-coccidioidal antibodies were retested quantitatively (*14*). Anti-coccidioidal immunoglobulin M (IgM) and IgG antibodies were measured by enzyme-linked immunoassay by using a commercial kit according to the manufacturer's instructions (Coccidioides EIA-Gold, Meridian Diagnostics, Cincinnati OH, USA) (*15*). An optical density ≥0.20 was considered positive. The relative sensitivity of these tests in patients with coccidioidal pneumonia has been previously analyzed (*15*).

Statistical Analysis

Patient and laboratory data were entered into a database (Access 2003, Microsoft Corp., Bellingham WA, USA), and statistical analysis was accomplished with SAS version 8.2 (SAS Institute, Inc., Cary, NC, USA). Differences between groups for categorical variables were compared with the χ^2 test and those for continuous variables were compared with the Wilcoxon sign-rank test and Wilcoxon rank-sum test as appropriate. Differences with p values <0.05 were considered significant.

Results

Of the 56 persons enrolled in this study, 1 did not provide a serum sample at baseline and was excluded from analysis. Fifty-five percent of the enrollees were male, and the median age was 48 years (interquartile range 33–63 years) (Table 1). Most (87%) persons were non-Hispanic whites, which reflected the demographics of the study-site sample. Patients with lower respiratory syndromes had a median of 3 additional inclusion criteria. Individual criteria were evaluation by a chest radiograph (82%), administration of an antimicrobial drug (80%), dyspnea on exertion (56%), pleurisy (44%), and multiple visits for the same condition (42%).

Frequency of Coccidioidomycosis as a Cause of CAP

Of the 55 persons who provided serum samples at baseline, 19 provided second serum samples 10–40 days later (median 18 days). Of these persons, 16 were positive by ≥1 serologic test (29%, 95% confidence interval [CI] 16%–44%). At baseline, 12 (75%) of the 16 were positive by multiple assays, and all but 3 had positive results for multiple serologic tests for both serum samples (Table 2). Of the remaining 36 persons, all had negative results for all tests at baseline, and a second serum sample obtained from 12 persons was also nonreactive.

Comparison of Clinical Characteristics between Groups

Demographic results for persons with and without valley fever are shown in Table 1. Length of exposure in the disease-endemic area was significantly shorter for patients with valley fever than for those who were seronegative (p = 0.043). The odds ratio for developing coccidioidomycosis in persons with exposure of <10 years to a disease-endemic area compared to those with a longer exposure time was 4.11 (95% CI 1.01–16.8). No other significant

Table 1. Demographic characteristics of the study sample*

Characteristic	Total (N = 55)	Persons with valley fever (n = 16)	Others (n = 39)
Male sex, no. (%)	30 (55)	8 (50)	22 (56)
Median age, y (IQR)	48 (33–63)	47 (30–57)	48 (33–63)
Race, no. (%)			
Non-Hispanic white	48 (87)	13 (81)	35 (90)
Hispanic	4 (3.6)	2 (13)	2 (5.1)
Asian	3 (5.5)	1 (6.3)	2 (5.1)
Median body mass index (IQR)	26 (22–31)	25 (22–30)	26 (23–31)
Median length of exposure in disease-endemic area, y (IQR)†	9 (5–24)	6.5 (3.5–10)	10 (6–26)
Coexisting condition			
COPD	2	0	2
Asthma	9	1	8
Lung disease	9	2	7
History of pneumonia	4	1	3
Renal disease	2	1	1
Liver disease	1	0	1
Immunocompromised	0	0	0
Rheumatologic	1	0	1

*IQR, interquartile range; COPD, chronic obstructive pulmonary disease.
†p = 0.043.

demographic differences were identified between the 2 groups. Twenty, 24, and 11 participants had age ranges of <40 years, 40–64 years, and ≥65 years, respectively. The percentage of participants in each of these age groups with valley fever was 30%, 29%, and 27%, respectively.

Respiratory, systemic, and musculoskeletal symptoms are shown in Table 3. Only myalgia showed a significant difference between the 2 groups. The SF-36 survey and respiratory infection severity scale did not identify additional differences. However, the Iowa Fatigue Scale survey median productivity domain score (maximum score = 10 indicates greatest reduction) was 7 for persons with valley fever compared with 4.5 for all other persons (p = 0.008).

The proportion of patients in whom a chest radiograph was obtained was similar between seropositive and seronegative patients (75% vs. 72%). However, for those from whom chest radiographs were obtained (n = 40), abnormalities were significantly more frequent in participants with valley fever (75% vs. 25%, p = 0.005). Radiographic abnormalities associated with coccidioidal infection included pulmonary infiltrates in 88% and hilar adenopathy in 1 participant.

Table 2. Serologic characteristics of the study sample*

Seropositive persons (N = 16)	No.
Baseline serum sample	
Positive by >1 method	11
IDTP only	1
EIA IgM only	1
EIA IgG only	2
Negative†	1
Second serum sample	
Positive by >1 method	8
Negative	0
Not obtained	8
Seronegative persons (N = 39)	
Baseline serum sample	39
Second serum sample	12
Second serum sample not obtained	27

*IDTP, immunodiffusion tube precipitin; EIA, enzyme immunoassay; IgM, immunoglobulin M.
†This person was subsequently positive by multiple assays on his second serum sample.

Use of Antimicrobial Drugs

Of the 55 persons who were seen in the primary care setting, 46 (84%) were prescribed antimicrobial medications; 13 received 2 consecutive courses of treatment, and 1 received 3 consecutive courses of treatment. No differences were seen between the seropositive and seronegative groups in either the proportion treated with antimicrobial drugs (81% vs. 85%) or the proportion treated with multiple courses of drugs (31% vs. 26%).

Follow-up of Persons with Valley Fever

All persons improved in the 6 months after enrollment. Only 1 received specific antifungal therapy (oral fluconazole for 1 month), and none required hospitalization. Eleven persons with valley fever repeated the symptom survey and the Iowa Fatigue Scale a median of 22 days after enrollment. Their responses indicated significant improvement for cough (p<0.016), fatigue (p<0.0039), cognition (p = 0.016), energy (p = 0.0015), and productivity (p = 0.062). Similar improvements were noted with the SF-36 survey and the Iowa Fatigue Scale readministered to 10 persons at the 6-month follow-up visit.

Discussion

Of the patients enrolled in our prospective study from select ambulatory care settings within the disease-endemic region, 29% were diagnosed serologically as having

coccidioidomycosis. Even if one takes into account the wide 95% CI (16%–44%), this number demonstrates a high proportion of CAP caused by this infection. Furthermore, although the serologic tests used for diagnosis are highly specific for coccidioidal infection (*12*,*13*,*16*–*18*), another study emphasized that in the first weeks of primary illness these tests frequently show negative results (*15*). In the current study, 1 of 13 participants not initially serologically positive had coccidioidal antibodies in a second serum sample. Twenty-seven persons serologically negative at enrollment did not return for retesting; thus, additional coccidioidal infections may have been identified in this group. Our results will likely provide an underestimate of the incidence in this group of patients, further strengthening the conclusion that valley fever is a common cause of CAP in persons exposed to *Coccidiodes* in a disease-endemic area.

The high frequency of valley fever as a cause of CAP found in this study is consistent with previous estimates of coccidioidomycosis as a dominant cause of CAP with exposure in disease-endemic areas. A similar estimate of 25% to 30% has been obtained retrospectively at the Southern Arizona Veterans Administration Health Care System in Tucson, Arizona (*7*). Conversely, a diagnosis of valley fever requires laboratory testing. That this practice may not be uniform among clinicians was shown in a retrospective analysis of physician-specific diagnoses at primary care clinics in Tucson, Arizona, in which the rate of diagnosing coccidioidomycosis varied between 0% and 25% among physicians within the same group practice (*7*). Similar differences might also account for the increasing case rate associated with patient age that was reported in a recent analysis of 2001 Arizona state statistics (*8*). Case rates for persons >44 years of age were nearly twice those for persons 21–44 years of age. In our study in which all persons were uniformly evaluated for valley fever, all age groups had similar rates (27.3%–30.0%). Furthermore, although not detailed in our results, severity of illness in terms of respiratory symptoms was less in elderly subjects. We interpret the differences between the state statistics and those of our study as indicating that older persons who develop an illness are more likely to have an exact diagnosis determined, underscoring underreporting of illness in some patient groups such as young adults.

A corollary to the high frequency of coccidioidomycosis seen in this study is that persons anywhere with CAP and a history of recent travel to south-central Arizona or other regions where coccidioidomycosis is highly endemic would be expected to have a similarly high risk. For this reason, obtaining a travel history for any patient with CAP is essential for early and accurate diagnosis of this disease, as well as for other regional problems such as severe acute respiratory syndrome (SARS), hantavirus pneumonia, and avian influenza. Although the Infectious Diseases Society of America practice guidelines for CAP currently recommend obtaining a complete travel history only in patients with refractory pneumonia (*19*), we recommend that the guidelines be revised to recommend obtaining a travel history at the first evaluation.

Table 3. Symptoms of the study sample at enrollment

Symptoms	Persons with valley fever (n = 16), no. (%)	Others (n = 39), no. (%)	p value
Respiratory			
Cough	11 (69)	35 (90)	0.10
Sputum production	8 (50)	28 (72)	0.21
Hemoptysis	1 (6.3)	3 (7.7)	1.0
Pleurisy	9 (56)	15 (38)	0.25
Dyspnea	10 (63)	21 (54)	0.77
Systemic			
Fever	9 (56)	19 (49)	0.77
Chills	9 (56)	17 (44)	0.55
Night sweats	9 (56)	21 (54)	1.0
Fatigue	16 (100)	34 (87)	0.31
Weight loss	3 (19)	7 (18)	1.0
Musculoskeletal			
Myalgia	11 (69)	9 (23)	0.0022
Arthralgia	7 (44)	11 (28)	0.35
Rash	3 (19)	3 (7.7)	0.34

Analysis of multiple symptoms at baseline showed several characteristics associated with coccidioidal infections. Both a shorter length of exposure in a disease-endemic region and a greater frequency of radiographic abnormalities were seen in persons with valley fever compared with those without valley fever. These associations were also evident in a previous report from a university health center (*5*). Symptoms of myalgia and reduced productivity were also evident with coccidioidal infection. However, none of these associations, alone or in combination, were of a sufficient magnitude to assist clinicians in the initial diagnosis. Therefore, our findings, as in the previous study (*5*), emphasize that laboratory testing at the initial physician visit is essential to identify patients with symptoms of CAP that are caused by valley fever.

A high proportion (81%) of persons with valley fever were prescribed an initial course of antimicrobial drugs. Of these, 12 patients, 3 of whom were diagnosed with valley fever, received 2 courses of these drugs. Although diagnosis of valley fever by serologic methods is frequently delayed by 3–5 days, use of antimicrobial drugs could still be avoided or stopped earlier in patients whose illness is determined to be caused by *Coccidioides* species.

The inclusion criteria used in this study were designed both to be broadly inclusive and to select patients with more severe illness. As such, they differ in some respects from commonly used entry criteria for clinical trials of new antimicrobial drugs as treatment for CAP. For example, we chose pleuritic chest pain and dyspnea at rest and fever as an entry requirement. 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*). When comparing our findings to those of other studies, the way in which patients were selected should be taken into account.

Several limitations of our methods deserve emphasis. Because our inclusion criteria were not standard, comparison of our results to those of other studies of CAP is difficult. Also, we did not have a diagnosis for patients without valley fever. Since this is a relatively small study, additional expanded studies may be useful, especially to extend observations to other groups such as children, the elderly, those requiring hospitalization, and residents elsewhere within disease-endemic regions. Future studies are also needed to determine best practices for management of primary coccidioidal infection and possible therapy with specific antifungal treatment. Such a high proportion of CAP caused by *Coccidioides* species should provide further impetus to conduct those studies.

This work was supported in part by the US Department of Veterans Affairs, the Department of Health and Human Services, and grant 1PO1AI061310-01 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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References

1. Pappagianis D. Epidemiology of coccidioidomycosis. Curr Top Med Mycol. 1988;2:199–238.
2. Snyder LS, Galgiani JN. Coccidioidomycosis: the initial pulmonary infection and beyond. Seminars in Respiratory and Critical Care Medicine. 1997;18:235–47.
3. Kerrick SS, Lundergan LL, Galgiani JN. Coccidioidomycosis at a university health service. Am Rev Respir Dis. 1985;131:100–2.
4. Lundergan LL, Kerrick SS, Galgiani JN. Coccidioidomycosis at a university outpatient clinic: a clinical description. In: Einstein HE, Catanzaro A, editors. Coccidioidomycosis. Proceedings of the Fourth International Conference. Washington: National Foundation for Infectious Diseases; 1985. p. 47–54.
5. Yozwiak ML, Lundergan LL, Kerrick SS, Galgiani JN. Symptoms and routine laboratory abnormalities associated with coccidioidomycosis. West J Med. 1988;149:419–21.
6. Dodge RR, Lebowitz MD, Barbee RA, Burrows B. Estimates of C. immitis infection by skin test reactivity in an endemic community. Am J Public Health. 1985;75:863–5.
7. Campion JM, Gardner M, Galgiani JN. Coccidioidomycosis (valley fever) in older adults: an increasing problem. Arizona Geriatrics Society Journal. 2003;8:3–12.
8. Park BJ, Sigel K, Vaz V, Komatsu K, McRill C, Phelan M, et al. An epidemic of coccidioidomycosis in Arizona associated with climatic changes, 1998–2001. J Infect Dis. 2005;191:1981–7.
9. Guyatt GH, Feeny DH, Patrick DL. Measuring health-related quality of life. Ann Intern Med. 1993;118:622–9.
10. Krupp LB, LaRocca NG, Muir-Nash J, Steinberg AD. The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus. Arch Neurol. 1989;46:1121–3.
11. Metlay JP, Fine MJ, Schulz R, Marrie TJ, Coley CM, Kapoor WN, et al. Measuring symptomatic and functional recovery in patients with community-acquired pneumonia. J Gen Intern Med. 1997;12:423–30.
12. Huppert M, Bailey JW. The use of immunodiffusion tests in coccidioidomycosis. II. An immunodiffusion test as a substitute for the tube precipitin test. Tech Bull. Regist. Med Technol. 1965;35:155–9.
13. Huppert M, Bailey JW. The use of immunodiffusion tests in coccidioidomycosis. I. The accuracy and reproducibility of the immunodiffusion test which correlates with complement fixation. Tech. Bull Regist Med Technol. 1965;35:150–4.
14. Wieden MA, Galgiani JN, Pappagianis D. Comparison of immunodiffusion techniques with standard complement fixation assay for quantitation of coccidioidal antibodies. J Clin Microbiol. 1983;18:529–34.
15. Wieden MA, Lundergan LL, Blum J, Delgado KL, Coolbaugh R, Howard R, et al. Detection of coccidioidal antibodies by 33-kDa spherule antigen, Coccidioides EIA, and standard serologic tests in sera from patients evaluated for coccidioidomycosis. J Infect Dis. 1996;173:1273–7.
16. Smith CE, Saito MT, Simons SA. Pattern of 39,500 serologic tests in coccidioidomycosis. JAMA. 1956;160:546–52.
17. Pappagianis D, Zimmer BL. Serology of coccidioidomycosis. Clin Microbiol Rev. 1990;3:247–68.
18. Smith CE. Coccidioidomycosis. In: Coates JB, Hoff EC, editors. Communicable diseases transmitted chiefly through respiratory and alimentary tracts. Washington: Office of the Surgeon General, Medical Department, US Army; 1958. p. 285–316.
19. Bartlett JG, Breiman RF, Mandell LA, File TM. Community-acquired pneumonia in adults: guidelines for management. The Infectious Diseases Society of America. Clin Infect Dis. 1998;26:811–38.

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Human Rotavirus Serotype G9, São Paulo, Brazil, 1996–2003

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A total of 3,101 fecal specimens were collected during an 8-year survey for rotavirus infection in São Paulo, Brazil. Group A rotavirus was detected in 774 (25.0%) specimens. Of these, 431 strains (55.7%) were analyzed for G and P types by reverse transcription–polymerase chain reaction; G1 was the predominant serotype (68.2%), followed by G9 (17.2%), G4 (6.3%), G2 (1.2%), G3 (0.7%), mixed infection (1.8%), and untypeable (4.6%). Both rotavirus G and P types could be established in 332 strains (77.0%). We identified the 4 most common strains worldwide: P[8]G1 (66.6%), P[4]G2 (1.0%), P[8]G3 (0.6%), and P[8]G4 (7.2%). Among the single G9 strains detected, VP4 genotyping showed that P[8]G9 was the most prevalent, followed by P[4]G9 and P[6]G9. The emergence and high frequency of rotavirus G9 in São Paulo, Brazil, and other parts of the world will affect the development and evaluation of future vaccines.

Group A rotavirus is the most common cause of acute gastroenteritis in infants and young children worldwide (*1*). More than 130 million cases of diarrhea each year are attributed to rotavirus. It is estimated to cause >400,000 deaths annually in children <5 years of age and is responsible for 2 million hospital admissions due to acute diarrhea worldwide. In developing countries, an estimated 1,205 children die from rotavirus disease each day, and 82% of these deaths occur in children in the poorest countries (*2*)

Rotavirus serotypes are determined by neutralizing antibody responses to each of the 2 outer capsids proteins, VP7 (G serotype) and VP4 (P serotype) (*1*). To date, 11 VP7 G serotypes and 13 P serotypes have been identified in humans. Serotypes G1, G2, G3, and G4 are frequently associated with diarrhea in humans and have become prime targets for vaccine development (*3,4*). The recent emergence and wide distribution of rotavirus G9 indicate that this serotype may become the fifth relevant strain (*5,6*). Unusual types of rotavirus have been described in certain settings. The G5 type has been reported in Brazil, Argentina, Paraguay, Cameroon, and the United Kingdom (*6,7*); G6 has been detected in Italy, Australia, India, the United States, Belgium, and Hungary; G8 has been frequently isolated in Africa and sporadically in other countries; rotavirus G10 specificity has been reported in the United Kingdom, India, Thailand, Paraguay, and Brazil (*6*); G11 type was recently detected in Dhaka, Bangladesh (*4*); and the G12 type has been detected in the Philippines (*8*), Thailand (*9*), the United States (*10*), India (*11*), Japan (*12*), Korea (*13*), Argentina (*14*), and Brazil (*15*).

The genotypes VP4 P[8] and P[4] are the most common P types that infect humans. The P[8] type is generally associated with VP7 types G1, G3, and G4, and the P[4] type is associated with G2 (*16*). Combined G and P genotyping may have advantages in identifying reassortants as unusual or new virus strains (*17*). Continued surveillance of the diverse rotavirus strains circulating in a community is crucial before developing a vaccine and during and after implementing an immunization program. Therefore, we describe the results of an 8-year surveillance study of G- and P-type rotavirus strains from persons with acute diarrhea in the state of São Paulo, Brazil.

Materials and Methods

From 1996 to 2003, a total of 3,101 fecal specimens were collected from children <5 years of age, school-age children (5–17 years), adults (18–59 years), and elderly patients (≥60 years) with acute gastroenteritis. These patients received treatment for diarrhea at the departments of public health or were admitted to hospitals in several cities in São Paulo State, in southeast Brazil. São Paulo

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State has an area of ≈248,800 km^2 and a population of 40 million (21.5% of the population of Brazil). Figure 1 shows main cities in São Paulo where samples were collected. Epidemiologic data (age, date of diarrhea onset, date of sample collection) were available from some patients. Specimens were stored at –20°C until tested for rotavirus and characterized. Study methods were approved by the ethical committee of Adolfo Lutz Institute.

All specimens were screened for rotavirus by using a commercial enzyme-linked immunosorbent assay (ELISA) (Premier Rotaclone, Meridian Diagnostics, Cincinnati, OH, USA) with monoclonal antibodies specific for group A human rotavirus, according to the manufacturer's protocol. Rotavirus double-stranded RNA (dsRNA) was extracted directly from stool by the TRIzol method (Invitrogen, Carlsbad, CA, USA) and precipitated with isopropanol. The extracted dsRNA was subjected to G and P typing by multiplex reverse transcription–polymerase chain reaction (RT-PCR) with type-specific primers. Consensus primers Beg9 and End9 were used in a first-round PCR (30 cycles) to amplify the full-length VP7 gene (1,062 bp); cDNA was used in a second-round PCR for G typing (25 cycles) with primer set aBT1 (G1), aCT2 (G2), aET3 (G3), aDT4 (G4), aFT9 (G9) and primer set FT5 (G5), DT6 (G6), HT8 (G8), ET10 (G10), BT11 (G11) (*18*,*19*). For P typing, consensus primers Con2 and Con3 were used in a first-round RT-PCR (30 cycles) to amplify the 876 bp of the VP8* region of the VP4 gene, and the second-round PCR (20 cycles) used primer set 1T-1 (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), 4T-1 (P[9]), 5T-1 (P[10]) (*20*). All PCR products were analyzed by electrophoresis in 1.2% agarose gels, containing 0.5 μg ethidium bromide per milliliter and visualized under UV illumination.

Figure 1. Map of São Paulo State, Brazil, indicating where fecal specimens were collected during the 8-year survey period.

Results

Rotavirus was detected in 774 (25.0%) of 3,101 specimens collected from children, adults, and elderly patients in São Paulo during an 8-year period. Rotavirus infection was found predominantly in the winter and in drier months. The incidence peaked in August (Figure 2). The age or date of birth was provided for 677 (87.5%) of 774 patients who tested positive for rotavirus. Rotavirus disease was detected mainly in children <2 years of age (463 [59.8%] of 774), and peaks of incidence occurred from 7 to 12 months (Figure 3). However, rotavirus infection also was detected in adults and elderly patients (55 [7.1%] of 774).

We randomly selected 431 rotavirus-positive samples (55.7%) for determination of G and P genotypes by an RT-PCR assay. G1 was the predominant serotype in these samples (294, 68.2%), followed by G9 (74, 17.2%), G4 (27, 6.3%), G2 (5, 1.2%), G3 (3, 0.7%), mixed infection (8, 1.8%), and untypeable (20, 4.6%) (Table 1). The distribution of rotavirus types in São Paulo during this 8-year period shows that G1 was the most prevalent genotype in most years, but it was displaced by G9 in 2002. Incidence of G2 and G3 serotypes was low during the period of analysis. Frequency of G4 serotype differed during the surveillance period; it was not detected in 1996 and 2003. We found several mixed infections from 2000 to 2003 (G1+G4, G1+G9, G2+G3, and G4+G9).

Both rotavirus G and P types could be established in 332 (77.0%) strains, and 17 different P and G associations were detected (Table 2). Of these, we identified the 4 most globally common strains, P[8]G1, P[8]G4, P[4]G2, and P[8]G3, which represented 75.3% of all typed rotavirus strains. Uncommon strains were also detected, including P[8]G9, P[4]G9, P[6]G9, P[4]G1, P[6]G1, P[6]G2, and P[4]G4. And combination P-G mixed infections were as diverse as P[8]G1+G4, P[8]G1+G9, P[6]G1+G9, P[4]G2+G3, and P[8]G4+G9.

Discussion

We detected rotaviruses in the specimens of 25.0% of patients with acute diarrhea, which is comparable to the

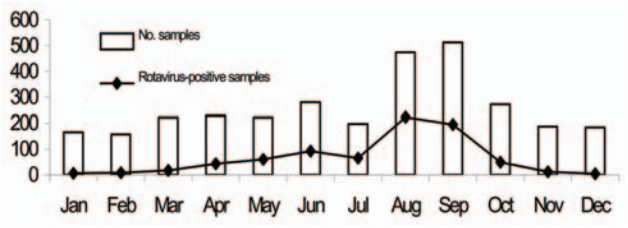

Figure 2. Temporal distribution of rotavirus strains from children, adults, and elderly patients with acute diarrhea, São Paulo, Brazil, 1996–2003.

prevalence seen in other studies in Brazil (*21*). Among children <5 years of age, we detected rotavirus infection mainly in those <2 years of age (81.0%) (data not shown); in adults, rotavirus was detected less frequently (7.1%). The finding of a low percentage of rotavirus infection among adults is likely because the disease is generally perceived to be a childhood infection (*22*). Common epidemiologic settings for rotavirus infection among adults include endemic disease, epidemic outbreak, travel-related infection, and child-to-adult transmission (*23*).

In studies performed at various locations in Brazil with diverse climatic conditions, rotavirus disease appears to occur year-round (*24*). In São Paulo State, however, infection occurred mainly during cooler and drier seasons; similar observations have been made in other countries with temperate climates (*16*).

During the 8-year period studied, the G1 type was the most prevalent rotavirus strain. The second most prevalent was the G9 type, which accounted for 17.2% of disease, followed by G4, G2, and G3, which are common around the world. G1 was the most prevalent type in most years; however, it was displaced by G9 during the 2002 season, when G9 accounted for 46.9% of typed isolates. The G9 type has been reported to be a common cause of diarrhea and has become the fifth most common serotype, which suggests that it may be a substantial cause of diarrhea in humans (*5*,*6*). This type has been detected in Brazil since 1997 (*25*,*26*).

Surveillance on rotavirus types has been performed in São Paulo for ≥18 years, from 1986 to 2003 (*27*,*28*). The first G9 type was isolated in 2000 and has been fluctuating in frequency since its emergence (Table 1). The G5 type, normally associated with animal rotavirus (pigs and horses), has been frequently detected in persons in Brazil and it was considered an endemic virus (*28*,*29*). Nevertheless, in our survey in São Paulo, G5 rotavirus was not detected. Its incidence in Brazil has been decreasing over the last few years, and it may be disappearing (*25*,*26*); this type of rotavirus is likely to be a cyclic form. G9 strains have also been detected in animals (lambs and pigs [*1*]); detection of animal rotavirus provides evidence for natural human-animal genetic reassortment (*30*). Surveillance programs for animal rotavirus may aid in the development of next-generation vaccines (*6*).

Characterization of rotavirus VP4 types showed various strains. In this study, the 4 most globally common strains, P[8]G1, P[4]G2, P[8]G3, and P[8]G4, represented 75.3% of all typed viruses. The most prevalent association was P[8]G1, followed by P[8]G9. Worldwide, the 4 predominant rotavirus genotypes make up nearly 90% of all rotavirus infections (*16*). In Brazil, epidemiologic data on the prevalence of G and P types have been collected since the 1980s (*27*). This study showed great diversity of rotavirus strains in São Paulo. The uncommon genotypes P[4]G1, P[4]G4, P[4]+P[6]G1, and P[6]+P[8]G1 were also seen in some cases, similar to results from other countries (*31*,*32*). In our study, the uncommon genotypes P[6]G1, P[6]G2, and P[6]G9 were detected in children with acute diarrhea with an average age of 14 months (data not shown). Many studies have shown that this type is often detected in very young children with diarrhea, which suggests that P[6] strains may promote infection at an early age. Originally, P[6] in association with rotavirus types G1–G4 was detected in asymptomatic neonates (*33*), but recent studies have also shown that P[6]G9 circulates in hospitalized children without diarrhea (*34*). These strains are considered naturally attenuated and have been used to develop a vaccine candidate. Worldwide, rotavirus strains with the P[6] genotype have been seen in children with diarrhea (*35*–*37*). In Brazil, other studies isolated the P[6] type from children with acute diarrhea (*26*–*28*). In our survey, several mixed G and P types also appeared but in a low percentage (2.6%). The detection of unusual strains and mixed infections in this study suggests a previously unrecognized diversity among Brazilian rotavirus infections (*28*).

Among the single G9 strains detected in this survey, VP4 genotyping showed that P[8]G9 was the most prevalent (75.7%), followed by P[4]G9 (5.4%) and P[6]G9 (1.4%) (data not shown). This diversity among G9 types has also been detected in other studies (*5*,*6*,*26*). Combined

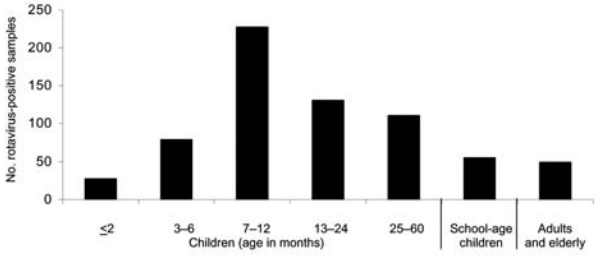

Figure 3. Rotavirus infection among children (<5 years of age), school-age children (5–17 years), adults (18–59 years), and elderly patients (≥60 years) with acute diarrhea, São Paulo, Brazil, 1996–2003.

Table 1. Distribution of rotavirus G types from children, adults, and elderly patients with acute diarrhea in São Paulo, Brazil, 1996–2003

Year	No. rotavirus isolates	No. (%) selected for genotyping	G1	G2	G3	G4	G9	Mixed*	Not typeable
1996	33	21 (63.3)	19 (90.5)	2 (9.5)	0	0	0	0	0
1997	121	48 (39.7)	45 (93.8)	0	0	3 (6.3)	0	0	1 (2.1)
1998	45	16 (35.6)	12 (75.0)	0	0	4 (25.0)	0	0	0
1999	99	56 (56.6)	46 (82.1)	0	0	10 (17.9)	0	0	2 (3.6)
2000	98	52 (53.1)	42 (80.8)	0	0	0	7 (13.4)	3 (5.8)	5 (9.6)
2001	57	46 (80.7)	28 (60.9)	2 (4.3)	1 (2.2)	6 (13.0)	4 (8.7)	1 (2.2)	1 (2.2)
2002	90	49 (54.4)	18 (36.7)	0	2 (4.1)	4 (8.2)	23 (46.9)	2 (4.1)	6 (12.2)
2003	231	127 (55.0)	84 (66.1)	1 (0.8)	0	0	40 (31.5)	2 (1.6)	5 (3.9)
Total	774	431 (55.7)	294 (68.2)	5 (1.2)	3 (0.7)	27 (6.3)	74 (17.2)	8 (1.8)	20 (4.6)

*Mixed infections: 2000, G1+G9 (n = 3); 2001, G2+G3 (n = 1); 2002, G1+G9 (n = 1) and G4+G9 (n = 1); 2003, G1+G9 (n = 2).

data from P and G typing are relevant to identify new strains that might have resulted from reassortment of genes between diverse human-human and human-animal rotaviruses (*38*).

Recently, 2 live rotavirus oral vaccines have been licensed in some countries and made available on the market, including a monovalent vaccine derived from the most common human rotavirus strain, P[8]G1, and a pentavalent vaccine based on a bovine strain, WC3, that contains 5 human-bovine reassortant viruses (G1, G2, G3, G4, and P[8]). Both vaccines have shown efficacy against severe rotavirus disease (*39*,*40*). In August 2005, the live, attenuated P[8]G1 human rotavirus vaccine was licensed in Brazil, the first country to introduce this vaccine into the public health network.

Our data show that challenges exist for the design of rotavirus vaccine for the Brazilian population and underscore that virus strain surveillance should be ongoing. Surveillance programs can establish whether G9 rotavirus strains will continue to rise in prevalence or whether they will follow a cyclical pattern of emergence, as has been shown for G1–G4. The composition of future rotavirus vaccines is likely to be formulated according to the geographic setting and the distribution of G and P strains.

Acknowledgments

We thank Carla Uchida Tanuma, Daniela Bernardes Borges, Fernanda Ferreira da Silva, Karla Tatiana Rubini, Raquel de Souza Pereira, and Tomoko Sekiya for their technical assistance.

This work was supported by Adolfo Lutz Institute, São Paulo State Secretary of Health, São Paulo.

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Table 2. Association of P- and G-type rotavirus strains from patients with acute diarrhea, São Paulo, Brazil, 1996–2003

P and G association	1996, n (%)	1997, n (%)	1998, n (%)	1999, n (%)	2000, n (%)	2001, n (%)	2002, n (%)	2003, n (%)	Total, n (%)
Common genotypes	17 (94.4)	32 (100)	15 (93.8)	50 (98.0)	34 (81.0)	21 (72.4)	16 (38.1)	65 (63.7)	250 (75.3)
P[8]G1	15 (83.3)	30 (93.7)	12 (75.0)	40 (78.4)	34 (81.0)	14 (48.3)	12 (28.5)	64 (62.7)	221 (66.6)
P[4]G2	2 (11.1)	0	0	0	0	0	0	1 (1.0)	3 (1.0)
P[8]G3	0	0	0	0	0	0	2 (4.8)	0	2 (0.6)
P[8]G4	0	2 (6.3)	3 (18.8)	10 (19.6)	0	7 (24.1)	2 (4.8)	0	24 (7.2)
Uncommon genotypes	1 (5.6)	0	1 (6.2)	1 (2.0)	0	6 (20.7)	3 (7.1)	1 (1.0)	13 (3.9)
P[4]G1	1 (5.6)	0	0	0	0	2 (7.0)	2 (4.8)	1 (1.0)	6 (1.8)
P[6]G1	0	0	0	0	0	1 (3.4)	0	0	1 (0.3)
P[4]+P[6]G1	0	0	0	0	0	0	1 (2.4)	0	1 (0.3)
P[6]+P[8]G1	0	0	0	1 (2.0)	0	1 (3.4)	0	0	2 (0.6)
P[6]G2	0	0	0	0	0	2 (7.0)	0	0	2 (0.6)
P[4]G4	0	0	1 (6.2)	0	0	0	0	0	1 (0.3)
G9 genotypes	0	0	0	0	5 (12.0)	1 (3.4)	21 (50.0)	34 (33.3)	61 (18.4)
P[4]G9	0	0	0	0	0	0	1 (2.4)	3 (2.9)	4 (1.2)
P[6]G9	0	0	0	0	0	0	0	1 (1.0)	1 (0.3)
P[8]G9	0	0	0	0	5 (12.0)	1 (3.4)	20 (47.6)	30 (29.4)	56 (16.9)
Mixed infection					3 (7.0)	1 (3.4)	2 (4.8)	2 (2.0)	8 (2.4)
P[8]G1+G9	0	0	0	0	3 (7.0)	0	0	1 (1.0)	5 (1.5)
P[6]G1+G9	0	0	0	0	0	0	1 (2.4)	0	1 (0.3)
P[4]G2+G3	0	0	0	0	0	1 (3.4)	0	0	1 (0.3)
P[8]G4+G9	0	0	0	0	0	0	1 (2.4)	0	1 (0.3)

characterization and epidemiology of rotavirus, norovirus, and enteroviruses.

References

1. Kapikian AZ, Hoshino Y, Chanock RM. Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, et al., editors. Fields virology. Volume 2. 4th ed. Philadelphia: Lippincott-Raven; 2001. p. 1787–833.
2. Parashar UD, Gibson CJ, Breese JS, Glass RI. Rotavirus and severe childhood diarrhea. Emerg Infect Dis. 2006;12:304–6
3. Hoshino Y, Kapikian AZ. Rotavirus serotypes: classification and importance in epidemiology, immunity, and vaccine development. J Health Popul Nutr. 2000;18:5–14
4. Rahman M, Matthijnssens J, Nahar S, Podder G, Sack DA, Azim T, et al. Characterization of a novel P[25], G11 human group a rotavirus. J Clin Microbiol. 2005;43:3208–12.
5. Castello AA, Arvay ML, Glass RI, Gentsch J. Rotavirus strain surveillance in Latin America. A review of the last nine years. Pediatr Infect Dis J. 2004;23:S168–72.
6. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol. 2005;15:29–56.
7. Beards G, Graham C. Temporal distribution of rotavirus G-serotypes in the West Midlands region of the United Kingdom, 1983–1994. J Diarrhoeal Dis Res. 1995;13:235–7.
8. Taniguchi K, Urasawa T, Kobayashi N, Gorziglia M, Urasawa S. Nucleotide sequence of VP4 and VP7 genes of human rotaviruses with subgroup I specificity and long RNA pattern: implication for new serotype specificity. J Virol. 1990;64:5640–4.
9. Pongsuwanna Y, Guntapong R, Chiwakul M, Tacharoenmuang R, Onvimala N, Wakuda M, et al. Detection of a human rotavirus with G12 and P[9] specificity in Thailand. J Clin Microbiol. 2002;40:1390–4.
10. Griffin DD, Nakagomi T, Hoshino Y, Nakagomi O, Kirkwood CD, Parashar UD, et al. Characterization of nontypeable rotavirus strains from the United Status: identification of a new rotavirus reassortant (P2A[6], G12) and rare P3[9] strains related to bovine rotavirus. Virology. 2002;294:256–69.
11. Das S, Varghese V, Chaudhury S, Barman P, Mahapatra S, Kojima K, et al. Emergence of novel human group A rotavirus G12 strain in India. J Clin Microbiol. 2003;41:2760–2.
12. Shinozaki K, Okada M, Nagashima S, Kaiho I, Taniguchi K. Characterization of human rotavirus strains with G12 and P[9] detected in Japan. J Med Virol. 2004;73:612–6.
13. Cheon DS, Lee K, Kim W, Lee S, Choi W, Ahn J, et al. Genetic analysis of the VP7 gene of unusual genotypes of human group A rotavirus strains circulating in Korea [abstract P37-3]. In: Abstracts of the 23rd Annual Meeting of the American Society for Virology; Montreal, Canada; 2004 Jul 10–14. American Society for Virology; 2004.
14. Castello AA, Jiang B, Glass RI, Glikmann G, Gentsch JR. Rotavirus G and P genotype prevalence in Argentina 1999–2003. Detection of P[9]G12 strains [abstract P37-4]. In: Abstracts of the 23rd Annual Meeting of the American Society for Virology; Montreal, Canada; 2004 Jul 10–14. American Society for Virology; 2004.
15. Timenetsky MCST, Carmona RCC, Morillo SG, Eduardo MBPE, Silva LJ. Incidence of rotavirus G and P Genotypes in children in southern Brazil. Emergence of genotype G9 [abstract V-330]. In: Abstracts of the International Congress of Virolgy, Joint Meeting of the 3 Divisions of the International Union of Microbiological Societies; San Francisco; 2005 Jul 23–28. International Union of Microbiological Societies; 2005.
16. Gentsch JR, Woods PA, Ramachadran M, Das BK, Leite JP, Alfieri A, et al. Review of G and P typing results from a global collection of rotavirus strains: implications for vaccine development. J Infect Dis. 1996;174:S30–6.
17. Fruhwirth M, Brosl S, Ellemunter H, Moll-Shuler I, Rohwedder A, Mutz I. Distribution of rotavirus VP4 genotypes and VP7 serotypes among nonhospitalized and hospitalized patients with gastroenteritis and patients with nosocomially acquired gastroenteritis in Austria. J Clin Microbiol. 2000;38:1804–6.
18. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, et al. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J Clin Microbiol. 1990;28:276–82.
19. Gouvea V, Santos N, Timenetsky MC. Identification of bovine and porcine rotavirus G types by PCR. J Clin Microbiol. 1994;32:1338–40.
20. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, et al. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol. 1992;30:1365–73.
21. Linhares AC. Rotavirus infection in Brazil: epidemiology and challenges for its control. Cad Saude Publica. 2000;16:629–46.
22. Fischer TK, Eugen-Oslen J, Pedersen AG, Mølbak K, Bottiger B, Rostgaard K, et al. Characterization of rotavirus strains in Danish population: high frequency of mixed infection and diversity within the VP4 gene of P[8] strains. J Clin Microbiol. 2005;43:1099–104.
23. Anderson EJ, Weber SG. Rotavirus infection in adults. Lancet Infect Dis. 2004;4:91–9.
24. Pereira HG, Linhares AC, Candeias JAN, Glass RI. National laboratory surveillance of viral agents of gastroenteritis in Brazil. Bull Pan Am Health Organ. 1993;27:224–33.
25. Santos N, Volotão EM, Soares C, Albuquerque MCM, Silva FM, Carvalho TRB, et al. Rotavirus strains bearing genotype G9 or P[9] recovered from Brazilian children with diarrhea from 1997–1999. J Clin Microbiol. 2001;39:1157–60.
26. Araújo IT, Ferreira MSR, Fialho AM, Assis RM, Cruz CM, Rocha M, et al. Rotavirus genotypes P[4]G9, P[6]G9, P[8]G9 in hospitalized children with acute gastroenteritis in Rio de Janeiro, Brazil. J Clin Microbiol. 2001;39:1999–2001.
27. Timenetsky MCS, Santos N, Gouvea V. Survey of rotavirus G and P types associated with human gastroenteritis in São Paulo, Brazil, from 1986 to 1992. J Clin Microbiol. 1994;32:2622–4.
28. Carmona RCC, Timenetsky MCST, Silva FF, Granato CFH. Characterization of rotavirus strains from hospitalized and outpatient children with acute diarrhea in São Paulo, Brazil. J Med Virol. 2004;74:166–72.
29. Gouvea V, Castro L, Timenetsky MC, Greenberg H, Santos N. Rotavirus serotype G5 associated with diarrhea in Brazilian children. J Clin Microbiol. 1994;32:1408–9.
30. Timenetsky MC, Gouvea V, Santos N, Carmona RCC, Hoshino Y. A novel human rotavirus serotype with dual G5–G11 specificity. J Gen Virol. 1997;78:1373–8.
31. Abdel-Haq NM, Thomas RA, Asmar BI, Zacharova V, Lyman WD. Increased prevalence of G1P[4] genotype among children with rotavirus-associated gastroenteritis in metropolitan Detroit. J Clin Microbiol. 2003;41:2680–2.
32. Iturriza-Gómara M, Isherwood B, Desselberger U, Gray J. Reassortment in vivo: driving force for diversity of human rotavirus strains isolated in the United Kingdom between 1995 and 1999. J Virol. 2001;75:3696–705.
33. Hoshino Y, Wyatt RG, Flores J, Midthun K, Kapikian AZ. Serotypic characterization of rotaviruses derived from asymptomatic human neonatal infections. J Clin Microbiol. 1985;21:425–30.
34. Cunliffe NA, Rogerson S, Dove W, Thindwa BD, Geensill J, Kirkwood CD, et al. Detection and characterization of rotaviruses in hospitalized neonates in Blantyre, Malawi. J Clin Microbiol. 2002;40:1534–7.
35. Cunliffe NA, Gondwe JS, Broadhead RL, Molyneux ME, Woods PA, Bresee JS, et al. Rotavirus G and P types in children with acute diarrhea in Blantre, Malawi, from 1997 to 1998: predominance of novel P[6]G8 strains. J Med Virol. 1999;57:308–12.

36. Trabelsi A, Peenze I, Pager C, Jeddi M, Steele D. Distribution of rotavirus VP7 serotypes and VP4 genotypes circulating in Sousse, Tunisia, from 1995 to 1999: emergence of natural human reassortants. J Clin Microbiol. 2000;38:3415–9.
37. Ramachandran M, Das BK, Vij A, Kumar R, Bhambal SS, Kesari N, et al. Unusual diversity of human rotavirus G and P genotypes in India. J Clin Microbiol. 1996;34:436–9.
38. Das S, Sen A, Uma G, Varghese V, Chaudhuri S, Bhattacharya K, et al. Genomic diversity of group A rotavirus strains infecting humans in Eastern India. J Clin Microbiol. 2002;40:146–9.
39. Ruiz-Palacios GM, Peres-Schael I, Velásquez FR, Abate H, Breuer T, Clemens SC, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med. 2006;354: 11–22.
40. Vesikari T, Matson DO, Dennehy P, Van Damme P, Santosham M, Rodriguez Z, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. N Engl J Med. 2006;354:23–33.

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Human Parechovirus Infections in Canada

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A new reverse transcription–polymerase chain reaction assay was developed for identification of 28 Canadian human parechovirus (HPeV) isolates, including 20 HPeV-1, 3 HPeV-2, and 5 HPeV-3, recovered from 1985 to 2004. All HPeV-1 isolates but 1 were genetically distinct from the Harris reference strain. One HPeV-2 isolate was related to the Williamson strain; the other 2 were related to the Connecticut strain. HPeV-3 isolates clustered together. Seventy-five percent of isolates were recovered during the typical enterovirus season. All patients but 1 were children with a mean age of 14.6 months, 6.3 months, and 0.7 months for HPeV-1, HPeV-2, and HPeV-3 patients, respectively. All HPeV-2– and HPeV-3–infected children were hospitalized with a diagnosis of viremia or sepsis. HPeV-1–infected children had bronchiolitis diagnosed in 50% of the cases, with few cases of pneumonia and enteritis. Two infected patients (1 child with leukemia and a 78-year-old woman) died of septic shock and severe pneumonia, respectively.

Picornaviruses constitute a diverse family of single-stranded positive-sense RNA viruses whose genome is packed into a nonenveloped icosahedral capsid (*1*). Within the *Picornaviridae* family, 5 genera are known to cause infections in humans: *Enterovirus*, *Hepatovirus*, *Rhinovirus*, *Kobuvirus*, and *Parechovirus* (*1*). The *Parechovirus* genus contains 3 human pathogens (HPeV-1, -2, and -3) as well as Ljungan virus, a rodent parechovirus isolated from bank voles (*2*). These viruses have several atypical biologic and molecular properties compared to other picornaviruses, such as unusual cytopathic effects and the lack of cleavage of the VP0 protein into VP4 and VP2, which results in a virion particle with only 3 capsid proteins rather than 4 (*3*).

Previous studies demonstrated that HPeV-1 (formerly echovirus 22) had a worldwide distribution and was associated with diseases similar to those caused by human enteroviruses (HEVs), i.e., gastroenteritis, respiratory diseases, aseptic meningitis, encephalitis, and neonatal sepsis–like syndromes (*3–6*). In general, HPeV-1 seems to be responsible for more gastrointestinal and respiratory syndromes and for fewer central nervous system (CNS) symptoms than enteroviruses (*3,6*). HPeV-2 (formerly echovirus 23) has been rarely reported, despite its early description in 1961 (*7*). In a retrospective Swedish study covering a period of >30 years, only 5 cases of HPeV-2 infections (including 4 cases of gastrointestinal symptoms and 1 case of respiratory symptoms) were reported (*8*), compared to 109 HPeV-1 infections during the same period (*9*). The third type of HPeV (HPeV-3) was reported in 2004 from stool specimen of a 1-year-old Japanese girl with transient paralysis (*10*). Subsequently, we reported 3 cases of sepsislike illnesses attributable to HPeV-3 infections in Canadian neonates (*11*). The association of HPeV-3 with 3 cases of sudden infant death syndrome was also suggested (*12*). In a recent Dutch study, HPeV-3 was shown to be more involved in CNS infections than HPeV-1 (*13*).

Similar to HEVs, HPeVs infections are commonly identified by virus isolation in cell culture, followed by neutralization typing (*6,9*). Isolation in cell culture is laborious and time-consuming. In addition, the method may lack sensitivity, thus leading to false-negative results (*14,15*). Because of increasing knowledge of genome sequences of HPeVs, development of molecular techniques such as reverse transcription–polymerase chain reaction (RT-PCR) could be an important alternative tool for specific and sensitive detection of these viruses. The increased sensitivity of RT-PCR compared to virus isolation has already been demonstrated for other clinically important picornaviruses such as enteroviruses and rhinoviruses (*16–19*). Nevertheless, HPeVs could not be detected by most molecular assays designed for HEV diagnosis because of considerable sequence differences between these 2 viral genera (*20–22*). In this study, we

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developed a single RT-PCR test for detecting the 3 HPeV types based on sequence alignments of HPeV-1, -2, and -3 reference strains and various clinical HPeV isolates. This test was applied for the identification of several putative HPeV isolates recovered in Quebec (Canada) during the last 2 decades (1985–2004).

Materials and Methods

Viral Strains and Cell Culture Procedures

A total of 30 HPeV strains, including the reference strains Harris (HPeV-1, ATCC VR-52), and Williamson (HPeV-2, ATCC VR-53), as well as 28 clinical HPeV isolates were used in this study (Table). Clinical specimens of various origins, such as nasopharyngeal aspirates (NPA), throat swabs, stools, cerebrospinal fluid (CSF), and endotracheal secretions (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no06/05-1675.htm#table_app), were first added to different continuous cell lines, including human lung adenocarcinoma (A-549), human rhabdosarcoma (RD), transformed human kidney (293), human colon adenocarcinoma (HT-29), human laryngeal carcinoma (Hep-2), human foreskin fibroblast, mink lung, African green monkey kidney (Vero), Madin Darby canine kidney , and rhesus monkey kidney (LLC-MK2) cells. The viral cultures were incubated for 3 weeks at 37°C in a 5% CO_2 atmosphere. Viral isolates with cytopathic effects (CPE) suggestive of HEV or HPeV were further analyzed by neutralization assays with Lim and Benyesh-Melnick antiserum pools A-H (National Institutes of Health, Bethesda, MD, USA) and specific antisera for HPeV-1 and -2 (MA Bioproducts, Walkersville, MD, USA).

Molecular Characterization

HPeV genomic RNA was isolated from the supernatant of infected cell cultures with the QIAamp Viral RNA kit (Qiagen, Mississauga, Ontario, Canada). cDNA was prepared by using the HPeVUniv5′ primer selected from the C-terminal region of the capsid VP0 encoding gene: 5′-GCT GAC CTA TGY ATC CCC TAT GT-3′ (nt 1358–1379, GenBank accession no. AJ998818) and the SuperScript II reverse transcriptase (Gibco BRL, Burlington, Ontario, Canada). Viral cDNA was then amplified by PCR by using the *Pfu* Turbo Polymerase (Stratagene, La Jolla, CA, USA) and primers HPeVUniv5′ and HPeVUniv3′ (selected from the N-terminal region of the capsid VP1 encoding gene): 5′-GTG AAC CCC AYG AAT TTT GGA A-3′ (nt 2351–2330, accession no. AJ998818). After a primary denaturation step at 95°C for 5 min, 35 cycles of denaturation at 94°C for 50 s, annealing at 58°C for 50 s, and extension at 72°C for 2 min were performed, followed by a final extension step at 72°C for 7 min. PCR products were analyzed by electrophoresis on a 1.2 % agarose gel and the expected amplicons of ≈1,000 bp were purified by using the GenElute PCR clean-up kit (Sigma-Aldrich, St-Louis, MO, USA). Nucleotide sequences of PCR products were determined with PCR primers by using an automated DNA sequencer (ABI Prism 377A; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were analyzed, and the deduced amino acid sequences were aligned by using ClustalW (available from http://www.infobiogen.fr/). The resulted alignment was analyzed by the neighbor-joining method with the MEGA2 (Biodesign Institute, Arizona State University, Phoenix, AZ, USA) program for the construction of the phylogenetic tree.

Table. Molecular and virologic characteristics of Canadian HPeV isolates*

Virus	HPeV type	% nt/aa identities†	Neutralization assay‡
Harris	1	NA	+
Williamson	2	NA	+
Can4541-85	1	76.7/89.9	+
Can-5188-85	1	76.8/89.9	+
Can11750-87	1	76.8/89.9	+
Can11758-87	1	76.8/89.9	+
Can29218-90	1	76.4/89.2	+
Can29192-90	1	75.0/89.6	+
Can40057-93	1	76.8/89.6	+
Can41934-93	1	77.1/89.6	+
Can61165-97	1	77.0/89.9	+
Can82753-01	1	76.5/89.2	+
Can81805-01	1	85.1/97.5	+
Can87376-02	1	76.4/89.6	+
Can87639-02	1	76.4/89.6	+
Can88461-02	1	77.1/89.9	+
Can88770-02	1	76.3/89.2	+
Can85372-02	1	76.5/89.2	+
Can84436-02	1	76.9/89.6	+
Can101909-04	1	76.6/89.6	+
Can102318-04	1	76.6/89.6	+
Can100121-04	1	77.0/89.2	+
Can82047-01	2	81.1/96.4	+
Can95219-03	2	67.8/72.5	–
Can95224-03	2	67.8/72.5	–
Can81235-01	3	95.3/96.1	–
Can81554-01	3	95.0/95.7	–
Can82853-01	3	95.5/96.4	–
Can99190-04	3	95.0/96.1	–
Can97858-04	3	95.0/96.4	–

*HPeV, human parechovirus; nt, nucleotide; aa, amino acid; NA, not applicable.
†% identities were calculated by comparison with Harris (accession no. S45208), Williamson (accession no. AJ005695) and A308/99 (accession no. AB084913) reference strains for HPeV-1, -2 and -3 isolates, respectively.
‡Neutralization assays were performed by using specific HPeV-1 and -2 (formerly echovirus 22 and 23, respectively) antisera (MA Bioproducts, Walkersville, MD, USA).

Clinical Data and Statistical Analysis

Demographic and clinical informations were retrospectively collected from clinical charts of hospitalized patients for whom a positive culture for HPeV was obtained in our laboratory in the past 20 years. The Mann-Whitney rank sum test was used to compare the mean age of patients infected with HPeV-1 versus HPeV-3; the Fisher exact test was used to compare HPeV-1 versus the 2 other HPeV types regarding the rate of underlying diseases and the nature of initial diagnosis.

Results

Virologic and Molecular Findings

Among the different cell lines used, HT-29 was found to be the most suitable for efficient isolation of HPeV-1 and -2. Both grew efficiently in this cell line with an evident CPE noted after a mean incubation time of 3.8 days (range 1–8 days). A presumptive distinction between HPeV- and HEV-induced CPE in this cell line was possible. Parechovirus-infected cells were large, regularly shaped spheres, whereas enterovirus-infected cells were rather small with an irregular shape (Figure 1A). On the other hand, HPeV-3 isolates only initially grew on LLC-MK2 cells after a mean incubation time of 16 days (range 14–17 days). On passage, however, these viruses grew rapidly (in ≈1–3 days) in the same cell line (Figure 1B) and in Vero cells. All tested HPeV-1 and only one third of HPeV-2 (Can82047-01) isolates were neutralized by specific antisera for HPEV-1 and -2 (Table).

All HPeV clinical isolates as well as the 2 HPeV-1 and -2 reference strains were efficiently amplified by our RT-PCR test, generating an amplicon of the expected size (≈1,000 bp). No PCR products were generated from enterovirus (coxsackievirus A4 and coxsackievirus B4) and rhinovirus (n = 2) clinical isolates. Conversely, our HPeV isolates were negative when tested by our HEV RT-PCR assay, which is based on the amplification of the 5′ noncoding region. Sequencing of HPeV PCR products confirmed the presence of the VP3 gene in our HPeV-1 (n = 20), HPeV-2 (n = 3) and HPeV-3 (n = 5) clinical isolates. Sequence analysis showed that HPeV-1 clinical isolates had 75.0% to 85.1% nt and 89.2 to 97.5% amino acid identities with the prototype Harris strain (accession no. S45208) (Table). HPeV-2 isolates had nucleotide and amino acid identities of 67.8% to 81.1% and 72.5% to 96.4%, respectively, with the prototype Williamson strain (accession no. AJ005695). Of note, Can95219-03 and Can95224-03 HPeV-2 isolates had the same sequence and were more related to the Connecticut CT-80-6760 HPeV-2 strain (accession no. AF055846) with which they had nucleotide and amino acid identities of 85.1% and 95.8%, respectively. HPeV-3 isolates had nucleotide and amino acid identities of 95.0% to 95.5% and 95.7% to 96.4%, respectively, with the Japanese A308/99 HPeV-3 isolate (accession no. AB084913).

Phylogenetic analysis confirmed the identities of HPeV isolates. The HPeV-1 isolate Can81805-01 was closely related to the reference Harris strain (lineage II), whereas the remaining 19 isolates from this study, as well as 3 other Japanese HPeV-1 isolates (A1087-99, A942-99, and A10987-00), were found to form a distinct cluster (lineage I) (Figure 2). The HPeV-2 isolate Can82047-01 was related to the reference Williamson strain (lineage I), whereas the other 2 HPeV-2 isolates, Can95219-01 and Can95224-01 HPeV-2, were somewhat related to the Connecticut CT80-6760 strain (lineage II). Finally, all HPeV-3 isolates formed a separate cluster that also included other Japanese

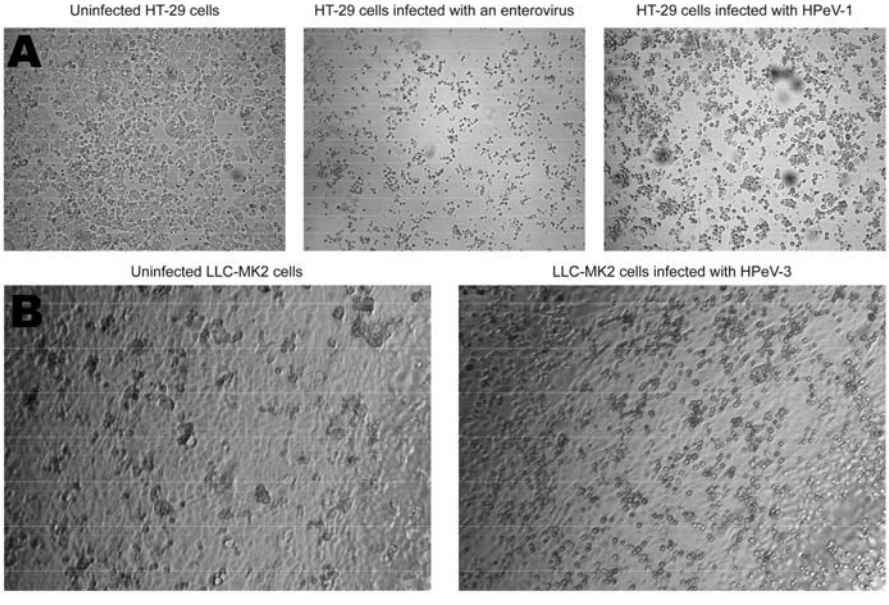

Figure 1. Cytopathic effects (CPEs) observed 3 days after infection of HT-29 cells with an enterovirus or a human parechovirus (HPeV)-1 isolate after 1 passage on this cell line (A); CPE observed 1 day after infection of LLC-MK2 cells with a HPeV-3 isolate after 3 passages on this cell line (B).

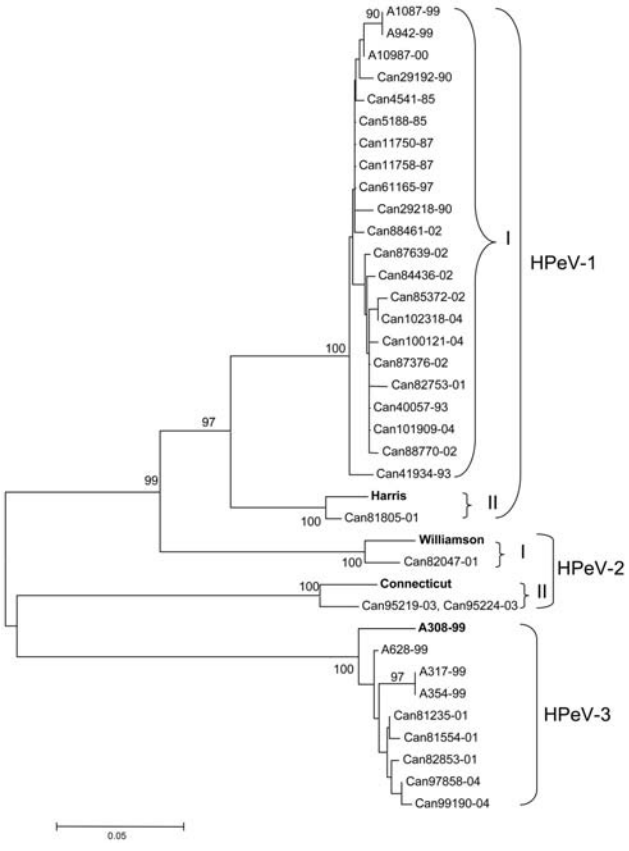

Figure 2. Phylogenetic analysis of Canadian human parechovirus (HPeV) isolates and HPeV-1 (Harris, GenBank accession no. S45208), HPeV-2 (Williamson, AJ005695, and Connecticut, AF055846), and HPeV-3 (A308-99, AB084913) reference strains based on the ClustalW alignment of the VP3 amino acid sequences. Japanese HPeV-1 (A1087-99, accession no. AB112485; A942-99, AB112486; and A10987-00, AB112487) and HPeV-3 (A628-99, accession no. AB112484; A317-99, AB112482, and A354-99, AB112483) isolates were also included in the analysis. The tree was constructed by using the neighbor-joining method with the MEGA 2 program. Bootstrap probabilities for 550 replicas are shown at the branch nodes. Only values of 70% to 100% are indicated. **Boldface** indicates HPeV reference strains.

HPeV-3 isolates (A308-99, A628-99, A317-99, and A354-99).

The alignment of the predicted amino acid sequences of the partial VP0–VP3 region of Canadian HPeV-1, -2 and -3 isolates with the reference strains showed differences in the VP0/VP3 cleavage site, which was N/N in Can952219-03 and Can95224-03 (HPeV-2), N/S in Connecticut CT80-6760, T/A in Williamson and Can82047-01 (HPeV-2), N/A in Harris and all Canadian HPeV-1 isolates, and N/G in all HPeV-3 isolates (Figure 3). The length of the N-terminal extension of the VP3 protein, which is a molecular feature of parechoviruses, was also shown to differ from 27 residues in HPeV-1 of lineage I to 34 residues in HPeV-2 of lineage II. HPeV-1 of lineage II and HPeV-2 of lineage I had an extension of 28 residues, whereas an extension of 30 residues was seen in all HPeV-3 isolates (Figure 3).

Clinical Manifestations of HPeV Infections

Twenty-eight HPeV isolates, recovered in the Quebec City area between 1985 and 2004, were available for this study (Table). This collection included 20 (71.4%) HPeV-1, 3 (10.7%) HPeV-2, and 5 (17.8%) HPeV-3. The viral isolates were recovered predominantly from NPAs (n = 16), but also from throat swab specimens (n = 2), endotracheal secretions (n = 2), stool samples (n = 7), and CSF (n = 1) (online Appendix Table). In addition, for 2 patients, a second concomitant HPeV isolate was recovered from another site (urine in patient 7 and NPA from patient 20). Complete clinical findings were obtained for 23 (82.1%) of the 28 patients, whereas partial information was available for the remaining 5 patients.

Twenty-seven (96.4%) of the 28 patients were children ≤4 years (mean 11.1 months, median 7.0 months, range 1 week to 4 years). Mean age was 14.6, 6.3, and 0.7 months for HPeV-1–, HPeV-2–, and HPeV-3–infected children, respectively ($p<0.001$ for comparison of HPeV-1 vs HPeV-3). The 78-year-old adult excreted HPeV-1 in her endotracheal secretions. The female-to-male ratio was 1.2 (54.2% vs. 45.8%). Seventy-five percent of all HPeV isolates were recovered during the typical enterovirus season, i.e., during summer (28.6%) and fall (46.4%). The epidemiology of the newly described HPeV-3 was similar to that of other HPeVs, with 80% of this viral serotype isolated during the summer-fall period.

Among patients for whom information was available, 33.3% (8/24) had an underlying disease, including 2 children with acute lymphoblastic leukemia (ALL) (online Appendix Table). Patients with underlying diseases represented 47.0% (8/17) of those who excreted HPeV-1 compared to none of the 7 HPeV-2–or HPeV-3–infected patients ($p = 0.18$ for comparison of HPeV-1 vs other HPeVs). Twenty-four (96.0%) of the 25 patients with available information were hospitalized (mean number of days 11.4, median 3.0, range 1–129). However, if the 3 patients with nosocomial HPeVs are excluded, the mean duration of hospitalization was 3.7 days; this finding was similar for the different HPeV types (mean of 3.3, 3.0, and 5.0 days for HPeV-1, -2 and -3, respectively). Only 3 (12.0%) of 25 patients were admitted to the intensive care unit. These 3 patients were infected with HPeV-1, and 2 of them (patients 6 and 26), a 4-year old boy with ALL and a 78-year-old woman, died of respiratory failure. A copathogen was found in 5 (20.8%) of 24 HPeV-infected patients, including 3 patients with human respiratory syncytial virus (HRSV), 1 with adenovirus, and 1 with human parainfluenza virus (HPIV) type 3.

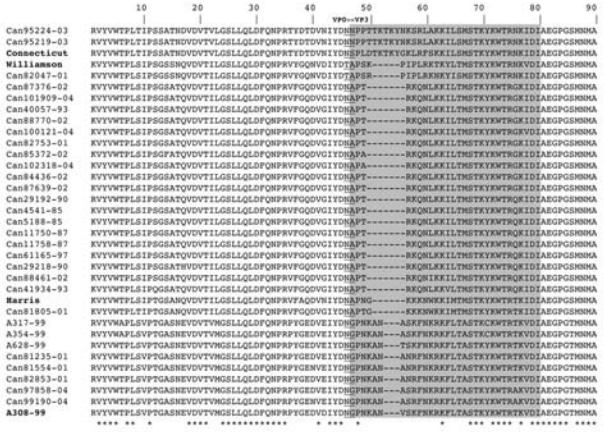

Figure 3. Comparison of the predicted partial VP0-VP3 capsid protein region of Canadian isolates with that of Harris (human parechovirus [HPeV]-1), Williamson and Connecticut (HPeV-2), and A308-99 (HPeV-3) strains. The aligned region contains 90 amino acids corresponding to residues 244 to 334 in the Harris polyprotein sequence (accession no. S45208). The VP3 N-terminal extension, which is specific to parechoviruses (21), is shaded. The cleavage site between VP0 and VP3 is underlined. Asterisks denote conserved residues. **Boldface** indicates HPeV reference strains.

Among the 24 children with available information, bronchiolitis, pneumonitis, or both, were reported as the final diagnosis in 50.0% of cases, whereas acute otitis media, sinusitis, and conjunctivitis were secondary diagnoses in 37.5%, 8.3%, and 4.2% of cases, respectively (online Appendix Table). In addition, the final diagnosis was reported as viremia/sepsis in 29.2% of children, whereas enteritis, de novo convulsions, and shock were found in 29.2%, 4.2%, and 4.2% of patients, respectively. Final diagnosis was unknown for 3 (11.1%) of 27 children (all 3 had positive stool viral cultures). All children (7/7) with HPeV-3 and HPeV-2 infections were admitted with a diagnosis of viremia or sepsis, and all received broad-spectrum antimicrobial drugs until results of bacterial cultures (blood, CSF, urine), whereas none (0/17) of the HPeV-1-infected children had such an initial diagnosis ($p = 0.001$ for comparison of HPeV-1 vs. other HPeVs). Conversely, bronchiolitis was found in 8 (47.0%) of 17 children with HPeV-1 infections and in none of the patients (0/7) infected with the other 2 serotypes ($p = 0.18$ for comparison of HPeV-1 vs other HPeVs). However, another viral pathogen (HRSV or HPIV-3) was found in addition to HPeV-1 in 4 (50%) of 8 children with bronchiolitis.

A few cases are particularly worth noting. As previously mentioned, patient 6 was the only child who died after HPeV infection in our cohort. At the time of his HPeV-1 infection, the 4-year-old boy was receiving consolidation chemotherapy for ALL. He was admitted with high fever (40°C), dry cough, and abdominal pain. Over a course of 4 to 5 days, the following conditions developed: bilateral pneumonitis, which required mechanical ventilation, hepatitis, and septic shock complicated by renal insufficiency, for which dialysis was necessary. He died of multiorgan failure while receiving treatment with amphotericin B, acyclovir, meropenem, and vancomycin; no autopsy was performed. Apart from isolation of HPeV-1 from a throat swab, no other microorganisms (bacteria, fungi, and viruses) were recovered from blood, urine, and endotracheal secretions. Also of interest are the clinical signs and symptoms of 1-month-old twins infected by HPeV-2, who were admitted to the hospital on the same day with high fever and irritability. In 1 case (patient 20), HPeV-2 was recovered from CSF and NPA samples, whereas the virus was only isolated in NPA from the other twin (patient 21). The viruses from the 2 children had identical VP3 gene sequences. Both infants received broad-spectrum antimicrobial drugs (ampicillin plus cefotaxime) for 3 days for suspected sepsis, and both had rapid clinical improvement. Patient 26 was the only adult infected with HPeV (type 1) in our study. This 78-year-old woman was admitted to the hospital for several fractures caused by a car accident. One month after admission, bilateral pneumonia developed. She eventually died of respiratory failure 43 days after admission. An endotracheal culture was positive for HPeV-1 12 days before her death; however, results of bacterial cultures were unavailable because part of her clinical chart was destroyed a few years after her death in 1987. No autopsy report was available.

Discussion

In this study, we retrospectively characterized 28 clinical HPeV isolates recovered during the last 2 decades in Québec, Canada, using a new RT-PCR assay. To date, our study is the only one that analyzed virologic and clinical data from the 3 HPeV serotypes. The study clearly demonstrated the importance of these pathogens and highlighted some clinical differences between infection caused by the 3 serotypes.

Virus isolation, followed by neutralization assays, a procedure that has been generally used for identification of HEVs and HPeVs, is laborious, time-consuming, and may lack sensitivity. Because of substantial sequence differences between HEVs and HPeVs, even within the conserved 5′ noncoding region (*20–22*), most molecular techniques designed for detection of HEVs cannot detect HPeVs (*13,14*). We developed a RT-PCR test for specifically detecting all 3 types of HPeV by using primers selected from the C-terminal and N-terminal regions of VP0 and VP1 HPeV capsid proteins, respectively. These regions were found to be conserved in various HPeV-1, -2, and -3 genomic sequences (data not shown). The PCR product of ≈1,000 bp encompasses the entire VP3 gene,

whose sequence differs substantially between the 3 HPeV types, allowing their discrimination. By the use of this test, we were able to identify HPeV-1 and -2 reference strains as well as 28 HPeV clinical isolates, in contrast to neutralization assays that failed to recognize 2 HPeV-2 isolates by using specific HPeV-1 and -2 antisera. This molecular test appears to be specific for HPeV since no amplifications were found with samples of related viruses, including enteroviruses and rhinoviruses.

By sequencing the PCR product, we could not only differentiate the 3 HPeV types but also make an intratypic discrimination. Our phylogenetic analysis demonstrated the existence of 2 clusters for HPeV-1 strains. The first contained a Can81805-01 isolate and was closely related to the Harris reference strain; the second cluster included the remaining 19 Canadian isolates as well as recent Japanese HPeV-1 isolates (Figure 2). A similar pattern was found in a VP1-based phylogenetic study of Dutch strains in which all HPeV-1 isolates formed a cluster that was distinct from that of the Harris reference strain (*13*). Thus, most recent European, Japanese, and Canadian HPeV-1 isolates appear to be, to a certain extent, genetically distinct from the only known HPeV-1 reference strain. Designs of molecular or serologic diagnostic tools should take this fact into consideration. Our molecular characterization also enabled us to detect and differentiate the 2 known HPeV-2 lineages with 1 isolate close to the Williamson reference strain and the other 2 isolates being more related to the Connecticut strain. On the other hand, our HPeV-3 were found to be rather genetically homogeneous and constituted a unique cluster, which also included the Japanese HPeV-3 strains. Since HPeVs are considered important nosocomial agents, differentiating viral strains for epidemiologic purposes may be relevant. Our molecular approach demonstrated the link between 2 HPeV-2 isolates recovered from twins (100% nt identity) and showed that all other HPeV-1 and -3 isolates were unrelated.

The HPeV-1 serotype was shown to be the most frequent, representing >70% of our HPeV isolates. The HPeV-2 serotype was the less frequent, with only 3 cases reported. A very limited number of HPeV-2 infections has also been reported by a Swedish group (*8*). In addition, in a recent Dutch study that reported 37 HPeV isolates recovered during the 2000–2005 period, no HPeV-2 isolates were recovered compared to 27 HPeV-1 and 10 HPeV-3 (*13*). The recently described HPeV-3 serotype, although less frequent than HPeV-1, appears to be more frequent than HPeV-2, both in our study and in the Dutch study (*13*).

HPeVs have been generally isolated from stool and NPA (*6–8*). Accordingly, our isolates were predominantly isolated from NPA (57%) and stool (25%) specimens; however, some isolates were also recovered from other types of samples, including throat swabs, endotracheal secretions, CSF, and urine, highlighting the need to collect multiple specimens for optimal detection of these viruses.

With the exception of one 78-year-old case-patient, all HPeV infections described in this retrospective study were in children ≤4 years, which confirms the particular importance of these pathogens in the pediatric population (*3*). However, only prospective evaluation will determine the true incidence of HPeV infections in both the pediatric and adult populations. Interestingly, all of our HPeV-3 cases involved neonates with a mean age of 0.7 month, whereas HPeV-1 infections occurred in significantly older children whose mean age was 14.6 months. A similar finding was reported in Dutch patients (*13*), and this situation could be related to the presence of variable proportions of maternal antibodies against these different viruses.

Our study showed that HPeVs seem to share with HEVs the same epidemiologic pattern, i.e., a peak during the summer-fall months. In addition, the epidemiology of HPeV-3 appears to be similar to that of the 2 other HPeV types in our study, whereas it was slightly different in the Dutch study (*13*). A better knowledge of the epidemiology of these viruses may have important consequences for diagnostic laboratories. A multiplex RT-PCR that allows the simultaneous detection of HEVs and HPeVs could be particularly useful during the summer-fall seasons if the epidemiologic pattern seen in our study is confirmed by others.

Most HPeV cases reported here were associated with hospitalizations (mean hospital stay 3.7 days). No differences were found between the 3 HPeV types in this aspect. Nevertheless, only HPeV-1 infections led to severe diseases, i.e., 3 patients were transferred to an intensive care unit. Our study also showed the high rate of underlying diseases in our infected patients, which were particularly frequent (≈50%) in HPeV-1 cases. Also, substantial differences in clinical signs and symptoms were seen between the HPeV types. All 7 patients with HPeV-2 and -3 infections were admitted with a diagnosis of sepsislike illness in contrast to none of the 20 HPeV-1 patients ($p = 0.001$). Sepsislike illness seems to be a notable clinical feature of HPeV-3 infection, as first reported by us (*11*) and recently by a Dutch group (*13*). This clinical variation may be explained by the younger age of HPeV-2– and -3–infected children.

HPeV-1 has been reported to cause mainly gastrointestinal and respiratory diseases and fewer CNS symptoms (*3,8,9,13*). Accordingly, bronchiolitis, pneumonitis, acute otitis media, and enteritis were frequently diagnosed in HPeV-1–infected children in our study, whereas this HPeV type was not involved in CNS infections. Of interest, bronchiolitis was seen only in HPeV-1–infected children, representing almost 50% of the cases. However, in 4 of

these 8 cases, a paramyxovirus (HRSV or HPIV) was found as copathogen; thus, the relative contribution of HPeV-1 in cases of bronchiolitis needs to be further studied. Our study also suggested that HPeV-1 could be involved in severe infections, as shown by 2 cases of bilateral pneumonia, leading to death in a 4-year-old boy with ALL and in a 78-year-old woman. Since no autopsy was performed, the role of HPeV-1 in these fatal cases cannot be confirmed, although no other microorganisms could be recovered premortem.

In conclusion, this study confirms that all 3 HPeVs are pathogens that are particularly important in the pediatric setting, that is, responsible for hospitalizations and some severe infections. HPeV infections could encompass a variety of clinical syndromes with respiratory, gastrointestinal, cerebral, and sepsislike diseases. Molecular assays now allow for their specific detection and should contribute to increased knowledge regarding their incidence, epidemiologic features, and clinical manifestations.

Acknowledgments

We are grateful to Suzanne Tessier and Marthe Gauthier for their valuable technical assistance.

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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: San Diego: Academic Press; 2000. p. 657–78.
2. Niklasson B, Kinnunen L, Hornfeldt B, Horling J, Benemar C, Hedlund KO, et al. A new picornavirus isolated from bank voles (*Clethriomys glareolus*). Virology. 1999;255:86–93.
3. Stanway G, Joki-Korpela P, Hyypia T. Human parechoviruses-biology and clinical significance. Rev Med Virol. 2000;10:57–69.
4. Koskiniemi M, Paeteau R, Linnavuori, K. Severe encephalitis associated with disseminated echovirus 22 infection. Scand J Infect Dis. 1989;21:463–6.
5. Figueroa JP, Ashley D, King D, Hull B. An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. J Med Virol. 1989;29:315–9.
6. Joki-Korpela P, Hyypia T. Diagnosis and epidemiology of echovirus 22 infections. Clin Infect Dis.1998;26:129–36.
7. Wigand R, Sabin AB. Properties of ECHO types 22, 23, and 24 viruses. Arch Gesamte Virusforsch. 1961;11:224–47.
8. Ehrnst A, Eriksson M. Echovirus type 23 observed as a nosocomial infection in infants. Scand J Infect Dis. 1996;28:205–6.
9. Ehrnst A, Eriksson M. Epidemiological features of echovirus type 22 infection. Scand J Infect Dis. 1993;25:275–81.
10. Ito M, Yamashita T, Tsuzuki H, Takeda N, Sakae K. Isolation and identification of a novel human parechovirus. J Gen Virol. 2004;85:391–8.
11. Boivin G, Abed Y, Boucher FD. Human parechovirus-3 and neonatal infections. Emerg Infect Dis. 2005;11:103–5.
12. Sedmack G, Jentzen J. Human parechovirus type 3 (HPeV-3) association with three infant deaths in Wisconsin from September 2003 through August 2004. Association of Public Health Laboratories–Infectious Disease Conference. Orlando, Florida, March 2–4, 2005. Abstract T-22.
13. Benshop KS, Shinkel J, Minnaar RP, Pajkrt D, Spanjerberg L, Kraaman HC, et al. Human parechovirus infections in Dutch children and the association between serotype and disease severity. Clin Infect Dis. 2006;42:204–10.
14. Legay V, Chomel JJ, Lina B. Specific RT-PCR procedure for the detection of human parechovirus type 1 genome in clinical samples. J Virol Methods. 2002;102:157–60.
15. Jokela P, Joki-Korpela P, Maaronen M, Glumoff V, Hyypia T. Detection of human picornaviruses by multiplex reverse transcription-PCR and liquid hybridization. J Clin Microbiol. 2005;43:1239–45.
16. Rotbart HA. Enzymatic amplification of the enteroviruses. J Clin Microbiol. 1990;28:438–42.
17. Guney C, Ozkaya E, Yapar M, Gumus I, Kubar A, Doganci L. Laboratory diagnosis of enteroviral infections of the central nervous system by using a nested RT-PCR assay. Diagn Microbiol Infect Dis. 2003;47:557–62.
18. Vuorinen T, Vainionpaa R, Hyypia T. Five years' experience of reverse-transcriptase polymerase chain reaction in daily diagnosis of enterovirus and rhinovirus infections. Clin Infect Dis. 2003;37:452–5.
19. Kares S, Lonnrot M, Vuorainen P, Oikarinen S, Tauriainen S, Hyoty H. Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using Light-Cycler. J Clin Virol. 2004;29:99–104.
20. Abed Y, Boivin G. Molecular characterization of a Canadian human parechovirus (HPeV)-3 isolate and its relationship to other HPeVs. J Med Virol. 2005;77:566–70.
21. Ghazi F, Hughes PJ, Hyypiä T, Stanway G. Molecular analysis of human parechovirus type 2 (formerly echovirus 23). J Gen Virol. 1998;79:2641–50.
22. Hyypiä T, Horsnell C, Maaronen M, Khan M, Kalkkinen N, Auvinen P, et al. A distinct picornavirus group identified by sequence analysis. Proc Natl Acad Sci U S A. 1992;89:8847–51.

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2,500-year Evolution of the Term Epidemic

Paul M.V. Martin* and Estelle Martin-Granel†

The term epidemic (from the Greek *epi* [on] plus *demos* [people]), first used by Homer, took its medical meaning when Hippocrates used it as the title of one of his famous treatises. At that time, epidemic was the name given to a collection of clinical syndromes, such as coughs or diarrheas, occurring and propagating in a given period at a given location. Over centuries, the form and meaning of the term have changed. Successive epidemics of plague in the Middle Ages contributed to the definition of an epidemic as the propagation of a single, well-defined disease. The meaning of the term continued to evolve in the 19th-century era of microbiology. Its most recent semantic evolution dates from the last quarter of the 20th century, and this evolution is likely to continue in the future.

At the start of the 21st century, epidemics of infectious diseases continue to be a threat to humanity. Severe acute respiratory syndrome, avian influenza, and HIV/AIDS have, in recent years, supported the reality of this threat. Civil wars and natural catastrophes are sometimes followed by epidemics. Climate change, tourism, the concentration of populations in refugee camps, the emergence of new human pathogens, and ecologic changes, which often accompany economic development, contribute to the emergence of infectious diseases and epidemics (*1*). Epidemics, however, have occurred throughout human history and have influenced that history. The term epidemic is ≈2,500 years old, but where does it come from?

Before Hippocrates

When works that put forward new ideas are translated, determining the original terminology (in Ancient Greek in this case) is not easy. In 430 BC, when Hippocrates was collecting the clinical observations he would publish in Epidemics, his treatise that forms the foundation of modern medicine, at least 3 terms were used in Ancient Greece to describe situations that resembled those described by Hippocrates: *nosos*, *phtoros*, and *loimos* (*2*).

Nosos, meaning disease, was used by Plato in the 4th century BC and clearly had the same meaning 2 centuries earlier in the works of Homer and Aeschylus. Nosos encompasses disease of the mind, body, and soul: physical, including epilepsy, and moral (i.e., psychological and psychiatric). Phtoros or *phthoros* means ruin, destruction, deterioration, damage, unhappiness, and loss, after war for example. The word was frequently used by Aeschylus and Aristophanes, was known in the 8th century BC, and was later used by Plato and Thucydides. Its meaning has remained general. Bailly translates loimos as plague or contagious scourge. Used by Esiodus in the 7th century BC and later by Sophocles and Herodotus, this term is ancient. Its translation as plague should be interpreted in the sense of a scourge rather than as the disease plague. In the Septuagint, a translation of the Old Testament into Greek by 70 Greek Jews from Alexandria, this word is used in the book of Kings to describe the 10 plagues of Egypt.

But the term epidemic already existed in 430 BC. The Greek word *epidemios* is constructed by combining the preposition *epi* (on) with the noun *demos* (people), but demos originally meant "the country" (inhabited by its people) before taking the connotation "the people" in classical Greek. Indeed, the word epidemios was used by Homer, 2 centuries before Hippocrates, in the Odyssey (canto I, verses 194 and 230), where it was used to mean "who is back home" and "who is in his country" in contrast to a voyager who is not: δὴ γάρ μιν ἔφανι ᾽ἐπιδήμιον εἶναι σὸν πατέρα, "because someone said that your father was back (home)" (canto I, verse 194). In this context, epidemios means indigenous or endemic. In the Iliad, Homer confirmed this meaning (canto XXIV, verse 262), by using

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also *polemos epidemios* to mean civil war: , "this one who ὃς πολέμου ἔραται ἐπιδημίου οκρυόευτος liked passionately the frightening civil war" (canto IX, verse 64). Later, Plato and Xenophon (400 BC) used the word to describe a stay in a country or the arrival of a person: Παριος ὅυ ἐγὼ ἠσθόμην ἐπιδημοῦντα , "a Parian who, I learned, was in town" (Plato, Apology, chapter I, paragraph 38). The verb *epidemeo* was used by Thucydides (460 BC–395 BC) to mean "to stay in one's own country," in contrast to *apodemeo*, "to be absent from one's country, to travel." For Plato, epidemeo meant "to return home after a voyage, to be in town." Later, the orators Demosthenes (384 BC–322 BC) and Eschines (390 BC–314 BC) used this word to refer to a stranger who came to a town with the intention of living there, and the verb epidemeo was used to mean "to reside." Typical of Greek semantics, epidemeo takes its meaning from the result of the action, rather than from the action itself. It relates to something that has already happened, with the implication that it had previously happened elsewhere. Authors before Hippocrates used epidemios for almost everything (persons, rain, rumors, war), except diseases. Hippocrates was the first to adapt this word as a medical term.

Hippocrates and the Term Epidemic

Written in the 5th century BC, Hippocrates' Corpus Hippocraticum contains 7 books, titled Epidemics (*3*). Hippocrates used the adjective epidemios (on the people) to mean "which circulates or propagates in a country" (*4*). This adjective gave rise to the noun in Greek, *epidemia*.

We do not know why Hippocrates chose epidemios to title his books instead of nosos, a well-established term meaning disease. Examining the meaning of the term before, during, and after his time may help us understand his choice. Schematically speaking, epidemios (or epidemeo) was used successively to mean "being at homeland" (Homer), "arriving in a country" or "going back to homeland" (Plato), and later "stranger coming in a city" (Demosthenes). Sophocles (495 BC–406 BC) used the adjective in Oedipus Tyrannos to refer to something (a rumor, noise, fame, or reputation) spreading in a country: εἶμ' Οἰδιπόδα ἐπὶ τ'αν ἐπίδαμον φ'ατιν , "I shall go (to make war) to Oedipus, against his fame which spread (in the country)" (verse 494). Oedipus Tyrannos was written at approximately the same time as Corpus Hippocraticum; consequently, we can infer that during Hippocrates' time, epidemios acquired a dynamic meaning, probably more adapted to describing a group of physical syndromes that circulate and propagate seasonally in a human population (i.e., on the people) than nosos, a term used to describe diseases at the individual level.

How epidemios, meaning "on the people," became adapted to mean "that which circulates or propagates in a country" is a crucial question. This evolution occurred during the second half of the 5th century (450 BC–400 BC), a period of intense activity in Greek literature, particularly with the prolificacy of Sophocles. But while nosos or loimos were frequently used, epidemios was not. In the Perseus Digital Library (www.perseus.tufts.edu), a database that does not yet include Hippocrates' works, the adjective epidemios was used only 9 times, including 4 times in Homer, in the 489 major referenced Greek texts (≈4.8 millions words, 0.02 occurrences per 10,000 words). Its Doric variants *epidamos* and *epidemos* were used 3 times. In comparison, nosos (disease) was used 712 times in the Perseus database (1.47 occurrences per 10,000 words). The verb epidemeo was used 144 times, primarily during the 4th century and always meaning "to live in or return to one's own country." This lack of material makes accurately exploring the reasons for the semantic evolution across centuries difficult. In Oedipus Tyrannos, Sophocles qualified the sense of epidemios as it referred to reputation or fame; fame naturally spreads in a country. But Hippocrates described a series of syndromes: καὶγαρ ἄλλως το νούσημα ἐπίδημον ἦν , "It is a fact that the disease was propagating in the country" (Epidemics, book I, chapter 3). Although Sophocles used epidemios once in that new sense, Hippocrates established a medical meaning for the term.

In Epidemics, books I and III constitute lists of diseases describing clinical cases. Hippocrates compared these cases and grouped them to generate series of similar cases. He adopted a classification approach, initially seeking clinical similarities between cases, thereby discovering, in addition to the notion of epidemic, the more fundamental concepts of symptom and syndrome. However, Hippocrates believed that prognosis was a major aspect of medicine. This belief led him to consider disease a dynamic process with its own progression, a temporal dimension, that represents a first nosologic evolution: syndromic groupings become diseases. Another of the books written by the physician from Kos—Airs, Waters, and Places—deals with the relationships between diseases and the environment, focusing particularly on the habitat of the patients and the season in which disease occurs. Hippocrates tried to determine the effect of environmental factors on what could be described as the distribution of diseases. He was, thus, more concerned about grouping together winter diseases or autumn diseases or diseases that occurred in a particular place or in persons whose way of life had changed than in identifying a large number of cases of the same disease in winter or autumn, at a particular place, or in association with a particular way of life. For Hippocrates, whose nosologic approach already contained a major element of preoccupation with the environment, the first meaning of epidemic was groups of cases

resembling each other clinically and the second meaning was groups of different diseases occurring at the same place or in the same season and sometimes spreading "on the people." Thus, Hippocrates applied the word epidemios to groupings of syndromes or diseases, with reference to atmospheric characteristics, seasons or geography, and sometimes propagation of a given syndrome in the human population.

Semantic confusion caused the great Emile Littré, who translated Hippocrates' works into French in the first half of the 19th century, to make a nosologic error. Hippocrates described what is known today, since the work of Littré, as the Cough of Perinthus. This account can be found in Epidemics book VI. Hippocrates described coughs that started toward the winter solstice and were accompanied by many symptoms: sore throat, leg paralysis, peripneumonia, problems with night vision, voice problems, difficulty swallowing, difficulty breathing, and aches. When Littré published his translation and commentaries on Epidemics in 1846, he mistakenly considered the Cough of Perinthus to be a single disease (*5*). This error made retrospectively diagnosing the diseases of Perinthus difficult, if not impossible. Moreover, as Littré saw this collection of illnesses as a single disease, he essentially turned it into an epidemic, probably because he had the modern sense of the term in mind and thought that Hippocrates had observed and described an epidemic illness unknown to modern medicine (*5*).

According to Grmek, "Littré took chapter VI, 7.1 as a general description of an epidemic in the sense of this word in the medical language of the 19th century rather than in the sense intrinsic to the works of Hippocrates. In the Corpus Hippocraticum, the noun 'epidemic' designates a collection of diseases observed at a given place, during a given period. A disease described as epidemic, such as epidemic cough, is a condition occurring from time to time in a given place, the appearance of which is closely linked to changes in season and climatic variations from year to year" (*5*).

Historians of medicine and philologists have over the years attributed the Cough of Perinthus to diphtheria, influenza, epidemic encephalitis, dengue fever, acute poliomyelitis, and many other diseases. However, a French physician named Chamseru, who practiced in the 18th century, almost a century before Littré, finally got to the bottom of what may be meant by the Cough of Perinthus, probably because the term epidemic had not yet taken on the meaning it had in Littré's time. According to Chamseru, the Cough of Perinthus could have encompassed several diseases, among them diphtheria, influenza, and whooping cough (*5*).

Thucydides and the First Descriptions of Epidemics

Thucydides (460 BC–395 BC) interrupted his account of the Peloponnesian War to describe the famous Plague of Athens, which occurred at the start of the summer in 430 BC. This description was long considered among the first descriptions of an epidemic. Indeed, whereas Thucydides used nosos, the term plague, which is used by all the translators of his work, is used in the sense of the Latin term *pestis*, a term with no clear etymology (*4*), meaning contagious disease, epidemic, or scourge. The description of the Plague of Athens, like that of the Cough of Perinthus by Hippocrates, is an essential text in the philologic and semantic study of epidemics (*5*). We must therefore consider, as for Littré's translation, the meaning that translators have assigned to the original description by Thucydides. Thucydides never used the term epidemic that Hippocrates was in the process of establishing. Under the term nosos, Thucydides described a series of clinical signs, which originated in the south of Ethiopia and propagated throughout Egypt, Libya, and then Greece. Thucydides used the words nosos, *kakos* (evil), *ponos* (pain), phtoros (ruin, destruction), and loimos (scourge) to describe what his translators call plagues. In her translation of Thucydides' works (*6*), published in 1991, in the chapter titled Second Invasion of Attica: the Plague of Athens (the original Greek work had no title), Jacqueline de Romilly translated nosos as disease or epidemic. Similarly, she translated loimos, kakos, and phtoros as disease or epidemic and the list of clinical signs (the original Greek meant "following these things") as symptoms. de Romilly rendered the text more elegant and accessible to 20th-century readers by this translation but gave the words used by Thucydides their 20th-century meaning rather than the meanings they had in the 5th century BC. Herein lies the principal problem of translation.

But was the Plague of Athens a true epidemic, in the modern sense? The death rate for the disease was extremely high, reaching up to 25% in 1 group of soldiers, and Pericles died of it. Historians have tried to understand the origin of this plague, and various diseases have been suggested, e.g., typhus, measles, smallpox, bubonic plague, ergotism, or an unknown disease. Thucydides wrote that all preexisting diseases were transformed into a plague and that persons in good health were affected in the absence of a predisposing cause (*7*). The large number of symptoms and of possible and probable causes rules out the possibility of an epidemic in the modern sense of the term. Instead, the Plague of Athens seems to have been the appearance of a large number of diseases that affected the population at the same time. Plague therefore has the same meaning here as epidemic in the works of Hippocrates. These 2 terms have been used in association or confused throughout

history. However, epidemic existed at this time, even if the notion of epidemic as we mean it in modern times had not yet emerged.

Evolution of the Term Epidemic

After the nonmedical use of the term epidemic by Homer, Sophocles, Plato, and Xenophon, Hippocrates gave it its medical meaning. However, the term has since undergone a long evolution. The adjective epidemios gave rise to the Greek noun *epidemia*. The Greek term epidemia in turn gave rise to the Latin term *epidimia* or *epidemia*. The term *ypidime* in Medieval French has its origins in these Latin words and went on to become *épydime* in the 14th century, *epidimie* in the 17th century, and then *epidémie* in the 18th century. Not until 22 centuries after Hippocrates, in the second half of the 19th century, were the terms *épidémiologie* (1855), *épidémiologique* (1878), and *épidémiologiste* (1896) coined in French and notions attached to them developed. Of course, at approximately the same time, corresponding terms appeared in the English language. The term epidemic and the terms linked to it therefore required an extremely long time to be constructed. This evolution is representative of the evolution of science and medicine over the centuries and reflects the semantic evolution of the term.

Semantic Evolution

In parallel with the evolution of the term epidemic itself, its meaning also changed over time. If we limit ourselves to the meaning that epidemic has acquired with respect to infectious diseases, we can identify 4 major steps in its semantic evolution in the medical sense. For Hippocrates, an epidemic meant a collection of syndromes occurring at a given place over a given period, e.g., winter coughs on the island of Kos or summer diarrheas on other islands. Much later, in the Middle Ages, the long and dramatic succession of waves of The Plague enabled physicians of the time to identify this disease with increasing precision and certainty; they began to recognize epidemics of the same, well-characterized disease. Then, with the historic contributions of Louis Pasteur and Robert Koch, epidemics of a characteristic disease could be attributed to the same microbe, which belonged to a given genus and species. The last stage in the semantic evolution of the term epidemic was the progressive acquisition of the notion that most epidemics were due to the expansion of a clone or clonal complex of bacteria or viruses known as the epidemic strain (*8*). More recently, microevolution of a clone of a bacterium (the epidemic strain) was shown to occur during an epidemic with person-to-person transmission (*9*). The Table summarizes these 4 major stages in the semantic evolution of the term epidemic.

In the second half of the 20th century, epidemic was also applied to noninfectious diseases, as in cancer epidemic or epidemic of obesity. The extension of the meaning to noninfectious causes refers to a disease that affects a large number of people, with a recent and substantial increase in the number of cases. This semantic extension of epidemic also concerns nonmedical events; the term is used by journalists to qualify anything that adversely affects a large number of persons or objects and propagates like a disease, such as crack cocaine or computer viruses.

What can we gain from investigating the origin and meaning of the word epidemic or from studying its semantic evolution? Beyond simply satisfying our curiosity, the slow evolution of the form and meaning of the term suggests that we still have much to learn about the concept of epidemic.

Acknowledgments

We thank Jean-Michel Alonso and Jean-Pierre Dedet for thorough reading and criticism of the manuscript.

Table. Semantic evolution of the term epidemic

Stage in evolution	Meaning	Use
Greek: *epi* (on) and *demos* (people) (6th century BC); *epidemios* used by Homer in the Odyssey	Who is in his country	Nonmedical use
Greek: Sophocles and Hippocrates (second half of the 5th century BC)	That which circulates and propagates in a country	First medical use
Greek: *epidemios* established by Hippocrates (430 BC) in the medical sense of a collection of syndromes	Sometimes spreading “on the people”	Epidemic of diarrhea
Medieval French: *ypidime* (1256 and later, *epidimie*)	Large number of cases of unique, well-characterized disease	Epidemic of cholera
19th century: *épidémie* (late 18th-century French) and *epidemic* (18th-century English)	Epidemics caused by a microbe belonging to a given genus and species	Epidemic of cholera due to *Vibrio cholerae*
End of 20th century	Clonal expansion of an epidemic strain, as defined with molecular markers	An epidemic due to *V. cholerae* El Tor, belonging to a defined ribotype or pulsotype

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References

1. Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis. 1995;1:7–14.
2. Bailly A. Dictionnaire grec – français. Paris: Payot; 1950.
3. Littré E. Oeuvres complètes d'Hippocrate. Volume II. Paris: Baillère; 1840. p. 598.
4. Rey A. Dictionnaire historique de la langue française. Paris: Dictionnaires Le Robert; 1992.
5. Grmek MD. Les maladies à l'aube de la civilisation occidentale. Paris: Payot; 1994.
6. de Romilly J. Thucydide, la guerre du Péloponnèse, livre II, texte établi et traduit par Jacqueline de Romilly. Paris: Les Belles Lettres; 1991.
7. Carmichael AG. Plague of Athens. In: Kiple KF, editor. The Cambridge world history of human disease. Cambridge: Cambridge University Press; 1993.
8. Wachsmuth K. Molecular epidemiology of bacterial infections: examples of methodology and of investigations of outbreaks. Rev Infect Dis. 1986;8:682–92.
9. Kriz P, Giogini D, Musilek M, Larribe M, Taha MK. Microevolution through DNA exchange among strains of *Neisseria meningitidis* isolated during an outbreak in the Czech Republic. Res Microbiol. 1999;150:273–80.

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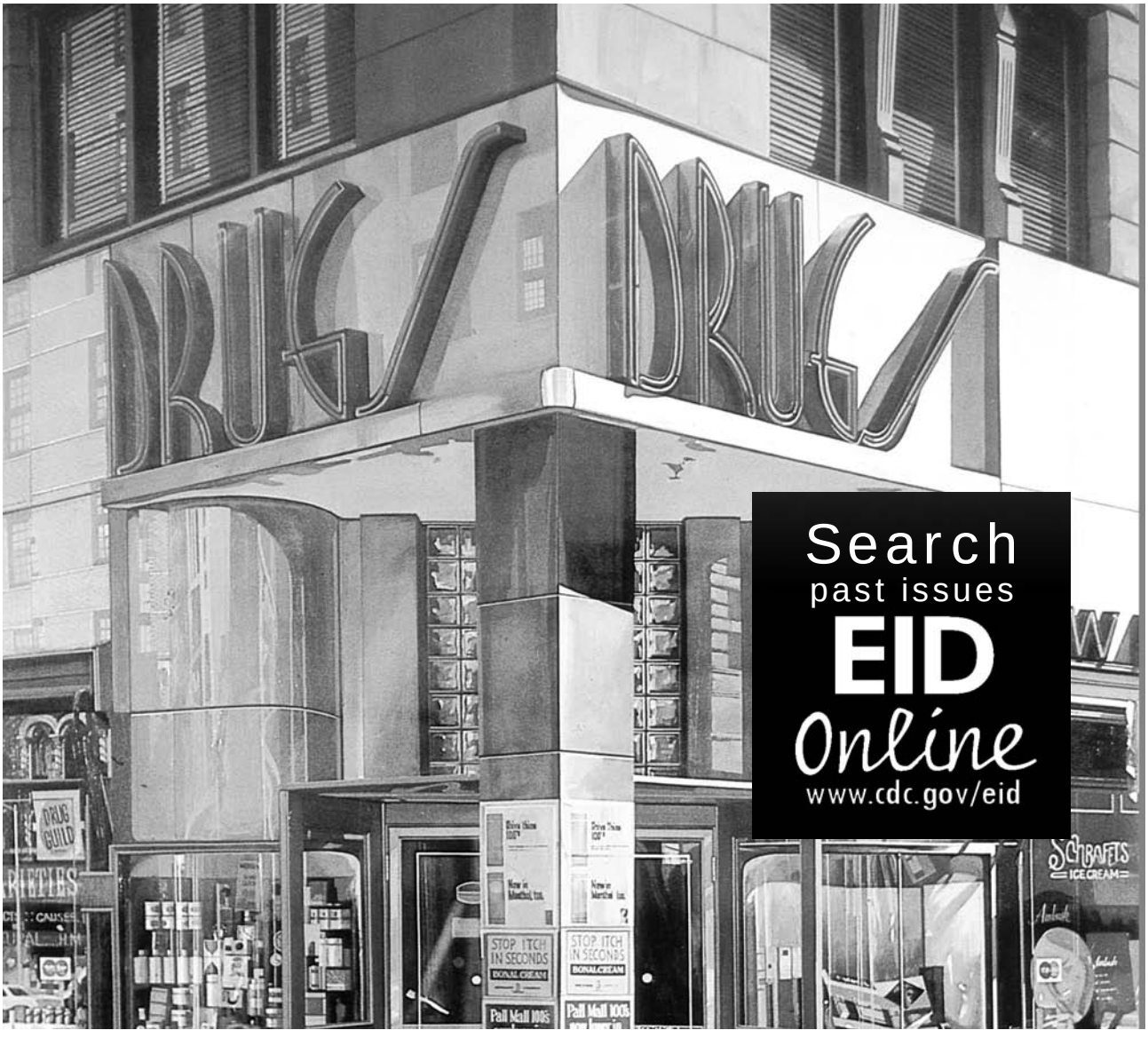

VIM-1 Metallo-β-lactamase in *Acinetobacter baumannii*

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In 2004 and 2005, 5 metallo-β-lactamase (MBL)-positive *Acinetobacter baumannii* isolates were found in 2 Greek hospitals. Isolates were unrelated and carried bla_{VIM-1} in a class 1 integron; $bla_{OXA-51-}$ and $bla_{OXA-58-like}$ carbapenemase genes were also detected. VIM-1 MBL in *Acinetobacter* spp. causes concern, given the increasing resistance of this species.

In the last few years, resistance to antibacterial drugs has been increasing in *Acinetobacter* spp., which will likely become a substantial treatment challenge in the future (*1*). Carbapenems have potent activity against *Acinetobacter* spp. and are usually the drugs of choice against multidrug-resistant *Acinetobacter baumannii* isolates. *Acinetobacter* spp. may develop resistance to carbapenems through various mechanisms, including class B and D carbapenemase production, decreased permeability, altered penicillin-binding proteins, and rarely, overexpression of efflux pumps (*2*,*3*).

In Europe, carbapenem resistance in *A. baumannii* has been sporadically attributed to the production of IMP-type metallo-β-lactamases (MBLs) and OXA-type carbapenemases (*4*). VIM-2–producing *Acinetobacter* spp. have been isolated in the Far East (*4*,*5*) and on 1 occasion in Germany (*6*). In this study, we report the appearance of the VIM-1 MBL determinant among *A. baumannii* in Greece.

The Study

We included in the study *A. baumannii* clinical isolates from tertiary care hospitals in 2 different Greek regions (Hippokration University Hospital, Thessaloniki, and University Hospital of Larissa, Thessalia) that were positive by the imipenem-EDTA double-disk synergy test (DDST) from March 2004 to March 2005. Bacteria were provisionally identified to the genus level by the Vitek 2 automated system (bioMérieux, Marcy l' Étoile, France) and the ATB 32GN system (bioMérieux). Antimicrobial drug susceptibility testing of the DDST-positive isolates for β-lactams (aztreonam, ceftazidime, cefepime, imipenem, meropenem, and piperacillin), β-lactam/β-lactamase inhibitor combinations (ampicillin/sulbactam, piperacillin/tazobactam), aminoglycosides (amikacin, gentamicin, netilmicin, and tobramycin), fluoroquinolones (ciprofloxacin and ofloxacin), and colistin was performed by Etest and Etest MBL (AB Biodisk, Solna, Sweden). The Clinical and Laboratory Standards Institute (CLSI) interpretative criteria were used (*7*), and *Pseudomonas aeruginosa* ATCC 27853 was used as control.

Polymerase chain reaction (PCR) testing of the synergy-positive isolates for carbapenemase genes was done by using consensus primers for bla_{IMP} (*8*), bla_{VIM} (*9*), bla_{SPM} (*10*), $bla_{OXA-23-like}$ (*11*), $bla_{OXA-24-like}$ (*11*), $bla_{OXA-58-like}$ (*11*), and $bla_{OXA-51-like}$ (*12*). Pulsed-field gel electrophoresis (PFGE) of *Apa*I-digested genomic DNA was performed in the bla_{VIM-1}-positive isolates, and the banding patterns were compared by using criteria proposed by Tenover et al. (*13*). The potential for conjugational transfer of imipenem resistance was examined in filter matings by using *Escherichia coli* 20R764 (lac^+ rif^r) as the recipient. Donor and recipient were mixed in a 1:5 ratio, and transconjugants were selected on MacConkey agar plates containing 100 μg/mL rifampicin and imipenem at concentrations of 0.5 to 2 μg/mL or 2 μg/mL ceftazidime.

Five *A. baumannii* clinical isolates that were MBL producers on the basis of DDST were detected among collections of isolates from patients hospitalized during the study period. Two of the isolates were recovered from blood cultures, 1 from bronchial secretions, 1 from a urine specimen, and 1 from cerebrospinal fluid; 2 isolates that had reduced susceptibility to carbapenems were not positive by the Etest MBL (Table). Imipenem MICs ranged from 4 to >32 μg/mL, while meropenem MICs were 2–32 μg/mL. *P. aeruginosa* ATCC 27853 was consistently characterized as having imipenem MIC of 2 μg/mL and meropenem MIC of 0.5 μg/mL. The 5 *A. baumannii* isolates were multidrug resistant; they showed resistance to all other antimicrobial drugs tested, with the exception of colistin.

We did not detect bla_{IMP}, bla_{SPM}, $bla_{OXA-23-like}$, or $bla_{OXA-24-like}$ in any of the 5 isolates, whereas bla_{VIM} was detected in all of them. In 2 isolates, $bla_{OXA-51-}$ and $bla_{OXA-58-like}$ genes were also simultaneously present, while 2 more carried a $bla_{OXA-51-like}$ gene (Table). By sequencing both strands of the entire bla_{VIM} amplicons (*14*), a bla_{VIM-1} sequence identical to that available in the database was identified. Sequencing $bla_{OXA-51-like}$ amplicons identified bla_{OXA-66} in all cases, while $bla_{OXA-58-like}$ alleles were classical bla_{OXA-58} in both cases. In 2 isolates, 1 from each region, PCR mapping of the integron that possibly carried bla_{VIM-1}, with primers 5′ CS and a set of primers for genes

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Table. Characteristics of bla_{VIM-1}-bearing *Acinetobacter baumannii* isolates*†

Isolate no.	Region of isolation	Material	PFGE type	Etest IMP MIC (μg/mL)	Etest MBL (IMP+EDTA)	Etest MER MIC (μg/mL)	$bla_{OXA-51-like}$ status	bla_{OXA-58} status
1	Thessaloniki	Blood	Ia	4	≤1	2	+	–
2	Thessalia	Bronchial fluid	II	32	2	4	+	+
3	Thessalia	CSF	III	4	≤1	2	–	–
4	Thessalia	Blood	IV	>32	1	32	+	–
5	Thessaloniki	Urine	Ib	8	≤1	4	+	+

*The 5 isolates exhibited resistance to all alternative antimicrobial drugs except colistin.
†PFGE, pulsed-field gel electrophoresis; IMP, imipenem; MBL, metallo-β-lactamase; MER, meropenem; CSF, cerebrospinal fluid.

aacA, *dhfrI*, *aadA*, *qacE*Δ1, and *sul*, showed a class 1 integron with a variable region including from 5′ to 3′ bla_{VIM-1}, *aacA7*, *dhfr*I, and *aadA1* gene cassettes. Sequencing of the overlapping PCR amplicons showed that this class 1 integron contained the *intI1* gene with a strong P1 promoter, an inactivated (without a GGG insertion) P2 promoter, an *attI1* site, and the bla_{VIM-1} gene cassette with its 59-base element identical to those reported previously in other gram-negative bacteria from Greece (*15*). PFGE showed that the 5 bla_{VIM-1}-positive isolates did not form a genetically homogeneous group; they belonged to 4 distinct types. The 2 isolates from Thessaloniki were subtypes of the same clone (Table, Figure). In none of the 5 isolates was bla_{VIM-1} transferable to the susceptible *E. coli* host after repeated conjugal experiments with imipenem or ceftazidime selection. The 5,387-bp nucleotide sequence of the integron structure reported in this study has been submitted to the EMBL/GenBank/DDBJ sequence databases and has been assigned accession no. DQ112355.

Conclusions

This is the first report of the VIM-1 determinant in *A. baumannii* in the world. Occurrence of VIM-2 MBL among *A. baumannii* and *Acinetobacter* genomospecies 3 isolates has previously been described among clinical isolates in Korea (*4*,*5*). In Europe, IMP-type enzymes had been reported in single *A. baumannii* isolates from some European regions (*4*) and VIM-2 from 1 German hospital (*6*), while MBLs have not been detected in *A. baumannii* from the United States despite carbapenem resistance there (*1*). Though anticipated because of the circulation of bla_{VIM} genes in several other gram-negative species in Europe and the ability of *Acinetobacter* spp. to acquire foreign DNA, this evolution is worrisome.

Retention of moderate susceptibility to carbapenems by bla_{VIM}-positive *A. baumannii* isolates in our study may seem unexpected, since MBLs hydrolyze these compounds. However, MBL production in gram-negative bacteria may not substantially increase carbapenem MICs without the simultaneous operation of other mechanisms, such as impaired permeability (*2*,*4*). Furthermore, 2 of the 4 strains carrying oxacillinase genes with carbapenemase properties had imipenem MICs not higher than the CLSI breakpoints for resistance. Recently, $bla_{OXA-51-like}$ genes have been shown to be possibly naturally occurring (*12*), while OXA-58 enzymes play a minor role in carbapenem resistance in *A. baumannii*, and strong promoter sequences are needed for higher levels of resistance to carbapenems (*2*).

During the last few years, *A. baumannii* has been increasingly isolated from severely ill patients, and its

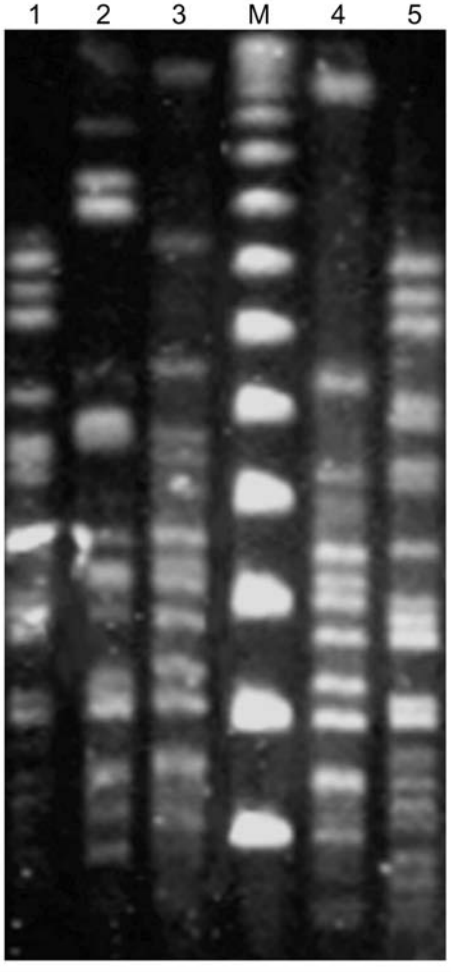

Figure. Pulsed-field gel electrophoresis of *Apa*I-restricted genomic DNA of the 5 bla_{VIM-1}-positive *Acinetobacter baumannii* isolates. The index numbers of the isolates are those listed in the Table. Lane M, molecular mass marker (48.5 kb).

usual cross-resistance to most available antimicrobial drugs, including carbapenems, poses substantial problems worldwide and especially in the United States (*1*). In New York, approximately two thirds of isolates are carbapenem resistant (*3*). In our region, *Acinetobacter* spp. are frequent nosocomial pathogens and are commonly multidrug resistant, which leads to the extensive use of carbapenems and, lately, polymyxins. The presence of MBLs among carbapenem-resistant *Acinetobacter* spp. from different Greek regions emphasizes the need for restricted use of carbapenems and early recognition of strains producing these enzymes. Although Etest MBL was reliable to detect VIM-2-producing *Acinetobacter* spp. in Korea (*5*), the assay seems unable to identify MBL-positive isolates exhibiting relatively low carbapenem MICs. Therefore, our diagnostic laboratories should screen *Acinetobacter* spp. with imipenem-EDTA DDST or alternative DDSTs, such as those using 2-mercaptopropionic acid, which appears to be more sensitive for detecting MBLs among *Acinetobacter* spp. (*4*). Whether carbapenems might be appropriate to treat infections with low-level carbapenem-resistant or susceptible bla_{VIM}-bearing *A. baumannii* isolates has yet to be determined.

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References

1. Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System. Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis. 2005;41:848–54.
2. Heritier C, Poirel L, Lambert T, Nordmann P. Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2005;49:3198–202.
3. Quale J, Bratu S, Landman D, Heddurshetti R. Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. Clin Infect Dis. 2003;37:214–20.
4. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-β-lactamases: the quiet before the storm? Clin Microbiol Rev. 2005;18:306–25.
5. Lee K, Lee WG, Uh U, Ha GY, Cho J, Chong Y, et al. VIM- and IMP-type metallo-β-lactamase-producing *Pseudomonas* spp. and *Acinetobacter* spp. in Korean hospitals. Emerg Infect Dis. 2003;9:868–71.
6. Toleman MA, Jones RN, Walsh TR. Hospital outbreak of an imipenem-resistant VIM-2 encoding *Acinetobacter* DNA group 14TU strain in a German teaching hospital. Clin Microbiol Infect. 2004;10(Suppl 3):48.
7. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M100-S15. Wayne (PA): The Institute; 2005.
8. Senda K, Arakawa Y, Ichiyama S, Nakashima K, Ito H, Ohsuka S, et al. PCR detection of metallo-β-lactamase gene (blaIMP) in gram-negative rods resistant to broad-spectrum β-lactams. J Clin Microbiol. 1996;34:2909–13.
9. Tsakris A, Pournaras S, Woodford N, Palepou MFI, Babini GS, Douboyas J, et al. Outbreak of infections caused by *Pseudomonas aeruginosa* producing VIM-1 carbapenemase in Greece. J Clin Microbiol. 2000;38:1290–2.
10. Toleman MA, Simm AM, Murphy TA, Gales AC, Biedenbach DJ, Jones RN, et al. Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J Antimicrob Chemother. 2002;50:673–9.
11. Poirel L, Marqué S, Héritier C, Segonds C, Chabanon G, Nordmann P. OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2005;49:202–8.
12. Héritier C, Poirel L, Fournier PE, Claverie JM, Raoult D, Nordmann P. Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2005;49:4174–9.
13. 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.
14. Yan JJ, Hsueh PR, Ko WC, Luh KT, Tsai SH, Wu HM. Metallo-â-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. Antimicrob Agents Chemother. 2001;45:2224–8.
15. Giakkoupi P, Xanthaki A, Kanelopoulou M, Vlahaki A, Miriagou V, Kontou S, et al. VIM-1 metallo-β-lactamase-producing *Klebsiella pneumoniae* strains in Greek hospitals. J Clin Microbiol. 2003;41: 3893–6.

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Acanthamoeba Encephalitis in Patient with Systemic Lupus, India

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We report a fatal case of encephalitis caused by *Acanthamoeba* in a 24-year-old woman from India with systemic lupus erythematosus. Diagnosis was made by identification of amebas in brain sections by immunofluorescence analysis and confirmed by demonstrating *Acanthamoeba* mitochondrial 16S rRNA gene DNA in brain tissue sections.

Acanthamoeba spp. are free-living amebae that cause granulomatous amebic encephalitis (GAE), most often in immunocompromised hosts, including HIV/AIDS and organ transplant patients and those receiving immunosuppressive medication (*1*). These organisms have also been associated with amebic keratitis, mainly in contact lens wearers, as well as with cutaneous, nasopharyngeal, and disseminated infections and amebic encephalitis.

Amebic encephalitis results from the hematogenous spread of the amebae from the initial portals of entry (skin, respiratory system) to the brain parenchyma. GAE is found worldwide (*1*); it develops as a subclinical infection and is difficult to diagnose because of vague symptoms. It is usually identified in a biopsy specimen of brain lesions or during postmortem examination.

Opportunistic infections are increasing because of HIV/AIDS, chemotherapeutic treatments, organ transplant procedures, and debilitating diseases. Although HIV/AIDS is under tenuous control in the industrialized world, it is a burgeoning public health crisis in the developing nations of Africa, Asia, and the Indian subcontinent. Amebic encephalitis cases likely are undetected in both industrialized and nonindustrialized nations. Acquainting clinicians with GAE is important so that they may include amebic encephalitis in differential diagnoses. We report a fatal case of *Acanthamoeba* encephalitis in a patient from India who was being treated for systemic lupus erythematosus with corticosteroids and methotrexate.

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The Case

The patient was a 24-year-old woman with a 2-year history of systemic lupus erythematosus, a history of central nervous system (CNS) involvement in the recent past, and autoimmune hemolytic anemia. She was taking chloroquine, prednisolone, and methotrexate. A month before her admission to the hospital, low-grade fever, joint pains, facial rash, and mouth ulcers developed. A day before admission she had generalized tonic clonic convulsions that progressed to status epilepticus, followed by loss of consciousness. On admission, she had a temperature of 40°C, pulse 110/min, blood pressure 126/80 mm Hg, and neck stiffness. She was stuporous and withdrawing to pain. Her cerebrospinal fluid (CSF) levels were the following: protein 174 mg/dL, glucose 42 mg/dL, erythrocytes 1/mm^3, and leukocytes 1/mm^3. Gram-stained CSF smears showed no bacteria, and CSF cultures were negative for bacteria and fungi. She was treated with ceftriaxone, phenytoin, and pulse steroids. On her second day in the hospital she was intubated for airway control. By that evening her pupils became small, quadriparesis developed, and she became deeply comatose.

Magnetic resonance imaging (MRI) showed multiple hypointense necrotic lesions of varying sizes on T2-weighted imaging. These lesions involved the supra- and the infratentorial compartments, with the largest lesion in the left cerebellar hemisphere (Figure 1A). Ascending transtentorial herniation, significant mass effect, distortion of the brainstem and fourth ventricle, moderate supratentorial ventricular dilatation, and associated sulcal/meningeal enhancement occurred. Similar lesions of varying intensity and size were seen in bilateral cerebral hemispheres, left caudate head, right thalamus, and right half of the pons (Figure 1B). Three-dimensional multivoxel spectroscopy through the left basal ganglia and right thalamic lesion showed elevated lipid areas with increased lactate in the basal ganglia. Choline levels were normal or minimally increased.

Emergency posterior fossa craniectomy and foramen magnum decompression were performed. The left cerebellar lesion had poor demarcation from the adjacent normal cerebellar tissue and necrotic material in the cavity. The tissue was soft, discolored with gray-black spots, and hyperemic. An external ventricular drain was placed, and dexamethasone treatment was started. However, the patient's condition continued to deteriorate and she died after 5 days.

Histopathologic analysis of CNS lesions showed foci of hemorrhagic necrosis. There was a modest chronic inflam-

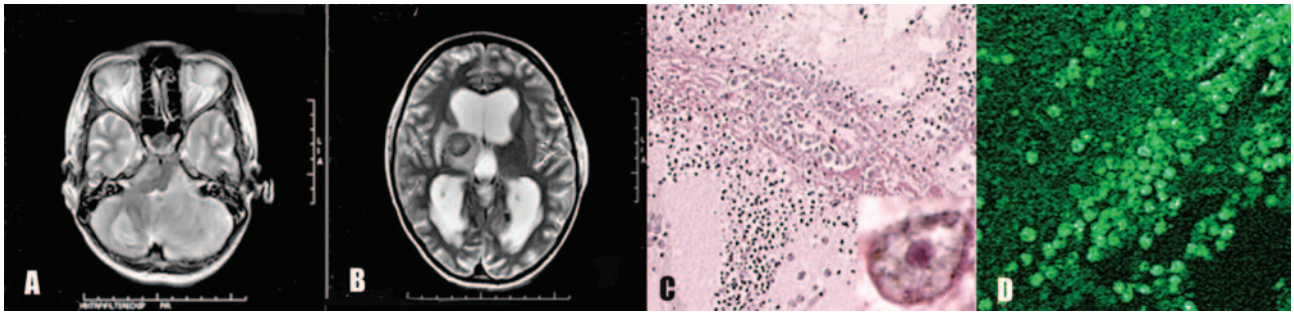

Figure 1. A) Magnetic resonance imaging (MRI) of the patient's brain showing a large lesion in the left cerebellar hemisphere. B) MRI in a different plane taken at the same time. Lesions are evident in the right thalamus and the right half of the pons. C) Blood vessel in brain parenchyma with large numbers of *Acanthamoeba* in the perivascular space (hematoxylin and eosin stained, magnification ×100). Inset, higher magnification (×1,000) showing nuclear morphology of the ameba. The dark-stained ameba nucleus with a central nucleolus is distinctive. D) Immunofluorescent staining of perivascular brain tissue showing many amebae (magnification ×100).

matory exudate composed mainly of lymphocytes, monocytes, a few plasma cells, and occasionally a few polymorphonuclear leukocytes. Amebic trophozoites were abundant, mainly around blood vessels, but few cysts were seen (Figure 1C). Amebae were identified by a nucleus containing a large central nucleolus (Figure 1C, inset). Formalin-fixed, paraffin-embedded sections were tested with rabbit antibodies to *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. Amebae were identified as *Acanthamoeba* on the basis of their reactivity in immunofluorescence analysis (Figure 1D).

DNA was extracted for amplification and sequencing from formalin-fixed brain tissue sections mounted on slides. Sections were deparaffinized, scraped from slides, suspended in lysis buffer, and tested by polymerase chain reaction (PCR) as previously described (*2*).

The primer set Aca16Sf1010 (5′-TTATATTGACTTGTACAGGTGCT-3′) and Aca16Sr1180 (5′-CATAATGATTTGACTTCTTCTCCT-3′), which was designed to give an amplimer of 161 bp, was used for detecting *Acanthamoeba* DNA (*3*). Testing for *Balamuthia* DNA was also included because this ameba can cause GAE (*2*). PCR results were positive for *Acanthamoeba* DNA (Figure 2, lanes 7–12) but negative for *Balamuthia* DNA (Figure 2, lanes 1–6).

Conclusions

Amebic encephalitis is caused by 3 different amebae: *Acanthamoeba* spp, *B. mandrillaris*, and *N. fowleri*. Since *Acanthamoeba* spp. and *B. mandrillaris* have similar forms and cause subacute, prolonged clinical courses, they have been misidentified in histopathologic examination of brain tissue (*4*). *N. fowleri* causes primary amebic meningoencephalitis, a fulminant disease often fatal within 7 to 10 days and usually associated with swimming or other water-related activities of otherwise immunocompetent persons, usually children or young adults (*1*). *Acanthamoeba* spp. are ubiquitous in soil and water and are found in the home environment, including water taps and sink drains, flowerpot soil, and aquariums. In healthcare settings, they have been isolated from hydrotherapy baths, dental irrigation equipment, humidifiers, cooling systems, ventilators, and intensive care units (*1*,*5*).

Encephalitis caused by *Acanthamoeba* is almost always fatal because of difficulty and delay in diagnosing the disease and lack of optimal antimicrobial therapy (*5*). The portal of entry of the ameba may be a break in the skin or the respiratory tract by inhalation of wind-blown cysts, with subsequent spread to the CNS through the circulatory system (*1*). In our patient, the portal of entry could not be determined.

Initial symptoms of GAE are vague and may mimic neurocysticercosis, tuberculoma, or brain tumor. Histopathologic analysis is the most common means of detecting amebae, but their appearance is often unfamiliar to pathologists, and they may be overlooked or misidentified (*1*). Either electron microscopy or immunostaining of brain tissue is necessary to distinguish *Acanthamoeba* spp. from *Balamuthia* (*1*). *Acanthamoeba* in our patient was identified by immunofluorescence testing using rabbit antibodies to *Acanthamoeba* (*1*,*5*). PCR was also used to detect *Acanthamoeba* DNA in brain tissue.

In almost all cases, persons with *Acanthamoeba* infections are immunocompromised (*1*). *Acanthamoeba* infections in persons with lupus erythematosus who were being treated with corticosteroids have been reported (*6*–*9*). Corticosteroids, besides reducing inflammation, impair the immune response, facilitating infection and disease caused by *Acanthamoeba* (*10*). In fixed brain tissue, *Acanthamoeba* and *B. mandrillaris* are similar and both form cysts in tissues (*4*,*5*). PCR has been used to identify ameba DNA in brain tissue and CSF of persons suspected of having balamuthiasis (*2*) and in brain tissue of a patient with primary amebic meningoencephalitis caused by *N. fowleri* (*11*).

In conclusion, *Acanthamoeba* infection was demonstrated by hematoxylin and eosin and immunostaining of

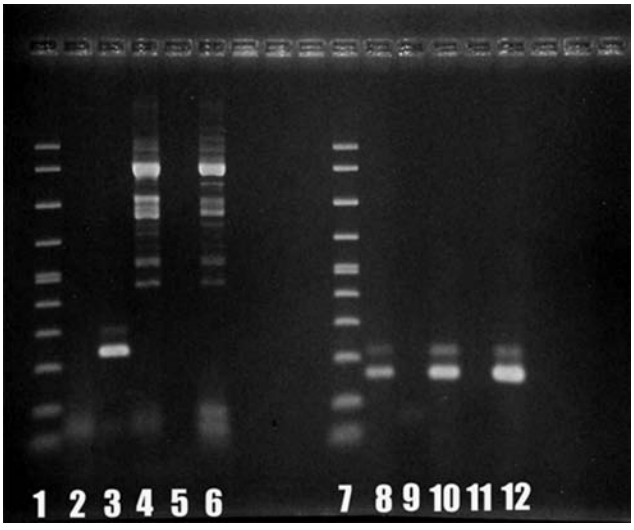

Figure 2. Results of polymerase chain reaction showing 230-bp and 161-bp bands for *Balamuthia* (lanes 1–6) and *Acanthamoeba* (lanes 7–12) mitochondrial 16S rRNA gene DNA. Lane 1, molecular mass marker; lane 2, absence of *Balamuthia* DNA in patient brain tissue (5-μL sample); lane 3, positive control (*Balamuthia* DNA); lane 4, negative control (*Acanthamoeba* DNA); lane 5, negative control (water); lane 6, absence of *Balamuthia* DNA in patient brain tissue (1-μL sample); lane 7, molecular mass marker; lane 8, *Acanthamoeba* DNA isolated from patient brain tissue (5-μL sample); lane 9, absence of *Balamuthia* DNA isolated from amebae in patient sample; lane 10, positive control (*Acanthamoeba* DNA); lane 11, negative control (water); lane 12, *Acanthamoeba* DNA isolated from patient brain tissue (1-μL sample).

brain tissue and PCR detection of ameba mitochondrial DNA in brain tissue. Treatment with corticosteroids and methotrexate likely facilitated development of amebic infection by compromising the patient's immune system.

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. Martinez AJ, Visvesvara GS. Free-living, amphizoic and opportunistic amebas. Brain Pathol. 1997;7:583–98.
2. Yagi S, Booton GC, Visvesvara GS, Schuster FL. Detection of *Balamuthia* mitochondrial 16S rRNA gene DNA in clinical specimens by PCR. J Clin Microbiol. 2005;43:3192–7.
3. Foreman O, Sykes J, Ball L, Yang N, De Cock H. Disseminated infection with *Balamthia mandrillaris* in a dog. Vet Pathol. 2004;41:506–10.
4. Visvesvara GS, Schuster FL, Martinez AJ. *Balamuthia mandrillaris*, N. G., N. Sp. agent of amebic meningoencephalitis in humans and other animals. J Eukaryot Microbiol. 1993;40:504–14.
5. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol. 2004;34:1001–27.
6. Bloch K, Schuster FL. Inability to make a premortem diagnosis of *Acanthamoeba* species infection in a patient with fatal granulomatous amebic encephalitis. J Clin Microbiol. 2005;43:3003–6.
7. Grunnet ML, Cannon GH, Kushner JP. Fulminant amebic meningoencephalitis due to *Acanthamoeba*. Neurology. 1981;31:174–6.
8. Koide J, Okusawa E, Ito T, Mori S, Takeuchi T, Itoyama S, et al. Granulomatous amoebic encephalitis caused by *Acanthamoeba* in patient with systemic lupus erythematosus. Clin Rheumatol. 1998;17:329–32.
9. Sangruchi T, Martinez AJ, Visvesvara GS. Spontaneous granulomatous amebic encephalitis: report of four cases from Thailand. Southeast Asian J Trop Med Public Health. 1994;25:309–13.
10. Martinez AJ. Acanthamoebiasis and immunosuppression. Case report. J Neuropathol Exp Neurol. 1982;41:548–57.
11. Cogo PE, Scalgia M, Gatti S, Rossetti F, Alaggio R, Laverda AM, et al. Fatal *Naegleria fowleri* meningoencephalitis, Italy. Emerg Infect Dis. 2004;10:1835–7.

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Bordetella pertussis, Finland and France

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We used pulsed-field gel electrophoresis analysis and genotyping to compare clinical isolates of *Bordetella pertussis* recovered since the early 1990s in Finland and France, 2 countries with similar histories of long-term mass vaccination with whole-cell pertussis vaccines. Isolates from both countries were similar genetically but varied temporally.

The introduction of whole-cell pertussis vaccines (wP) from the 1940s to the 1960s in many countries resulted in a dramatic decrease in illness and death from pertussis. However, pertussis remains a considerable public health problem worldwide. Indeed, the disease continues to be endemic in vaccinated populations in Europe, Australia, Canada, and the United States, with cyclic increases at 3- to 5-year intervals, despite high vaccination coverage (*1,2*). One of the major causes is waning of vaccine-induced immunity with time (*1,3*), but increased disease surveillance and use of biologic diagnosis are also implicated. However, changes in the agent of the disease, *Bordetella pertussis*, are of some concern. This bacterium expresses adhesins such as filamentous hemagglutinin, pertactin, fimbriae (FIM), and toxins such as pertussis toxin, and adenylate cyclase-hemolysin (*4*). Recently, circulating isolates were found to differ from the strains used for the wP vaccines in the world (*5–13*). These observed changes might modify the properties of the isolates and affect the efficacy of pertussis vaccines.

In Europe, heterogeneity is high in epidemic situations with respect to wP vaccines used and vaccination history and strategy. However, Finland and France have implemented similar mass wP vaccination programs with high coverage for several decades. Since 1952, children in Finland have been vaccinated with combined diphtheria-tetanus wP vaccine (DTwP) at 3, 4, and 5 months and from 20 to 24 months of age. The wP vaccine contains 2 strains and has remained unchanged since 1976. Since 1959, children in France have been vaccinated with DTwP-inactivated polio vaccine at 3, 4, and 5 months and from 16 to 18 months of age. The vaccine calendar for primary vaccination was changed to 2, 3, and 4 months of age in 1995. The same wP vaccine, composed of 2 strains, has been used for >40 years. In both Finland and France, incidence of pertussis has increased and the disease has shifted to older age groups, especially adolescents and adults. The cycles of pertussis disease are observed every 3–5 years (*5,13*). The aim of this study was to analyze and compare the isolates circulating in the 2 European countries with long-term and intensive vaccination.

B. pertussis isolates were selected from collections of the Finnish Pertussis Reference Laboratory of the National Public Health Institute (Turku, Finland) and the French Pertussis National Center of Reference (Paris, France). Of the 503 Finnish isolates recovered from 1991 to 2004, 64 were selected either because they represent all available isolates from 1 community (Paimio) or they were recovered from a geographic area as wide as possible. In addition, 6 isolates from a school outbreak that occurred in Heinavesi (Finland) in 1982 were included to study the changes over time. Of the 1,049 French isolates recovered from 1991 to 2004, we selected 61 because they are representative of the French collection from a temporal and geographic viewpoint and they correlate with the different cycles of pertussis observed in France and Finland.

All Finnish and French isolates showed a high similarity with a minimum of 80.3% overall relatedness by using pulsed-field gel electrophoresis (PFGE) analysis after digestion of *B. pertussis* genomic DNA with *Xba*I restriction enzyme (*14*) (Appendix Figure, available online at http://www.cdc.gov/ncidod/EID/vol12no06/05-1283_app.htm). Most Finnish and French isolates fell into PFGE groups III, IV, and V (Appendix Figure and Figure, panel A) corresponding to the isolates circulating in Europe from 1999 to 2001 (*14*). A new PFGE group (VII) was identified among Finnish isolates recovered in 2004 (Appendix Figure and Figure, panel A). This new profile, with 97.3% level of relatedness (Appendix Figure), might represent an emerging group. The new profile was further confirmed with PFGE by using the second restriction endonuclease *Spe*I (data not shown), as previously recommended (*5,14*). We cannot say whether PFGE VII isolates are actually emerging or whether evidence for their emergence is anecdotal, as is the case with French PFGE VI isolates, which represent only 0.6% of French isolates (data not shown).

We previously showed that the major PFGE group circulating in Europe from 1999 to 2001 is group IV (*14*). In France and in Finland, the PFGE group IV was overrepresented from 1992 to 2004 with 85.3% level of relatedness, confirming the limited polymorphism of *B. pertussis*. PFGE group IV was subdivided into 3 subgroups, α, β, and γ, whose frequencies varied between countries (*14*). In our study, we show that, as in France (*5*) the PFGE groups of the isolates circulating in Finland vary temporally with

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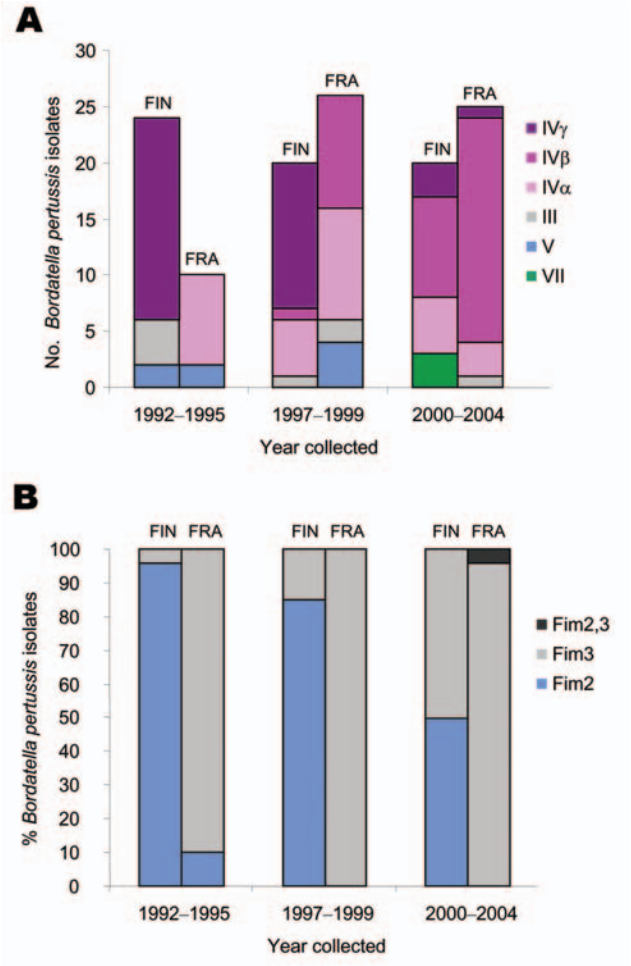

Figure. A) Pulsed-field gel electrophoresis profile repartition of *Bordetella pertussis* isolates by year and by country. B) Fimbriae expression of *B. pertussis* isolates by year and by country. FIN, Finland; FRA, France

the cycles of the disease (Appendix Figure and Figure, Panel A). However, the frequency of the isolate subgroups circulating in Finland and France was different. In fact, the major subgroup detected in Finland between 1992 and 1999 was IVγ; since 2000, subgroups IVα and IVβ have been found (Figure, Panel A). This circumstance is well illustrated among the isolates recovered in Paimio, where 99% of isolates with IVγ were circulating in 1992 whereas, in 2004, 42% of subgroup IVβ, 33% of subgroup IVα, 25% of the new group VII, and none of IVγ were circulating in Paimio. Subgroups IVα and β, absent from 1992 to 1994, are now circulating. However, subgroup IVα is not a new subgroup in Finland since the 6 isolates collected in Heinavesi in 1982 exhibit this profile (Appendix Figure).

The analysis also included genotyping of the genes encoding pertussis toxin S1 subunit (*ptxA*) and pertactin (*prn*) and serotyping of FIM, performed as described previously (*5,13*). The sequence of *ptxA* is the same (*ptxA* allele type 1) for all Finnish and French isolates. The same types of *prn* alleles are also harbored by Finnish and French isolates (Appendix Figure). The emergence of isolates harboring *ptxA1* and *prn2* or *prn3* alleles in both countries might be explained by the fact that the wP vaccine strains used in both countries harbor *ptxA2 or A4* and *prn1* alleles (*13,15*). A similar hypothesis might be proposed for the expression of FIM. In fact, most of the Finnish isolates collected from 1991 to 2004 express FIM2, whereas in France most of the isolates express FIM3 (Figure, Panel B). The differences in the expression of FIM between Finnish and French isolates might reflect the difference in strains used for Finnish and French wP vaccines. The Finnish wP vaccine contains 2 strains expressing FIM2,3 and FIM3, whereas the French wP vaccine includes 2 strains expressing FIM2,3 and FIM2 (*13,15*). A marked shift of predominant serotype from FIM2 to FIM3 has been observed in Finland since 1999, although the wP vaccine remained the same. The emergence of isolates with FIM3 and PFGE subgroup IVβ, a new subgroup found in Finland, might be due to the increase in the frequency of this subgroup in the neighboring countries and the increased mobility of people within the European Union in the last decades.

We show that the *B. pertussis* isolates circulating in 2 countries with a long history of wP vaccination are genetically close. In the 2 countries, similar PFGE groups and subgroups are present, but their frequencies were different in the 1990s. Further, the subgroup emerging according to the cycles of pertussis in each country varies. The difference observed in frequency of subgroups could be due to herd immunity or human density of the populations concerned. Does this herd immunity vary depending on the human genetic population concerned or are the vaccine strains used expressing similar factors but not at the same level (e.g., FIM2 vs. FIM3)? This question needs further investigation.

These 2 countries have started using acellular pertussis vaccines, France since 2002 and Finland since 2005. Continued monitoring of the circulating isolates will be important.

We thank the European Commission of Life Program (QLK2-CT-2001-01819, EUpertstrain), GlaxoSmithKline, and Sanofi-Pasteur laboratories for supporting this work financially. This work was also financially supported by Institut Pasteur Foundation and FRE-CNRS 2849 for France and the Special Governmental Fund for University Hospitals (EVO) for Finland.

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References

1. Wirsing von Konig CH, Halperin S, Riffelmann M, and Guiso N. Pertussis of adults and infants. Lancet Infect Dis.2002;2:744–50.
2. Tanaka M, Vitek CR, Pascual FB, Bisgard KM, Tate JE, and Murphy TV. Trends in pertussis among infants in the United States, 1980-1999. Jama.2003;290:2968–75.
3. Forsyth K, Tan T, von Konig CH, Caro JJ, and Plotkin S. Potential strategies to reduce the burden of pertussis. Aims, scope and findings of the global pertussis initiative. Pediatr Infect Dis J.2005;24:S69–74.
4. Mattoo S, and Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev.2005;18:326–82.
5. Weber C, Boursaux-Eude C, Coralie G, Caro V, and Guiso N. Polymorphism of *Bordetella pertussis* isolates circulating the last ten years in France, where a single effective whole-cell vaccine has been used for more than thirty years. J. Clin. Microbiol.2001;39:4396–4403.
6. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HGJ, Gaastra W, and Willems RJL. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in the Netherlands: Temporal trends and evidence for vaccine-driven evolution. Infect. Immun.1998;66:670–5.
7. Gzyl A, Augustynowicz E, Van Loo I, and Slusarczyk J. Temporal nucleotide changes in pertactin and pertussis toxin genes in *Bordetella pertussis* strains isolated from clinical cases in Poland. Vaccine.2002;20:299–303.
8. Fry NK, Neal S, Harrison TG, Miller E, Matthews R, and George RC. Genotypic variation in the *Bordetella pertussis* Virulence Factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect. Immun.2001;69:5520–8.
9. Mastrantonio P, Spigaglia P, van Oirschot H, van der Heide HGJ, Heuvelman K, Stefanelli P, et al. Antigenic variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children. Microbiol.1999;145:2069–75.
10. Poynten M, McIntyre PB, Mooi FR, Heuvelman KJ, and Gilbert GL. Temporal trends in circulating *Bordetella pertussis* strains in Australia. Epidemiol Infect.2004;132:185–93.
11. Peppler MS, Kuny S, Nevesinjac A, Rogers C, De Moissac YR, Knowles K, et al. Strain variation among *Bordetella pertussis* isolates from Quebec and Alberta provinces of Canada from 1985 to 1994. J Clin Microbiol.2003;41:334–47.
12. Hardwick TH, Cassiday P, Weyant RS, Bisgard KM, and Sanden GN. Changes in predominance and diversity of genomic subtypes of *Bordetella pertussis* isolated in the United States, 1935 to 1999. Emerging Infect. Dis.2002;8:44–9.
13. Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, Hallander H, et al. Strain variation among *Bordetella pertussis* isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J Clin Microbiol.2005;43:3681–7.
14. Caro V, Njamkepo E, Van Amersfoorth SC, Mooi FR, Advani A, Hallander HO, et al. Pulsed-field gel electrophoresis analysis of *Bordetella pertussis* populations in various European countries with different vaccine policies. Microbes Infect.2005;7:976–82.
15. Njamkepo E, Rimlinger F, Thiberge S, and Guiso N. Thirty-five years' experience with the whole-cell pertussis vaccine in France: vaccine strains analysis and immunogenicity. Vaccine.2002; 20:1290–4.

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Guillain-Barré Syndrome, Greater Paris Area

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We studied 263 cases of Guillain-Barré syndrome from 1996 to 2001, 40% of which were associated with a known causative agent, mainly *Campylobacter jejuni* (22%) or cytomegalovirus (15%). The cases with no known agent (60%) peaked in winter, and half were preceded by respiratory infection, influenzalike syndrome, or gastrointestinal illness.

Guillain-Barré syndrome (GBS) is a state of acute flaccid paralysis thought, in most cases, to result from an aberrant immune response triggered by microbial infections (*1*). Studies in Western countries have reported evidence of recent infection with *Campylobacter jejuni* in 15% to 40% of GBS cases and with cytomegalovirus (CMV) in 5% to 20% of cases (*1–5*). Recent infection with Epstein-Barr virus (EBV) or *Mycoplasma pneumoniae* was less frequent (1%–2% each) (*1–5*). No agent was identified in 60% to 70% of cases, although the patients often had a history of respiratory or gastrointestinal infection (*1*,*2*).

Previous studies have failed to identify any clear seasonal distribution of GBS cases in Europe and North America. It has been suggested that this failure to demonstrate seasonality in GBS is because most prevalent antecedent infections have inverse seasonal distributions (*6*). We tested this hypothesis to provide new insight into infectious agents associated with GBS in Western countries.

The Study

All GBS patients admitted to the medical intensive care unit of Raymond Poincaré Hospital in Garches, France, from January 1996 to December 2001 were included in the study. GBS was diagnosed according to the criteria of Asbury and Cornblath (*7*). The following data were collected at the time of hospital admission: time from onset of neurologic signs of GBS to admission; history of infections in the 2 months preceding the onset of neurologic signs; and time from the infectious event to onset of neurologic signs.

Serum samples were collected at hospital admission. Serum antibodies against *C. jejuni* and *M. pneumoniae* were assayed with complement fixation tests (Institut Virion, Würzburg, Germany); cutoff titers (*C. jejuni* 20; *M. pneumoniae* 80) were selected to give >95% specificity on the basis of data provided by the manufacturer. Serum samples were tested for immunoglobulin M (IgM) and IgG antibodies to CMV with the miniVIDAS system (bioMérieux, Marcy l'Etoile, France). IgG avidity was measured in samples positive for IgM by using the Enzygnost anti-CMV/IgG test (Dade Behring S.A., Paris la Défense, France) and 8 mol/L urea. Recent CMV infection was identified by detection of IgM with IgG avidity <35% (*8*). Serum antibodies against EBV were detected with commercial dot blot tests (ImmunoDOT EBV MONO M and G kits, GenBio, San Diego, CA, USA). Recent EBV infection was identified by detecting IgM antibodies to viral capsid antigen. IgM and IgG antibodies against gangliosides GM1 and GM2 were identified by an enzyme immunoassay (GanglioCombi, Bühlmann Laboratories AG, Schönenbuch, Switzerland) and an immunodot blot assay (*9*).

Statistical analyses were performed with the R 2.0.1 statistical package (R Development Core Team, Vienna, Austria). Groups were compared in pairs, and the Hochberg method for multiple testing was used to correct p values (*10*). Categorical variables were compared by Fisher exact test, and continuous variables were compared by Student *t* test or Wilcoxon rank sum test. Seasonal trends for GBS cases were analyzed by using the method of Jones et al. (*11*). The number of harmonics (seasonality periods) was determined by using the Akaike information criterion. All tests were 2-tailed, and a p value <0.05 was considered significant.

During the study period, 279 consecutive patients with GBS were admitted to our center. Sixteen patients were excluded because of missing clinical data or serum samples; 263 were included in the study. On the basis of an annual incidence of 1.2 to 1.9 GBS cases per 100,000 persons (*12*) in a population of 10.952 million people (*13*), we estimated that 130–210 GBS cases occurred annually in the greater Paris area during the study period. Thus, this study included 20%–30% of all estimated GBS cases in this area.

We observed serologic evidence of recent infection with *C. jejuni* in 58 patients (21.9%), CMV in 40 (15.1%) patients, *M. pneumoniae* in 6 (2.3%) patients, and EBV in 3 (1.15%) patients. Recent infection with *C. jejuni* and CMV was observed in 1 patient. Thus, 106 cases (40%) had ≥1 known agent of GBS (known agent group), and 157 cases (60%) had no known agent (unknown agent group) (Table). Most patients in the *C. jejuni* group were male,

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Table. Characteristics of patients with Guillain-Barré syndrome*

Characteristic	All patients	*Campylobacter jejuni*	CMV	Unknown agent	p value†	p value‡
No.	263	58	40	157		
Sex, no. (%)					0.53	0.31
Female	112 (43)	21 (36)	22 (55)	66 (42)		
Male	151 (57)	37 (64)	18 (45)	91 (58)		
Mean age, y (SD)	48.7 (18.3)	51.3 (19.1)	35.9 (12.0)	51.2 (17.9)	0.94	<0.0001
Median days from infectious event to neurologic signs (IQR)	8 (4–15)	7 (4–14.2)	10 (4.5–18)	8 (5–15)	0.45	0.55
Median days from neurologic signs to hospital admission (IQR)	5.5 (3–9)	4 (2.5–7)	7 (3–11)	6 (3–10)	0.11	0.27
Infectious event, no. (%)					0.0048	0.25
None	99 (38)	13 (22)	14 (35)	73 (47)		
≥1	164 (62)	45 (78)	26 (65)	84 (53)		
Infectious event, no. (%)					0.0049	0.026
GI	61 (37)	30 (67)	3(11)	27 (31)		
URTI	37 (23)	6 (13)	9 (35)	21 (25)		
LRTI	29 (12)	2 (4)	1 (4)	12 (14)		
Influenzalike	30 (18)	5 (11)	6 (23)	18 (21)		
Others	16 (10)	2 (4)	7 (27)	6 (7)		
Only motor symptoms, no. (%)	101 (39)	31 (54)	11 (28)	57 (37)	0.056	0.35
Mechanical ventilation, no. (%)	87 (33)	23 (40)	15 (38)	43 (27)	0.20	0.49
Antibodies to gangliosides, no. (%)						
GM1	30 (13)	24 (44)	0	5 (4)	<0.0001	0.59
GM2	15 (6)	0	15 (47)	0	1.0	<0.0001

*CMV, cytomegalovirus; SD, standard deviation, IQR, interquartile range; GI, gastrointestinal illness; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.
†Adjusted for unknown agent versus *C. jejuni*.
‡Adjusted for unknown agent versus CMV.

were ≥50 years of age, had a history of gastrointestinal illness (Figure 1), and exhibited a severe motor form of GBS with serum IgG antibodies against ganglioside GM1. Patients in the CMV group were significantly younger (p<0.0001), more likely to have respiratory or influenzalike symptoms than gastrointestinal symptoms (p<0.0001) before the onset of GBS symptoms (Figure 1), and showed a longer time from first neurologic signs to hospital admission (p = 0.048). These patients rarely showed a pure motor form of GBS (p = 0.037) and frequently had IgM antibodies against GM2 but did not have IgG antibodies against GM1 (p<0.0001).

Patients in the unknown agent group were older than those in the CMV group (p<0.0001), less likely to have had a history of infectious events than patients in the *C. jejuni* group (p = 0.0048), and had a significantly different antiganglioside response than those in *C. jejuni* and CMV groups (p<0.0001 in each case) (Table). The unknown agent group had a higher proportion of patients with gastrointestinal illness than did the CMV group (p = 0.045) and a higher proportion of patients with respiratory tract or influenzalike symptoms than the *C. jejuni* group (p = 0.0024) (Figure 1).

No seasonal variation was found for all patients combined (data not shown). However, this apparent absence of variation masked a substantial seasonal difference for the known agent and unknown agent groups. In the known agent group, 60% of cases occurred in spring and summer; only 16% occurred in winter. In the unknown agent group, only 17% of cases occurred in summer; 37% occurred in winter.

We used the method of Jones et al. (*11*) to test the seasonality of incidence. No seasonality was detected for the groups all cases, known agent, and *C. jejuni* (Figure 2). For the unknown agent group, a model with 1 harmonic

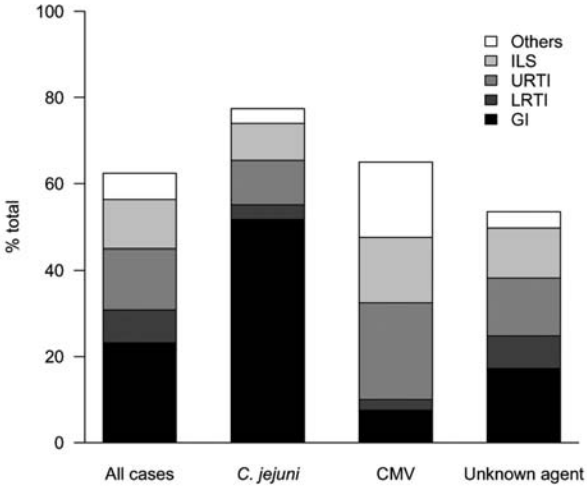

Figure 1. Distribution of preceding infectious events in patients with Guillain-Barré syndrome. ILS, influenzalike syndrome; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; GI, gastrointestinal illness; CMV, cytomegalovirus infection.

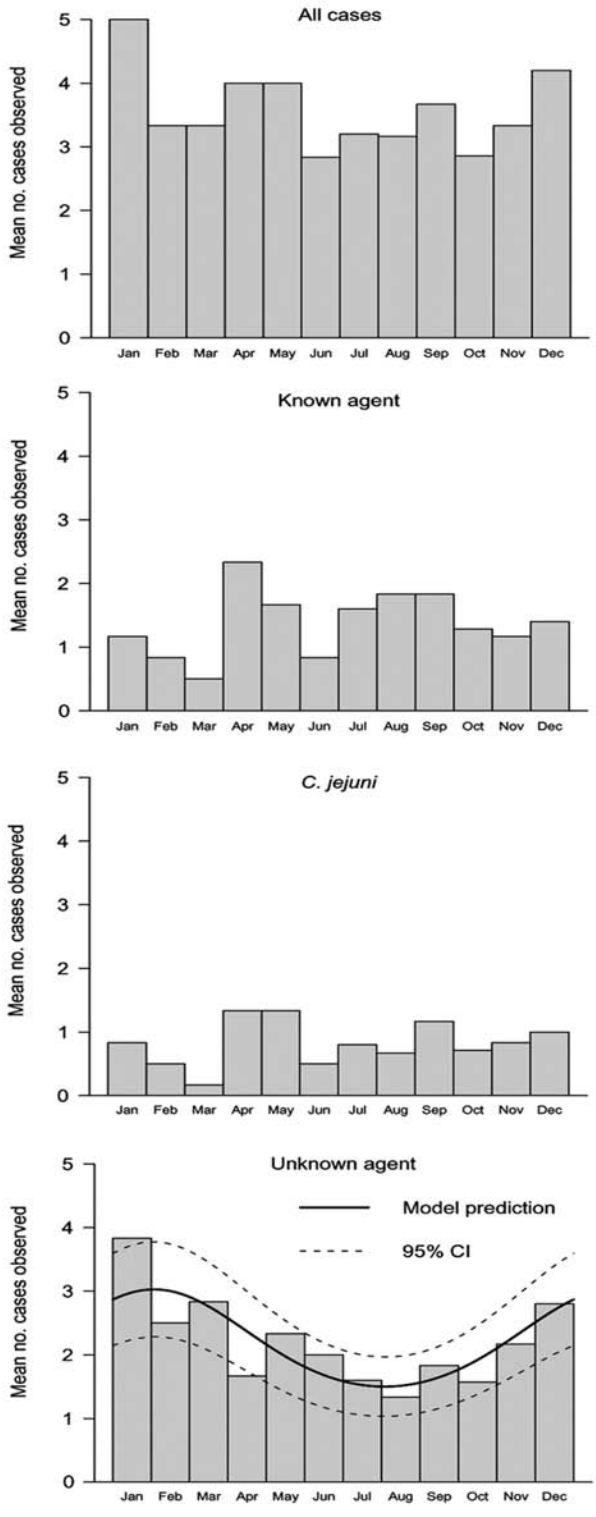

Figure 2. Seasonal distribution of preceding infectious agents by month for the study period (1996–2001). For the unknown agent group, the solid line represents the seasonal model prediction and the dashed lines represent its pointwise 95% confidence interval (CI).

(annual seasonality) gave a significantly better fit than a model without harmonics ($p = 0.0089$, by likelihood ratio test); additional harmonics did not improve the fit of the model. Since no significant linear trend was found ($p = 0.49$), this element was removed for model prediction. This best-fit, single-harmonic model indicated that incidence was highest at the beginning of February and lowest at the beginning of August (Figure 2).

Conclusions

This study provides new data about GBS patients not associated with known etiologic agents, which account for most patients in Western Europe (*2*,*14*). We have shown that GBS cases of unknown cause were more common in winter, with a peak incidence at the beginning of February. Moreover, in ≈50% of the patients, GBS symptoms were preceded by respiratory infection, influenzalike syndrome, or gastrointestinal illness. Together with the seasonality of cases, this finding suggests the involvement of winter infectious agents, probably respiratory or enteric viruses.

Acknowledgments

We thank Isabelle Sénégas for assistance and Marie-Hélène Canneson for technical assistance.

This work was supported by the Laboratoire Français du Fractionnement et des Biotechnologies.

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References

1. Hughes RA, Hadden RD, Gregson NA, Smith KJ. Pathogenesis of Guillain-Barré syndrome. J Neuroimmunol. 1999;100:74–97.
2. Hadden RD, Karch H, Hartung HP, Zielasek J, Weissbrich B, Schubert J, et al. Preceding infections, immune factors, and outcome in Guillain-Barré syndrome. Neurology. 2001;56:758–65.
3. Rees JH, Soudain SE, Gregson NA, Hughes RA. *Campylobacter jejuni* infection and Guillain-Barré syndrome. N Engl J Med. 1995;333:1374–9.
4. Visser LH, van der Meche FG, Meulstee J, Rothbarth PP, Jacobs BC, Schmitz PI, et al. Cytomegalovirus infection and Guillain-Barré syndrome: the clinical, electrophysiologic, and prognostic features. Dutch Guillain-Barré Study Group. Neurology. 1996;47:668–73.
5. Jacobs BC, Rothbarth PH, van der Meche FG, Herbrink P, Schmitz PI, de Klerk MA, et al. The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. Neurology. 1998;51:1110–5.
6. Hughes RA, Rees JH. Clinical and epidemiologic features of Guillain-Barré syndrome. J Infect Dis. 1997;176(Suppl 2):S92–8.
7. Asbury AK, Cornblath DR. Assessment of current diagnostic criteria for Guillain-Barré syndrome. Ann Neurol. 1990;27:S21–4.
8. Grangeot-Keros L, Mayaux MJ, Lebon P, Freymuth F, Eugene G, Stricker R, et al. Value of cytomegalovirus (CMV) IgG avidity index for the diagnosis of primary CMV infection in pregnant women. J Infect Dis. 1997;175:944–6.

9. Chabraoui F, Derrington EA, Mallie-Didier F, Confavreux C, Quincy C, Caudie C. Dot-blot immunodetection of antibodies against GM1 and other gangliosides on PVDF-P membranes. J Immunol Methods. 1993;165:225–30.
10. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika. 1988;75:800–3.
11. Jones RH, Ford PM, Hamman RF. Seasonality comparisons among groups using incidence data. Biometrics. 1988;44:1131–44.
12. Hughes RA, Cornblath DR. Guillain-Barré syndrome. Lancet. 2005;366:1653–66.
13. French Population Census. March 1999. The results. Paris: National Institute for Statistics and Economic Studies. Version 2.30.07. 2005 Dec 12 [cited 2006 Feb 6]. Available from http://www.recensement.insee.fr/RP99
14. Van Koningsveld R, van Doorn PA, Schmitz PI, Ang CW, van der Meche FG. Mild forms of Guillain-Barré syndrome in an epidemiologic survey in The Netherlands. Neurology. 2000;54:620–5.

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Francisella tularensis in Rodents, China

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A total of 420 rodents in China were examined for *Francisella tularensis* by polymerase chain reaction. The infection rates were 4.76% in total, and 11.65%, 10.00%, 6.56%, 1.77%, and 0% in Jilin, Xinjiang, Heilongjiang, Inner Mongolia, and Zhejiang, respectively. Sequence analysis showed that all the detected agents belonged to *F. tularensis* subsp. *holarctica*.

Tularemia is a disease caused by *Francisella tularensis*, a gram-negative, facultative intracellular bacterium. *F. tularensis* is generally believed to be confined to the Northern Hemisphere and has been reported in many American and Eurasian countries, for example, the United States, Mexico, Canada, Japan, the former Soviet Union, Spain, Sweden, and Norway (*1*). Terrestrial and aquatic mammals such as ground squirrels, rabbits, hares, voles, and water rats are animal reservoirs for transmission of *F. tularensis* to humans (*2*).

In China, *F. tularensis* was isolated in Daurian ground squirrels (*Spermophilus dauricus*) from Tongliao, Inner Mongolia Autonomous Region, in 1957. An outbreak of tularemia caused by contact with infected hares was first reported in Heilongjiang Province in 1959 (*3*). Thereafter, 6 cases were diagnosed in Qinghai Province in 1965 (*4*). Epidemiologic investigation identified several natural foci of the disease in Tibet from 1962 to 1972 and in Xinjiang Autonomous Region in 1986 (*5,6*), where *F. tularensis* was isolated from patients, *Ixodes liberelis* and *Dermacentor marginatus* ticks, and woolly hares (*Lepus oiostolos*). The latest outbreak occurred in 1986 at a food-processing factory in Shandong Province, where 31 of 36 workers who slaughtered hares became ill (*7*). Since then, no cases of *F. tularensis* infection have been reported in either humans or animal hosts. Whether the foci have become quiescent or the disease is underreported is unclear because tularemia is not a reportable disease in China. The objectives of this study were to investigate the presence of *F. tularensis* in rodents and to determine the subspecies type of the agent in China.

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The Study

During the spring and summer seasons of 2004 and 2005, rodents were captured with baited snap traps at 5 sites (Figure): Inner Mongolia Autonomous Region, Heilongjiang Province, Jilin Province, Xinjiang Autonomous Region, and Zhejiang Province. The first 3 sites are forested highlands in northeastern China. The study site in Xinjiang Autonomous Region is grassland in northwestern China. The study site in Zhejiang Province is wooded foothills in southern China.

After the species of the captured rodents was determined, a small piece of the spleen (500 mg) from each animal was used to extract DNA. Briefly, each spleen specimen was crushed with Trizol (Invitrogen, Carlsbad, CA, USA) to separate DNA from RNA after centrifugation, according to the manufacturer's instructions. Nested polymerase chain reaction (PCR) that targeted the *FopA* gene of *F. tularensis* was first performed as described previously (*8*). To further identify the agents in the samples by nested PCR and determine their genotype, PCR using the primer pair of C1 and C4 was performed to amplify the *ppI*-helicase region of *F. tularensis* gene structure (*9,10*). Then the products were gel purified by using Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with an automated DNA sequencer (ABI Prism 377, Perkin-Elmer, Foster City, CA, USA). To minimize contamination, DNA extraction, reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms.

Of 420 rodents tested by nested PCR, 20 were positive for the *FopA* gene of *F. tularensis*. The overall infection rate was 4.76% with a 95% confidence interval (CI) of 2.72%–6.78%. A total of 14 species of rodents were iden-

Figure. Study sites, People's Republic of China, 2004–2005.

tified in the study. Seven species, including *Apodemus peninsulae*, *A. agrarius*, *Cricetus migratorius*, *C. triton*, *Eutamias sibiricus*, *Meriones lybicus*, and *Clethrionomys rufocanus*, were positive for *F. tularensis* (Table). Although *E. sibiricus* and *M. lybicus* were most likely to be infected, with positive rates of 25% and 22.2%, respectively, no significant difference in infection rate was found among the 7 positive rodent species ($\chi^2 = 11.82$, degrees of freedom [*df*] = 6, p = 0.066).

The prevalence of *F. tularensis* in rodents varied with the geographic origin (Table). The infection rate was highest in rodents in Jilin Province (11.65%), followed by Xinjiang Autonomous Region (10.00%), Heilongjiang Province (6.54%), and Inner Mongolia Autonomous Region (1.76%). No *F. tularensis* infection was found in rodents collected from Zhejiang Province. The geographic difference in infection rate was significant ($\chi^2 = 20.91$, *df* = 4, p = 0.0003). PCR assay, targeting the *ppI*-helicase region in combination with sequence analysis, identified *F. tularensis* in rodents in China as type B. The nucleotide sequences of the 20 positive specimens were identical to the published sequences of *F. tularensis* subsp. *holarctica* strain (GenBank accession no. AF247642.2).

Conclusions

This study is the first PCR-based study on *F. tularensis* infection in rodents in China. Heilongjiang Province, Inner Mongolia, and Xinjiang Autonomous Regions were recognized as the natural foci of tularemia ≈40 years ago. The high prevalence of *F. tularensis* infection in rodents indicates that tularemia natural foci still exist in these areas. *F. tularensis* was detected for the first time in Jilin Province, which borders Heilongjiang Province and had similar landscape characteristics. Whether this newly described natural focus in Jilin Province is associated with human infection should be further investigated. In any case, this finding demonstrates that *F. tularensis* seems to be distributed more widely in China than expected. The extensive presence of *F. tularensis* indicates a potential threat to human health. The fact that rodents trapped from Zhejiang Province (southern China) were negative for the bacterium verifies our belief that *F. tularensis* is present only in northern China. The geographic variation in infection rate is likely attributable to the difference in biologic characteristics of each study site or a selection bias. Further studies are needed to clarify this question.

Rodents are efficient natural reservoirs for *F. tularensis* (*2*,*11*). In recent years, reports on human tularemia transmitted from small animals have been increasing (*12*). In this study, several rodent species were found to harbor *F. tularensis*, but which species are the main hosts in China is still unknown because no significant difference in infection rate was observed among rodents, regardless of their geographic origin. Systematic epidemiologic studies are required to investigate characteristics of natural foci and the role of both wild and domestic animals in transmission of *F. tularensis* to humans.

This work was supported by the National 973 Program of China (2002CB513201).

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References

1. Tärnvik A., Berglund L. Tularemia. Eur Respir J. 2003;21:361–73.

Table. Results of detection for *Francisella tularensis* in rodents by species and geographic origin in China

Rodent species	No. positive/no. detected (%)					
	Jilin	Heilongjiang	Inner Mongolia	Xinjiang	Zhejiang	Total
Apodemus peninsulae	9/43 (20.93)	0/129	0/5	–*	–	9/60 (15)
A. agrarius	0/24	0/12	2/36 (5.56)	–	0/7 (0)	2/79 (2.5)
Cricetus barabensis	–	–	0/5	–	–	0/5
Cricetus migratorius	–	–	–	1/4 (25)	0/13(0)	1/17 (5.88)
Cricetus triton	1/5 (20)	–	–	–	–	1/5 (20)
Clethrionomys rutilus	–	–	0/13	–	–	0/13
Clethrionomys rufocanus	0/23	4/37(10.81)	0/2	–	–	4/62 (6.45)
Rattus losea	–	–	–	–	0/30	0/30
R. norvegicus	0/1		0/11	0/1	0/3	0/16
R. confucianus	–	–	–	–	0/70	0/70
Mus musculus	0/4(0)	–	–	0/11	–	0/15
Microtus maximoviczii	–	–	0/35	–	–	0/35
Meriones lybicus	–	–	–	1/4 (25)	–	1/4 (25)
Eutamias sibiricus	2/3 (66.67)	–	0/6	–	–	2/9 (22.22)
Total	12/103 (11.65)	4/61 (6.56)	2/113 (1.77)	2/20 (10)	0/123	20/420 (4.76)

*–, no rodents captured.

2. Ellis J, Oyston PCF, Green M. Tularemia. Clin Microbiol Rev. 2002;15:631–46.
3. Kang CG. An epidemiological investigation on the first epidemic of human tularemia in China. Chin J Epidemiol. 1980;1:248–51.
4. Zhu HJ, Wang WH, Zhou AQ. Six cases report of tularemia. Chin J Epidemiol. 1980;1:251–3.
5. Li L, Kou ZM. Preliminary study of tularemia in Ta-cheng District of the Xinjiang Uygur Autonomous Region. Chin J Epidemiol. 1985;6:20–2.
6. Guo CH, Zeng QG, Wang SC, Shi TF, Wu FL, Jia MH. Preliminary study of tularemia in Tibet Autonomous Region. Chin J Epidemiol. 1981;2:176–81.
7. Pen ZC, Gen YL, Xiao YT, Cheng BY. The investigation of the first outbreak of tularemia in Shandong Peninsula. Chin J Epidemiol. 1987;8:261–3.
8. Fulop M, Leslie D, Titball R. A rapid, highly sensitive method for the detection of *Francisella tularensis* in clinical samples using the polymerase chain reaction. Am J Trop Med Hyg. 1996;54:364–6.
9. Goethert HK, Shani I, Telford SR. Genotypic diversity of *Francisella tularensis* infecting *Dermacentor variabilis* ticks on Martha's Vineyard, Massachusetts. J Clin Microbiol. 2004;42:4968–73.
10. Liu W, He J, Duan Q, Zhang PH, Zhao QM, Wu XM, et al. Genotyping of *Francisella tularensis* strains isolated from China by short-sequence tandem repeat region analysis. Chin J Microbiol Immunol. 2003;23:826–8.
11. Kortepeter MG, Parker GW. Potential biological weapons threats. Emerg Infect Dis. 1999;5:523–7.
12. Avashia SB, Petersen JM, Lindley CM, Schriefer ME, DeMarcus KL, Kim DK, et al. First reported prairie dog-to human tularemia transmission, Texas, 2002. Emerg Infect Dis. 2004;10:483–6.

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Preventing Zoonotic Influenza Virus Infection

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We evaluated 49 swine industry workers and 79 nonexposed controls for antibodies to swine influenza viruses. Multivariate modeling showed that workers who seldom used gloves (odds ratio [OR] 30.3) or who smoked (OR 18.7) most frequently had evidence of previous H1N1 swine virus. These findings may be valuable in planning for pandemic influenza.

In the United States, influenza viruses are estimated to cause 36,000 human deaths and 200,000 hospitalizations annually (*1–5*). The current outbreaks of avian influenza in Asia and Eastern Europe remind us of the zoonotic potential of these viruses. Swine cells express sialic acids that can be receptors for swine, human, and avian influenza strains and facilitate cross-species influenza transmission and the genesis of novel influenza strains. Reported cases of human-to-swine and swine-to-human influenza transmission illustrate this potential (*6,7*).

Persons who work in enclosed livestock buildings (confinement workers) have among the highest risk of becoming infected with swine influenza virus. Their work involves close contact with many swine, including sick ones. The purpose of this cross-sectional study was to learn if these workers had evidence of previous swine influenza virus infection and, if so, to determine factors that cause them to be at increased risk.

The Study

Iowa is the top swine-producing state in the United States and markets ≈25 million swine a year. From November 2004 to March 2005, we recruited confinement workers. Site selection was based on the authors' community contacts and opportunities to invite workers to participate. Local veterinary clinics advertised the study and permitted enrollment at their facilities. This study was approved by the University of Iowa's institutional review board.

Persons were eligible to participate in the study if they had worked in a swine confinement facility in the past 12 months. Participants completed a questionnaire and permitted blood sample collection on enrollment. The questionnaire captured demographic, medical, and occupational data including influenza immunization history, swine occupational exposures, and use of protective equipment (gloves and masks). Nonexposed controls were enrolled during a concurrent study of University of Iowa faculty, staff, and students (*8*).

Serum samples were studied by using a hemagglutination inhibition (HI) assay against 2 recently circulating swine strains, A/Swine/WI/238/97 (H1N1) and A/Swine/WI/R33F/01 (H1N2), and 1 human influenza virus strain, A/New Caledonia/20/99 (H1N1). The swine H1N1 strain represents a lineage of virus that has been circulating among US swine for 70 years. The swine H1N2 strain first appeared in US swine in 1999. HI titer results are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.65% solution of guinea pig erythrocytes for human influenza and 0.5% solution of turkey erythrocytes for swine influenza.

Specimen laboratory results were examined for their statistical association with demographic, immunization, occupational, and other behavioral risk factors. Confinement workers were queried about the nature of their work and whether they had used protective equipment. Because incidence of high titers was low or nonexistent in most groups, H1N1 titers >10 were grouped. The resulting categories were <10, 10, and >10. Wilcoxon rank sum and χ^2 statistic or 2-sided Fisher exact test were used to access bivariate risk factor associations. Depending on the nature of the data and modeling assumptions, proportional odds modeling or logistic regression was used to adjust for multiple risk factors. Final multivariate models were designed by using a saturated model and manual backwards elimination. Analyses were performed by using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). Questionnaires were made available in both English and Spanish. Site selections were based on personal contacts in 3 completely different areas.

Forty-nine confinement workers and 79 nonexposed controls were enrolled in the study. The distribution of ages was similar for the 2 groups, but the confinement workers were more likely to be male and Hispanic and less likely to have received influenza vaccination (Table 1).

Swine confinement workers were categorized by type of work, frequency of contact with swine, use of gloves, and use of masks. The question "When working with sick or diseased swine, how often do you wear gloves?" explained the most variation in swine H1N1 antibody titers and was included in the best fit model. Workers who sometimes or never used gloves were significantly more likely (odds ratio [OR] 30.3, 95% confidence interval [CI]

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3.8–243.5) to have elevated titers than the nonexposed controls (Table 2). These workers also were significantly more likely (OR 12.7, 95% CI 1.1–151.1) (data not shown) to have elevated titers than the other confinement workers who used gloves most of the time or always. Workers who reported smoking also had high OR (data not shown) for elevated titers.

Multivariate analysis also showed that persons who had received the 2003–04 influenza vaccine were significantly more likely to have elevated titers (≥10) against swine H1N1 virus (Table 2) as well as swine H1N2 (data not shown). Although cross-reaction with 1 of the viruses in the 2003–04 vaccine or a circulating influenza virus may explain this occurrence, higher titers would have been

Table 1. Characteristics of swine workers and controls, Iowa, 2004–2005

		Study sample, n (%)	
Variable	Total	Swine workers (n = 49)	Controls (n = 79)*
Age group (y)			
<29	46	15 (30.6)	31 (39.2)
29–42	40	12 (24.5)	28 (35.4)
>42	42	22 (44.9)	20 (25.3)
Mean age (y)		40.3	35.3
Sex†			
Male	63	37 (75.5)	26 (32.9)
Female	65	12 (24.5)	53 (67.1)
Race†			
American Indian/Alaska Native	1	0	1 (1.3)
Asian	2	1 (2)	1 (1.3)
African American	18	0	18 (22.8)
Native Hawaiian/Other Pacific Islander	2	0	2 (2.5)
White	105	48 (98)	57 (72.2)
Ethnicity†			
Hispanic/Latino	12	10 (21.7)	2 (2.5)
Non-Hispanic/Latino	113	36 (78.3)	77 (97.5)
Served in military			
Yes	12	5 (10.4)	7 (8.9)
No	115	43 (89.6)	72 (91.1)
Take medications that weaken immune system			
Yes	3	2 (4.2)	1 (1.3)
No	122	44 (91.7)	78 (98.7)
Do not know	2	2 (4.2)	0 (0)
Have heart or vascular disease			
Yes	3	2 (4.2)	1 (1.3)
No	124	46 (95.8)	78 (98.7)
Have any chronic lung problems such as asthma or emphysema			
Yes	5	2 (4.2)	3 (3.8)
No	122	46 (95.8)	76 (96.2)
Received the 2001–02 influenza vaccine			
Yes	37	6 (12.5)	31 (39.2)
No	90	42 (87.5)	48 (60.8)
Received the 2002–03 influenza vaccine†			
Yes	36	5 (10.4)	31 (39.2)
No	91	43 (89.6)	48 (60.8)
Received the 2003–04 influenza vaccine†			
Yes	43	8 (16.7)	35 (44.3)
No	84	40 (83.3)	44 (55.7)
Received the 1976–77 influenza vaccine†			
Yes	2	1 (2.1)	1 (1.3)
No	119	41 (85.4)	78 (98.7)
Do not know	6	6 (12.5)	0
Smoked ≥5 packs of cigarettes in past year†			
Yes	14	9 (18.4)	5 (6.3)
No	114	40 (81.6)	74 (93.7)

*Not exposed to swine.
†Significantly different than controls at α = 0.05.

Table 2. Odds ratios for increased serologic response against swine H1N1 influenza virus by hemagglutination inhibition assay

Variable	n	Swine H1N1*			
		Titer ≥10, n (%)	Titer ≥20, n (%)	Bivariate OR (95% CI)	Multivariate OR (95% CI)
Age group (y)					
<29	40	3 (7.5)	1 (2.5)	1.2 (0.2–6.1)	3.5 (0.4–30.6)
29–42	46	3 (6.5)	1 (2.2)	Reference	Reference
>42	42	9 (22)	6 (14.6)	4.2 (1.1–16.8)†	6.1 (0.9–41.3)
Sex					
Male	63	13 (21)	7 (11.3)	8.4 (1.8–38.7)†	7 (0.9–52.1)
Female	65	2 (3.1)	1 (1.5)	Reference	Reference
Swine exposure					
Swine workers occasionally or never use gloves	34	12 (35.3)	7 (20.6)	21 (4.4–100.8)†	30.3 (3.8–243.5)†
Swine workers usually or always use gloves	14	1 (7.1)	0	2.8 (0.2–34.2)	2.4 (0.1–40.9)
Controls not exposed to swine	79	2 (2.6)	1 (1.3)	Reference	Reference
Smoked ≥5 packs of cigarettes in past year					
Yes	14	4 (28.6)	3 (21.4)	4 (1.1–14.5)†	18.7 (2.5–141.3)†
No	114	11 (9.7)	5 (4.4)	Reference	Reference
Received 2002–03 influenza vaccine					
Yes	36	4 (11.4)	1 (2.9)	1 (0.3–3.4)	–
No	91	10 (11)	7 (7.7)	Reference	–
Received 2003–04 influenza vaccine					
Yes	43	6 (14.3)	3 (7.1)	1.6 (0.5–4.8)	16.3 (2.5–107.4)†
No	84	8 (9.5)	5 (6)	Reference	Reference
Elevated titer human H1N1 (≥40)					
Positive	39	2 (5.3)	1 (2.6)	0.3 (0.1–1.5)	–
Negative	89	13 (14.6)	7 (7.9)	Reference	–

*OR, odds ratio; CI, confidence interval; by using proportional odds model, these titers were grouped: <10, 10, >10.
†Significant odds for increased serologic response, p<0.05.

expected for all vaccinated persons (including controls), but such higher titers were not observed (Figure). We suggest that this result represents other behavior or health-related confounders not identified in the questionnaire for this study.

Conclusions

These data suggest, like previous studies (*8–10*), that swine confinement workers are at increased risk for zoonotic influenza infection. However, our data are among the first to evaluate swine confinement workers, our sample size was small (not likely representative of all swine workers), and exposure data were self-reported. Confinement workers, in contrast to other swine occupations, are difficult to reach because of language barriers, on-farm policies regarding visitors (biosecurity protocols), and lack of trust in the public health sector.

Several studies have documented smoking as a risk factor for human influenza virus infection (*11–13*). However, we believe our data are the first evidence that smoking also increases the risk for swine influenza virus infections. We believe that this increased risk may be because the workers' oral mucosa are exposed to swine influenza virus after handling pigs.

This study's chief unique contribution is the evidence that use of gloves during swine confinement work noticeably decreases the risk for swine influenza virus infection. Thus, a simple personal protective measure might do much to reduce swine-to-human virus transmission. Future larger studies of swine confinement workers are needed to validate our findings and to better quantify risk factors for this population.

Individual behavior strongly influences influenza virus transmission (*5*). The national strategy for pandemic influenza highlights worker education and emphasizes individual responsibilities in preventing the spread of infection (*14*). Should a virulent, novel zoonotic influenza virus enter swine confinement facilities and spread among concentrated swine populations, the impact would be grave. Surveillance for zoonotic influenza virus therefore must be routinely conducted among agricultural workers. Also, use of personal protective equipment, frequent hand

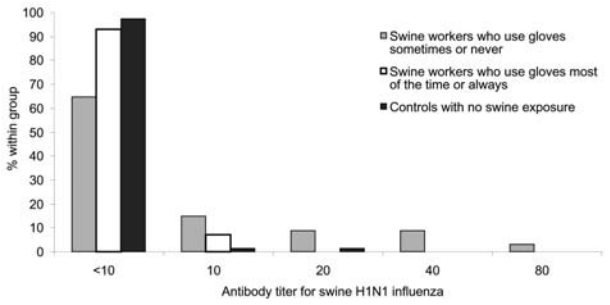

Figure. Variation in serologic response against swine H1N1 influenza virus and frequency of glove use by swine workers.

washing, and restrictions on smoking in or around swine facilities should be encouraged. Further, such workers should be included in state and federal pandemic plans as a high-risk group designated to receive annual influenza vaccines and antiviral drugs during pandemics.

Acknowledgments

We thank the veterinarians and staff from Valley Veterinary Center and the Winthrop Veterinary Clinic for use of their facilities for this project; James Miller and Hector Hernandez for their assistance in recruiting study subjects; the phlebotomists who volunteered to help with this project, especially Amy Kapanka and Carrie Ducommun; Kendall Myers for her assistance with study data; Christopher Olsen for providing swine viruses; Alexander Klimov for providing human viruses; and James Roth and the staff at the Center for Food Security and Public Health for their support.

This work was funded by the University of Iowa's Center for Emerging Infectious Diseases. Serologic assay work was funded in part by a grant from the National Institutes of Allergy and Infectious Diseases (NIAID-R21 AI059214-01).

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References

1. Murphy B, Webster RG. Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, et al., editors. Fields virology. 3rd ed. New York: Raven Press; 1996. p. 1397–445.
2. Simonsen L, Clarke MJ, Williamson GD, Stroup DF, Arden NH, Schonberger LB. The impact of influenza epidemics on mortality: introducing a severity index. Am J Public Health. 1997;87:1944–50.
3. Bridges CB, Fukuda K, Cox NJ, Singleton JA. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2001;50(RR-4):1–44.
4. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, et al. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. 2003;289:179–86.
5. Homeland Security Council. National strategy for pandemic influenza. 2005 [cited 2006 Apr 14]. Available from http://www.whitehouse.gov/homeland/pandemic-influenza.html#section3
6. Alexander DJ, Brown IH. Recent zoonoses caused by influenza A viruses. Rev Sci Tech. 2000;19:197–225.
7. Brown IH. The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol. 2000;74:29–46.
8. Myers K, Olsen C, Setterquist S, Capuano A, Donham K, Thacker E, et al. Evidence of swine influenza virus infection among swine workers. Clin Infect Dis. 2006:42;14–20.
9. Woods GT, Schnurrenberger PR, Martin RJ, Tompkins WA. Swine influenza virus in swine and man in Illinois. J Occup Med. 1981;23:263–7.
10. Olsen CW, Brammer L, Easterday BC, Arden N, Belay E, Baker I, et al. Serologic evidence of H1 swine influenza virus infection in swine farm residents and employees. Emerg Infect Dis. 2002;8:814–9.
11. Finklea JF, Sandifer SH, Smith DD. Cigarette smoking and epidemic influenza. Am J Epidemiol. 1969;90:390–9.
12. Arcavi L, Benowitz NL. Cigarette smoking and infection. Arch Intern Med. 2004;164:2206–16.
13. Kark JD, Lebiush M, Rannon L. Cigarette smoking as a risk factor for epidemic a(h1n1) influenza in young men. N Engl J Med. 1982;307:1042–6.
14. US Department of Health and Human Services. HHS Pandemic Influenza Plan 2005 [cited 2006 Apr 14]. Available from http://www.hhs.gov/pandemicflu/plan/pdf/HHSPandemicInfluenzaPlan.pdf

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Antiretroviral Drug Resistance and Routine Therapy, Cameroon

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Among 128 patients routinely receiving highly active antiretroviral therapy in an HIV/AIDS outpatient clinic in Cameroon, 16.4% had drug resistance after a median of 10 months. Of these, 12.5% had resistance to nucleoside reverse transcriptase inhibitors (NRTIs), 10.2% to non-NRTIs, and 2.3% to protease inhibitors.

HIV drug resistance is a major threat to the scaling up of antiretroviral therapy (ART) in developing countries (the World Health Organization/United Nations Programme on HIV/AIDS "3 by 5" Initiative) (*1*), especially in Africa (*2*). Inadequate clinical and biological follow-up has been linked to high rates of drug resistance (>50% after 8 to 20 months) in Gabon (*3*), Côte d'Ivoire (*4*), and Uganda (*5*). In a recent study in public and private health care clinics in Douala, the economic capital of Cameroon, we found that the clinical and biological follow-up and drug supply were irregular and that many patients interrupted their treatment (*6*). Data on drug resistance in the routine care setting are urgently required to design large, effective ART programs. We describe the frequency and nature of major genotypic mutations conferring resistance to antiretroviral drugs among patients treated in a routine HIV/AIDS outpatient clinic in Yaoundé, the political capital of Cameroon.

The Study

We conducted a cross-sectional survey from January 2002 to January 2004 among HIV-1–infected patients managed at the Central Hospital. The patients had to pay for their drugs (US $23–$100 monthly) and laboratory tests (US $58–$85 per viral load assay and $19–$27 per CD4 cell count). Consequently, follow-up was often irregular. All patients who were given ART for at least 3 months were eligible for the study. Approximately 15%–20% of eligible patients refused or were not asked (physicians forgot) to participate. Blood samples were not available for 9 other patients. The Cameroon national ethics committee approved the study protocol, and patients gave their informed consent. Basic demographic and medical data were recorded on a standard questionnaire.

HIV was typed in each patient (HIV-1 group M, N, or O, or HIV-2) with an in-house enzyme-linked immunosorbent assay (ELISA) based on V3 loop peptides (*7*). Genotypic resistance to antiretroviral drugs was studied by sequencing the protease and reverse transcriptase genes with group M- or O-specific primers, depending on the serotyping results (*8*); samples that could not be typed with ELISA were tested with both group M and O primers. Briefly, viral RNA was extracted from plasma with the QIAamp Viral RNA minikit (Qiagen, Courtaboeuf, France) and reverse transcribed to cDNA by using Expand RT (Boehringer, Mannheim, Germany) and a reverse primer. An 1,800-bp fragment encompassing the protease and reverse transcriptase genes was amplified by nested polymerase chain reaction and directly sequenced with an ABI PrISM Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer, Roissy, France). Genetic subtypes were determined by phylogenetic tree analysis with the Clustal W program (*8*). The deduced amino acid sequences were compared with a reference sequence to detect mutations associated with resistance. Mutations were classified as minor or major, by using the September 2004 version of the French National Agency for Research on AIDS consensus statements on antiretroviral drug resistance (http://www.hivfrenchresistance.org). A susceptible strain based on absence of major drug resistance mutations by genotyping or a strain that could not be amplified for genotyping was considered nonresistant.

One hundred twenty-eight HIV-1–infected patients received ART for a median of 10 months (interquartile range [IQR] 7–18). Median age was 39 years (IQR 33–46); 70 (54.7%) of the patients were women. In addition to nucleoside reverse transcriptase inhibitors (NRTIs), 94 patients (73.4%) had received non-NRTIs (59 patients received only efavirenz, 30 received only nevirapine, and 5 received both) and 53 patients (41.4%) had received protease inhibitors (PIs, 50 patients received only indinavir, 2 received only nelfinavir, and l received both); 19 patients had received both non-NRTIs and PIs. Two patients (1.6%) initially received only 2 NRTIs (lamivudine and zidovudine for 7 months in 1 case; stavudine and didanosine for 14 months in the other). Samples from 113 patients (88.3%) reacted with group M peptides, 3 samples (2.3%) reacted with group O peptides, and 2 other samples (1.6%)

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reacted with both group M and O peptides. Ten samples did not react with group M, N, or O or HIV-2 peptides. Thirty-five samples could be amplified, and all were characterized in the pol gene. The circulating recombinant form (CRF) 02-AG strain predominated (22 patients, 62.9%); the other 13 patients had subtype A (1), D (2) or F2 (3), or CRF01-AE (2), CRF02-AG/F (2), CRF11-cpx (2), or CRF13-cpx (1).

Major genotypic mutations associated with antiretroviral drugs resistance were detected in 21 patients (16.4%, 95% confidence interval 10.5–24.0). The characteristics of these patients are shown in the Table. Sixteen patients (12.5%) had resistance to NRTIs (Figure) due to the mutations M184V (15 patients), M184I (1), T215Y (1), T215F (3), K65R (2), and Q151M (1); thymidine analog mutations M41L (2), D67N (2), K70R (3), K219Q (1), and K219E (1) were also detected. Thirteen patients (10.2%) had resistance to non-NRTIs due to the mutations K103N (11), K101E (1), Y181C (1), Y188L (2), G190E (1), and P225H (2). Three patients (2.3%) had resistance to PIs due to the mutations V82A (2 patients) and N88D (1). The 2 patients treated for a time with only 2 NRTIs (patients 2-59 and 2-84, Table) had several major genotypic mutations but had received ART for 52 and 48 months, respectively.

Conclusions

This observational study showed that 16.4% of patients receiving ART in a routine care setting in Cameroon had drug resistance after a median of 10 months. The rate of resistance was lower than that observed in earlier studies in Côte d'Ivoire (*4*), Gabon (*3*), and Uganda (*5*). Several factors could explain this finding. First, a history of suboptimal therapy was rare: only 2 patients had received a 2-drug regimen, and none had received single-agent therapy. Second, 90% of our patients began receiving ART after a national consensus conference held in June 2000 had standardized the drugs supply, drugs regimen, and clinical and biological follow-up. Third, the physicians were trained and experienced in ART use. Fourth, the cost of drugs and laboratory tests has fallen in recent years in Cameroon, a

Table. Antiretroviral drug resistance in 21 patients receiving multiple ART in a routine care setting in Cameroon*

Patient no.	Age	Sex	Antiretroviral drugs received	Months from start of ART	Drug resistance	Major genotypic mutations	Subtype pol
2-29	46	F	3TC, ZDV, EFV	33	3TC, FTC, EFV, NVP	M184V, K103N, P225H	CRF02-AG
2-44	49	F	3TC, ZDV, EFV	10	3TC, FTC, EFV, NVP	M184V, (K70R), K103N, Y188L	CRF02-AG
2-47	42	M	3TC, ZDV, IDV	10	3TC, FTC	M184V	CRF02-AG
2-59	36	F	3TC, ZDV, EFV, IDV	52	3TC, ZDV, d4T, FTC, EFV, NVP	M184V, T215F, (M41L), K103N	CRF02-AG
2-66	36	M	3TC, ZDV, d4T, ddI, EFV	21	3TC, FTC, EFV, NVP	M184V, K103N	CRF02-AG
2-70	30	M	d4T, ddI, EFV	6	EFV, NVP	K103N	CRF02-AG/F
2-75	37	F	3TC, d4T, IDV	9	3TC, FTC	M184V	A
2-76	34	M	3TC, d4T, EFV	10	3TC, ZDV, d4T, FTC, EFV, NVP	M184V, T215Y, K103N	F2
2-84	51	M	3TC, ZDV, d4T, ddI, NFV	48	NRTIs, NFV	K65R, M184V, Q151M, N88D	D
2-91	44	M	3TC, d4T, EFV, IDV	6	EFV, NVP	G190E	CRF02-AG/F
2-98	32	F	d4T, ddI, IDV	7	IDV, RTV	V82A	D
22-2	42	M	3TC, ZDV, EFV	14	3TC, FTC, EFV, NVP	M184I, (M41L), K101E, K103N	CRF02-AG
22-9	33	F	3TC, ZDV, d4T, ddI, EFV, IDV	31	3TC, FTC, EFV, NVP	M184V, K103N, P225H	CRF11-cpx
22-25	30	F	3TC, ZDV, EFV, IDV	18	3TC, FTC	M184V	CRF02-AG
22-31	42	M	3TC, d4T, IDV	8	3TC, FTC	M184V	CRF02-AG
22-33	41	F	3TC, ZDV, IDV	18	3TC, FTC	M184V	CRF02-AG
22-35	58	M	3TC, ZDV, IDV	17	ATV, IDV, RTV	V82A	CRF01-AE
22-47A	48	F	3TC, ZDV, EFV, IDV	45	3TC, ZDV, d4T, FTC, EFV, NVP	M184V, T215F, (D67N, K70R, K219Q), K103N, Y188L	CRF02-AG
22-50	32	M	3TC, d4T, NVP	6	3TC, FTC, TDF, (ABC, ddI), EFV, NVP	K65R, M184V, Y181C	CRF01-AE
22-57	53	M	3TC, ZDV, EFV	7	EFV, NVP	K103N	CRF02-AG
22l-75	50	F	3TC, ZDV, d4T, ddI, EFV, IDV	29	3TC, ZDV, d4T, FTC, EFV, NVP	M184V, T215F, (D67N, K70R, K219E), K103N	CRF02-AG

*ART, antiretroviral therapy; 3TC, lamivudine; ZDV, zidovudine; EFV, efavirenz; FTC, emtricitabine; NVP, nevirapine; IDV, indinavir; d4T, stavudine; ddI, didanosine; NFV, nelfinavir; NRTIs, nucleoside reverse transcriptase inhibitors; ATV, atazanavir; RTV, ritonavir; TDF, tenofovir; SQV, saquinavir; ABC, abacavir. Resistances in parentheses indicate possible resistances. Mutations in parentheses indicate thymidine analogue mutations.

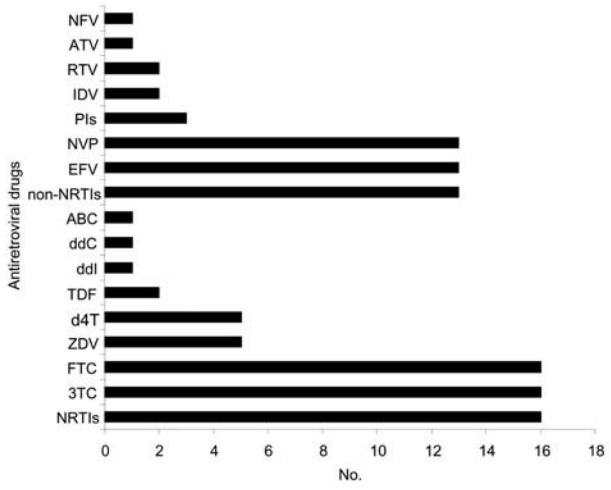

Figure. Patients with resistance to antiretroviral drugs. NFV, nelfinavir; ATV, atazanavir; RTV, ritonavir; IDV, indinavir; PIs, protease inhibitors; NVP, nevirapine; EFV, efavirenz; non-NRTIs, non-nucleoside reverse transcriptase inhibitors; ABC, abacavir; ddC, zalcitabine; ddI, didanosine; TDF, tenofovir; d4T, stavudine; ZDV, zidovudine; FTC, emtricitabine; 3TC, lamivudine; NRTIs, nucleoside reverse transcriptase inhibitors.

fact that favors adherence to therapy. Our methods could also account for the difference: our median follow-up period was substantially less than that in the studies in Gabon and Uganda, so that our patients had less time for resistance to develop, and our assumption that nonamplification was equivalent to nonresistance could have led to an undercount of resistant strains. Lower rates of resistance were achieved in pilot studies in Cameroon (*9*) and Senegal (*10*,*11*), thanks to measures favoring adherence to therapy, such as provision of drugs and laboratory follow-up at no cost (or for a limited charge), and psychosocial support (counseling, access to discussion groups, and active search for patients who did not attend scheduled clinical visits, biological examinations, or drug dispensing sessions).

Resistance most often involved lamivudine (12.5%; and emtricitabine, due to mutation M184V/I related to lamivudine pressure [emtricitabine was not used in Cameroon]), efavirenz, and nevirapine (10.2%). These drugs are widely used in Cameroon in either individual formulations or fixed-dose combinations (lamivudine/zidovudine, lamivudine/stavudine, lamivudine/stavudine/nevirapine, and lamivudine/zidovudine/nevirapine). The fixed-dose combination of lamivudine/stavudine/nevirapine is now the most frequently prescribed drug in Cameroon and other African countries (*12*). In our study, 19 patients (14.8%) had resistance to ≥1 component on this fixed-dose combination, and high rates of resistance could compromise the use of this inexpensive (US $4.5 monthly) and convenient drug. Frequent resistance to nevirapine could also compromise the use of this drug for preventing mother-child transmission (most such programs in Africa, including in Cameroon, are based on nevirapine).

Our study showed a relatively low level of resistance after a median duration of 10 months' treatment in a routine care setting, but we could not evaluate the association of resistance with adherence, support, or prescribing practices. The differences in methods among the African cross-sectional studies of resistance, including our own and the others referenced, make comparisons among countries difficult, although some differences are likely due to prescribing practices, drug availability, support for adherence, and follow-up. More extensive prospective studies that use standardized methods could provide comparable estimates of resistance seen at specific times (e.g., 6, 12, and 24 months after ART begins) in different countries and delineate ART program factors associated with a low prevalence of resistance.

Acknowledgments

We thank all the patients and staff who participated in the study.

This study was supported by a grant from the French National Agency for Research on AIDS (ANRS 1257) and the Institut de Recherche pour le Développement.

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References

1. World Health Organization/United Nations Programme on HIV/AIDS. WHO-UNAIDS report. Treating 3 million by 2005: making it happen. [cited 2005 Jun 16]. Available from http://www.who.int/3by5/publications/documents/en/3by5StrategyMakingItHappen.pdf
2. Stevens W, Kaye S, Corrah T. Antiretroviral therapy in Africa. BMJ. 2004;328:280–2.
3. Vergne L, Malonga-Louellet G, Mistoul I, Mavoungou R, Mansaray H, Peeters M, et al. Resistance to antiretroviral treatment in Gabon: need for implementation of guidelines on antiretroviral therapy use and HIV-1 drug resistance monitoring in developing countries. J Acquir Immune Defic Syndr. 2002;29:165–8.
4. Adjé C, Cheingsong R, Roels TH, Maurice C, Djomand G, Verbiest W, et al. High prevalence of genotypic and phenotypic HIV-1 drug-resistant strains among patients receiving antiretroviral therapy in Abidjan, Côte d'Ivoire. J Acquir Immune Defic Syndr. 2001;26:501–6.
5. Richard N, Juntilla M, Abraha A, Demers K, Paxinos E, Galovich J, et al. High prevalence of antiretroviral resistance in treated Ugandans infected with non-subtype B human immunodeficiency virus type 1. AIDS Res Hum Retroviruses. 2004;20:355–64.
6. Laurent C, Meilo H, Guiard-Schmid JB, Mapoure Y, Noël JM, M'Bangué M, et al. Antiretroviral therapy in public and private routine health care clinics in Cameroon: lessons from the Douala antiretroviral (DARVIR) initiative. Clin Infect Dis. 2005;41:108–11.

7. Vergne L, Bourgeois A, Mpoudi-Ngole E, Mougnutou R, Mbuagbaw J, Liegeois F, et al. Biological and genetic characteristics of HIV infections in Cameroon reveals dual group M and O infections and a correlation between SI-inducing phenotype of the predominant CRF02_AG variant and disease stage. Virology. 2003;310:254–66.
8. Vergne L, Peeters M, Mpoudi-Ngole E, Bourgeois A, Liegeois F, Toure-Kane C, et al. Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients. J Clin Microbiol. 2000;38:3919–25.
9. Bourgeois A, Laurent C, Mougnutou R, Nkoué N, Lactuock B, Ciaffi L, et al. Field assessment of generic antiretroviral drugs: a prospective cohort study in Cameroon. Antivir Ther. 2005;10:335–41.
10. Vergne L, Touré Kane C, Laurent C, Diakhaté N, Ngom Gueye NF, Gueye PM, et al. Low rate of genotypic HIV-1 drug-resistant strains in the Senegalese government initiative of access to antiretroviral therapy. AIDS. 2003;17(Suppl 3):S31–8.
11. Lanièce I, Ciss M, Desclaux A, Diop K, Mbodj F, Ndiaye B, et al. Adherence to HAART and its principal determinants in a cohort of Senegalese adults. AIDS. 2003;17(Suppl 3):S103–8.
12. Laurent C, Kouanfack C, Koulla-Shiro S, Nkoué N, Bourgeois A, Calmy A, et al. Effectiveness and safety of a generic fixed-dose combination of nevirapine, stavudine, and lamivudine in HIV-1-infected adults in Cameroon: open-label multicentre trial. Lancet. 2004;364:29–34.

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Cowpox Virus Transmission from Rats to Monkeys, the Netherlands

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We report an outbreak of cowpox virus among monkeys at a sanctuary for exotic animals. Serologic analysis and polymerase chain reaction were performed on blood and swab samples from different rodent species trapped at the sanctuary during the outbreak. Sequence comparison and serologic results showed that brown rats (*Rattus norvegicus*) transmitted the virus to monkeys.

Cowpox virus (CPXV) is a member of the genus *Orthopoxvirus*, family *Poxviridae*, and is antigenically and genetically related to variola virus, vaccinia virus, and monkeypox virus (MPXV). With the eradication of smallpox, routine vaccination with vaccinia virus ceased, which created a niche for animal poxviruses to infect humans. However, cowpox is a rare zoonosis, and infection of immunocompetent persons usually results in localized lesions mainly on fingers, hands, or face. However, in immunocompromised patients, severe generalized infections have been documented (*1*,*2*).

The reservoir hosts of CPXV are wild rodents; cows, domestic cats, and humans are incidental hosts. In Europe, bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) constitute the main reservoirs (*3*), whereas CPXV was sporadically detected in rats (*Rattus norvegicus*) (*4*,*5*). Domestic cats play a role in transmission of CPXV to humans (*6*,*7*). Direct transmission of CPXV from rodents to humans has also been documented (*3*,*5*). In the United States, prairie dogs (*Cynomys ludovicianus*) have been suggested as a potential reservoir for MPXV and are susceptible to CPXV infection by wild rodents (*8*). We report an outbreak of CPXV in nonhuman primates through contact with infected brown rats.

The Study

In September 2003, three Barbary macaques (*Macaca sylvanus*) at a sanctuary for exotic animals in Almere, the Netherlands, showed multifocal gingival, buccal, and lingual lesions. Typical intranuclear inclusions were found by histologic analysis, and poxlike particles were found by transmission electron microscopy of 6 biopsy specimens from buccal lesions of the same animals. Because of concerns that these macaques were infected with MPXV, additional biopsy specimens of poxlike lesions were obtained for virus isolation and polymerase chain reaction (PCR) studies.

Vero cells were infected with homogenized biopsy samples from the 3 macaques, and cells were monitored daily for appearance of cytopathic changes. Three days after infection, cells showed cytopathic effects characterized by plaques of rounded cells with prominent cytoplasmic bridging and syncytia formation. To confirm the isolation of a poxvirus, an immunofluorescence test was conducted with human antivaccinia serum (diluted 1:1,000) and goat antihuman immunoglobulin G (IgG) (diluted 1:500, Dako, Roskilde, Denmark). Diffuse cytoplasmic fluorescence confirmed an orthopoxvirus.

Isolates were further characterized by PCR and sequence analysis with primers for the hemagglutinin gene (*9*). Melting curve and sequence analyses confirmed the presence of an orthopoxvirus, most likely CPXV (Figure). Because this PCR assay was designed to differentiate variola virus from other orthopoxviruses but not among nonvariola orthopoxviruses, we developed a CPXV-specific PCR by using nested primer sets within the A-type inclusion protein (ATI) gene. PCR was conducted by using external primers (ATIF1) 5′-GAACTTAATAAGTGTTTCGATA-3′ (forward primer) and (ATIR1) 5′-CAGTAACGTCGGACGATGGAGG-3′ (reverse primer) with nested forward primer ATIF2 5′-GAGGAAGTTAAGAGATTGCGTC-3′ and reverse primer ATIR1. The nucleotide sequences are available from GenBank. Nucleotide sequencing confirmed that the virus isolated from Barbary macaques was a CPXV.

Since all macaques were in the center before disease manifested and they had not been in contact with other animals, other monkeys were tested for CPXV infection by serologic analysis. Serum samples from 16 Barbary macaques (*Macaca sylvanus*), 2 pig-tailed macaques (*M. nemestrina*), 2 squirrel monkeys (*Saimiri sciureus*), 2 Japanese macaques (*M. fuscata*), 6 cynomolgus macaques (*M. fascicularis*), 2 Hamadryas baboons (*Papio hamadryas*), 4 rhesus macaques (*M. mulatta*), and 1 vervet (*Cercopithecus aethiops*) were tested with a virus neutralization test (VNT) using a CPXV strain isolated in this study (CPXVmac). Neutralizing titers were determined after 5 days on the basis of complete reduction of a cytopathic effect. At the end of the outbreak, neutralizing serum antibodies were detected in 9 Barbary macaques,

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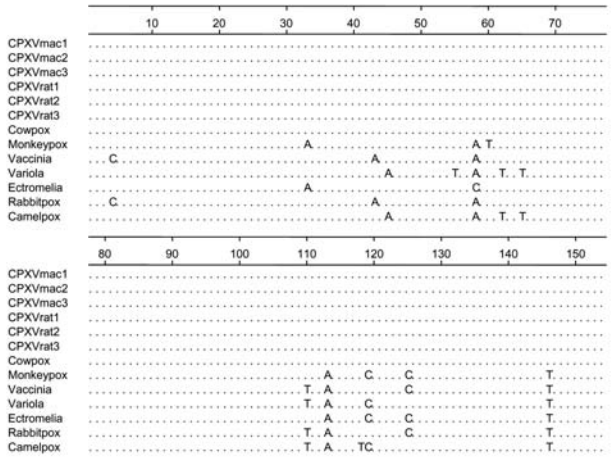

Figure. Sequence alignment of the partial hemagglutinin gene of cowpox viruses (CPXV) isolated from Barbary macaques and brown rats. CPXV-2001 strain was isolated from a rat in 2001 from the Netherlands (*5*).

2 pig-tailed macaques, 1 Japanese macaque, 3 cynomolgus macaques, and 1 rhesus monkey. This finding suggested that that all of these animals had been exposed to CXPV. Retrospective serosurveillance showed that only 1 Barbary macaque was seropositive at the start of the outbreak. No swab samples from the animals were available for culture or PCR analysis.

To identify the possible reservoir of the CPXV infection, animals known to be susceptible to CPXV and housed at the sanctuary at the time of the outbreak were tested. These included 4 domestic cats (*Felis catus*), 2 red squirrels (*Sciurus vulgaris*), and 6 prairie dogs (*C. ludovicianus*). In addition, 32 wood mice (*A. sylvaticus*) and 34 rats (*R. norvegicus*) trapped in the area of the sanctuary were tested by serologic analysis and PCR. Only 1 of the tested cats had neutralizing serum antibodies (titer 20) to CPXV. Throat swabs of all 4 cats obtained during the outbreak were negative by both PCRs. These results suggest that cats were not infected by CPXV at the time of the outbreak. Prairie dogs housed at the sanctuary at the time of the outbreak were not infected, as shown by negative PCR results on throat swabs and the absence of orthopoxvirus-specific antibodies.

In contrast, PCR and virus culture showed that 56% of rats tested were infected with CPXV (Table). Sequence comparison of the hemagglutinin and ATI genes of 19 CPXV rat isolates with the Barbary macaque isolates showed identical sequences, which indicated that rats were the most probable source of infection. No significant differences were observed between the genes of CPXVs isolated during this outbreak and previous isolates (Figure).

Since serum samples collected from trapped dead rats could not be tested by VNT, we developed an indirect enzyme-linked immunosorbent assay (ELISA) based on vaccinia virus, which was validated with sera of monkeys infected with vaccinia virus. Briefly, vaccinia virus was treated with 2% Triton X-100, and 100 ng was added to wells of high-binding plates (Costar, Corning Inc., Corning, NY, USA). Two-fold dilutions of sera starting at 1:20 were added to the plates, and antibodies were detected with horseradish peroxidase–labeled protein A (1:1,000 dilution, Zymed Laboratories, South San Francisco, CA, USA). Titers were expressed as the reciprocal of the highest serum dilution for which an optical density at 450 nm (OD_{450}) was >3× the OD_{450} of the negative control. Of 19 rats that tested positive by PCR, only 3 were seropositive, which suggested that CPXV was spreading actively in the rat population. Swabs of all mice tested were negative by both PCRs, and only 2 of 32 mice tested were seropositive by ELISA, indicating that mice were not the source of infection.

Conclusions

This is the first report describing CPXV infection in captive monkeys. Wild brown rats captured at the sanctuary

Table. Results for animals tested for cowpox virus infection, the Netherlands, 2003*

Animal tested	VNT	ELISA	Virus isolation	PCR
Barbary macaque (*Macaca sylvanus*)	9/16	9/16	ND	ND
Pig-tailed macaque (*M. nemestrina*)	2/2	2/2	ND	ND
Squirrel monkey (*Saimiri sciureus*)	0/2	0/2	ND	ND
Japanese macaque (*M. fuscata*)	1/2	1/2	ND	ND
Cynomolgus macaque (*M. fascicularis*)	3/6	4/6	ND	ND
Hamadryas baboon (*Papio hamadryas*)	0/2	0/2	ND	ND
Rhesus macaque (*M. mulatta*)	1/4	2/4	ND	ND
Vervet (*Cercopithecus aethiops*)	0/1	0/1	ND	ND
Domestic cat (*Felis catus*)	1/4	3/4	0/4	0/4
Red squirrel (*Sciurus vulgaris*)	0/2	0/2	0/2	0/2
Prairie dog (*Cynomys ludovicianus*)	0/6	0/6	0/6	0/6
Wood mouse (*Apodemus sylvaticus*)	0/32	2/32	0/32	0/32
Brown rat (*Rattus norvegicus*)	ND	11/34	16/28	19/34

*Values are no. positive/no. tested. VNT, virus neutralization test; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction, ND, not determined.

were infected with CPXV and assumed to be the most probable source of infection. Whether multiple contacts of infected rats with the monkeys contributed to the outbreak or monkey-to-monkey transmission occurred efficiently is not clear. Furthermore, whether monkeys were infected by rats through direct contact or infected excreta is unclear (*10*). Longitudinal field studies are required to clarify if rats could be the principal reservoirs for CPXV. Circulation of CPXV in wild and captive animals, together with decreased immunity against orthopoxviruses in the community, may put animal trappers and handlers at risk for CPXV infection. Our findings show the threat of orthopoxviruses that can cross species barriers, which indicates the importance of developing new vaccines and antiviral drugs against orthopoxvirus infection of humans.

Acknowledgments

We thank Thijs Kuiken for critical comments on the manuscript, Edwin Fries and Penelopie Koraka for technical assistance, and Herman Meyer for confirming the PCR results.

Dr Martina is a virologist in the department of virology at Erasmus Medical Center Rotterdam. His research interests include epidemiology of emerging viruses, pathogenesis, and vaccinology of flaviviruses.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

References

1. Pelkonen PM, Tarvainen K, Hynninen A, Kallio ER, Henttonen K, Palva A, et al. Cowpox with severe generalized eruption, Finland. Emerg Infect Dis. 2003;9:1458–61.
2. Czerny CP, Eis-Hubinge AM, Mayr A, Schneweis KE, Pfeiffer B. Animal poxviruses transmitted from cat to man: current event with lethal end. Zentralbl Veterinarmed. 1991;38:421–31.
3. Chantrey J, Meyer H, Baxby D, Begon M, Bown KJ, Hazel SM, et al. Cowpox: reservoir hosts and geographic range. Epidemiol Infect. 1999;122:455–60.
4. Marennikova SS, Shelukhina EM. White rats as source of pox infection in carnivora of the family *Felidae*. Acta Virol. 1976;20:442.
5. Wolfs TF, Wagenaar JA, Niesters HG, Osterhaus AD. Rat-to-human transmission of cowpox infection. Emerg Infect Dis. 2002;8:1495–6.
6. Baxby D, Bennett M. Cowpox: a re-evaluation of the risks of human cowpox based on new epidemiological information. Arch Virol. 1997;13:1–12.
7. Baxby D, Bennett M, Getty B. Human cowpox 1969–93: a review based on 54 cases. Br J Dermatol. 1994;131:598–607.
8. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, et al. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med. 2004;350:342–50.
9. Espy MJ, Cockerill FR III, Meyer RF, Bowen MD, Poland GA, Hadfield TL, et al. Detection of smallpox virus DNA by LightCycler PCR. J Clin Microbiol. 2002;40:1985–8.
10. Maiboroda AD. Experimental infection of Norwegian rats (*Rattus norvegicus*) with ratpox virus. Acta Virol. 1982;26:288–91.

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Pasture Types and *Echinococcus multilocularis*, Tibetan Communities

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Our study showed that open pastures had more small mammal burrows than fenced pastures in Tibetan pastoralist communities in 2003. This characteristic was linked to a higher prevalence of *Echinococcus multilocularis* in dogs and indicates that pasture type may affect *E. multilocularis* transmission.

Human alveolar echinococcosis (AE) is an infection caused by *Echinococcus multilocularis*, a highly pathogenic cestode. Foxes are frequently definitive hosts (adult stage), and small mammals are intermediate hosts (larval stage or metacestode). Human AE, albeit restricted to localized endemic areas, is a public health concern in central Europe (*1*). In western China (*2*,*3*), dogs are definitive hosts of AE and have transmitted infection to humans more often than was realized historically (*4*). Recent mass ultrasound screenings in Tibetan pastoral communities of Sichuan Province, People's Republic of China, documented an average AE prevalence of 2% (*5*) and a maximum prevalence of 14.3% (*6*) in humans.

Since the 1980s, partial fencing of pastures around Tibetan pastoral winter settlements has become common because of changes in land property regulations (*7*). In a previous study, we showed that partial fencing promoted AE transmission in these communities (*8*). This increased disease prevalence is likely due to the greater population of small mammal intermediate hosts of the parasite on the Tibetan plateau and leads to more infection in community dogs (*8*). When yak, sheep, and horse grazing lowers the height of vegetation, *Ochotona curzoniae*, a very susceptible host for *E. multilocularis*, may be found at greater densities than on natural meadows (*9*). This study was designed to investigate the effect of partial fencing on the general abundance of small mammals in the Tibetan pastoralist winter settlements and its potential consequences for contamination pressure. The study was approved by the ethical committees of Sichuan Institute of Parasitic Diseases and all collaborating investigators.

Based on documented high prevalence of AE and observed fencing practices in the area (*8*), Qiwu, Yiniu, and Xiazha townships in Shiqu County of Ganzi Tibetan Autonomous Prefecture, located at a mean elevation of 4,200 m in northwest Sichuan Province, were selected as study sites to carry out investigations in spring and autumn 2003. For the 3 townships, the populations were 2,238, 2,515, and 2,471 and the areas 1,046 km^2, 955 km^2, and 834 km^2, respectively. Thirty kilometers of transect over 30 settlements in the 3 townships (18 villages), which were randomly selected according to landscape patterns, i.e., valley, valley entrance, piedmont, and flat land, were surveyed. Small mammal populations were monitored by using index methods. These methods are based on detecting surface indicators of small mammals, i.e., holes and feces, and are used to link small mammals and their habitats on large areas (*10–12*). Sampling was performed by 2 investigators walking along a 1-km transect drawn across each settlement, according to a standardized protocol. Along each transect, 50 areas were sampled for small mammal burrows; each area was 200 m^2. Areas of fenced pastures were measured in 22 settlements by using a global positioning system (GPS) (GPS 12, Garmin International Inc., Olathe, KS, USA). In 15 settlements in which dog feces samplings were conducted in Yiniu and Xiazha townships, feces specimens were collected from dogs after purging with arecoline, according to the recommendations of World Animal Health Organization/World Health Organization (*13*), and droppings were collected from the ground when accessible (*4*). Helminths found in the feces were removed, counted, and placed in 10% formal saline or 85% ethanol. Copro-polymerase chain reaction testing was conducted by using species-specific primers for *E. multilocularis* DNA amplification according to Dinkel et al. (*14*) as modified by van der Giessen et al. (*15*).

The distribution of small mammal burrows was highly skewed. Kolmogorov-Smirnov test indicated that the data did not fit a normal distribution ($p<0.001$ in both cases) either inside or outside fenced pastures. Normality was not obtained after Box-Cox transformations. Thus, the burrow density of small mammals was compared between open and fenced pastures by using nonparametric tests that considered landscape factor. Spearman correlation tests were used to quantify the relationship between the burrow

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Table 1. Comparisons of open and fenced pastures small mammal burrow densities, stratified by landscapes*

Landscape	Pasture type	No. observations	Mean rank of densities	Sum rank of densities	Mann-Whitney U	Z	Asymptomatic p (2-tailed)
Valley	Open	616	439.07	270,464.50	63,715.500	–2.784	0.005
	Fenced	234	389.79	91,210.50			
Flat land	Open	175	109.38	19,141.00	634.000	–5.819	<0.001
	Fenced	25	38.36	959.00			
Piedmont	Open	155	96.91	15,020.50	2,930.500	–1.643	0.100
	Fenced	45	112.88	5,079.50			
Valley entrance	Open	180	103.83	18,690.00	1,200.000	–2.833	0.005
	Fenced	20	70.50	1,410.00			

*Density, no. burrows per 200 m^2 of pasture.

density of small mammals on open pastures and the surface of fenced pastures in settlements in which the fenced areas were all measured, controlling for the landscape factor. A multiple logistic regression model was used to relate median burrow density of small mammals to dog infection in the settlements. The dependent variable was a presence/absence vector (0/1) (dog was negative or positive for *E. multilocularis*). Independent variables included dog's age and sex, droppings collected versus purged fecal samples, and median density of small mammal burrows. All these analyses used SPSS release 10 (SPSS, Chicago, IL, USA).

Landscape type influenced the abundance of small mammal burrows ($p<0.001$). Post hoc Tukey multiple comparison test on ranks confirmed that the densities of small mammal burrows were different among different landscape types ($p<0.05$), except for the comparison between flatland and piedmont. The burrow densities of small mammals on open pastures were significantly higher than those on fenced pastures in 3 of 4 landscapes (Table 1).

The Spearman correlations showed larger fenced areas associated with higher density of small mammal burrows in the open pastures in all landscape types (Table 2). The relationships between the area of fenced pastures and the burrow density of small mammals inside the fenced pastures in the 4 landscapes were not significant (valley [r_s =–0.08, p = 0.32], flatland [r_s =–0.46, p = 0.02], piedmont [statistics not applicable because of 3 observations only], and valley entrance [r_s = 0.08, p = 0.736]), except for flatland.

Feces samples, of which 159 (63.1%) were purged, were obtained from 252 dogs (mean age 4.4 years; 183 males). *E. multilocularis* infection rate was 16.7% (42/252); the infection rate was 18.2% (29/159) for purged samples and 14.0% (13/93) for sampled droppings. Multiple logistic regressions showed that the median density of small mammal burrows in the open pastures was significantly positively related to dog infection (p = 0.003, odds ratio 1.05, 95% confidence interval 1.02–1.08). No correlation to dog age (p = 0.52), sex (p = 0.78), or sample collection method was seen (p = 0.380).

The higher median burrow density of small mammals was linked to a higher prevalence of *E. multilocularis* in dogs in these Tibetan pastoralist communities. Thus, partial fencing increases populations of potentially susceptible small mammal species in open pastures and consequently higher contamination pressure by dogs.

In a previous study, we showed that partial fencing around Tibetan settlements in winter pasture was significantly and independently associated with the risk for human AE in surveyed villages (*8*). We assumed that the underlying reason might be overgrazing, exacerbated by reducing communal pastures near the settlements because of introduction of partial fencing around group tenure pastures acquired by Tibetan pastoralist families. Overgrazing may have promoted population outbreaks of small mammal intermediate hosts of the parasite and increased the density of the small mammal intermediate host, especially *O. curzoniae*. This increase consequently favored higher dog *E. multilocularis* infection and, thus, transmission to humans (*8*). This study supports this hypothesis.

This study was supported by the Sichuan Provincial Health Department, China, and the US National Institutes of Health/National Science Foundation (Ecology of Infectious Diseases program, grant #1565). Q.W. was supported by a PhD grant from the French Embassy in Beijing.

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Table 2. Relationship between surface of fenced pastures and densities of small mammal burrows in open pastures*

Characteristic	Valley	Flat land	Piedmont	Valley entrance
No. observations	490	126	147	130
Correlation coefficient	0.382	0.312	0.471	0.296
p (2-tailed)	<0.001	<0.001	<0.001	0.001

*Spearman correlations.

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References

1. Kern P, Bardonnet K, Renner E, Auer H, Pawlowski Z, Ammann RW, et al. European echinococcosis registry: human alveolar echinococcosis, Europe, 1982–2000. Emerg Infect Dis. 2003;9:343–9.
2. Ito A, Urbani C, Qiu JM, Vuitton DA, Qiu D, Heath DD, et al. Control of echinococcosis and cysticercosis: a public health challenge to international cooperation in PR China. Acta Trop. 2003;86:3–17.
3. Vuitton DA, Zhou H, Bresson-Hadni S, Wang Q, Piarroux M, Raoul F, et al. Epidemiology of alveolar echinococcosis with particular reference to China and Europe. Parasitology. 2003;127:S87–107.
4. Budke CM, Campos-Ponce M, Wang Q, Torgerson PR. A canine purgation study and risk factor analysis for echinococcosis in a high endemic region of the Tibetan plateau. Vet Parasitol. 2005;127:43–9.
5. Wang Q, Qiu JM, Schantz P, He JG, Ito A, Liu FJ. Risk factors for development of human hydatidosis among people whose family raises livestock in Western Sichuan Province, China. Chinese Journal of Parasite Diseases and Parasitology. 2001;19:289–93.
6. Tiaoying L, Jiamin Q, Wen Y, Craig PS, Xingwang C, Ning X, et al. Echinococcosis in Tibetan populations, western Sichuan Province, China. Emerg Infect Dis. 2005;11:1866–73.
7. Editorial commission of Shiqu County record. Chengdu (People's Republic of China): The People's Publication House of Sichuan Province. 2000; p. 55–137.
8. Wang Q, Vuitton DA, Qiu J, Giraudoux P, Xiao Y, Schantz PM, et al. Fenced pasture: a possible risk factor for human alveolar echinococcosis in Tibetan pastoralist communities of Sichuan, China. Acta Trop. 2004;90:285–93.
9. Zhang Y, Fan N, Wang Q, Jing Z. The changing ecological process of rodent communities during rodent pest managements in alpine meadow. Acta Theriologica Sinica. 1998;18:137–43.
10. Giraudoux P, Quéré JP, Delattre P, Bao G, Wang X, Shi D, et al. Distribution of small mammals along a deforestation gradient in south Gansu, China. Acta Theriologica. 1998;43:349–62.
11. Giraudoux P, Pradier B, Delattre P, Deblay S, Salvi D, Defaut R, et al. Estimation of water vole abundance, by using surface indices. Acta Theriologica. 1995;40:77–96.
12. Delattre P, De Sousa B, Fichet E, Quéré JP, Giraudoux P. Vole outbreaks in a landscape context: evidence from a six year study of *Microtus arvalis*. Landscape Ecology. 1999;14:401–12.
13. Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. WHO/OIE manual on echinococcosis in humans and animals. A public health problem of global concern. Paris: The World Health Organization: 2001.
14. 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.
15. van der Giessen JW, Rombout YB, Franchimont JH, Limper LP, Homan WL. Detection of *Echinococcus multilocularis* in foxes in The Netherlands. Vet Parasitol. 1999;82:49–57.

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Class 1 Integrons in Resistant *Escherichia coli* and *Klebsiella* spp., US Hospitals

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We examined *Escherichia coli* and *Klebsiella* spp. from US hospitals for class 1 integrons. Of 320 isolates, 181 (57%) were positive; association of integrons with resistance varied by drug and organism. Thus, determining integron epidemiology will improve understanding of how antibacterial resistance determinants spread in the United States.

Integrons are genetic elements, located on the bacterial chromosome or a plasmid, that often carry genetic determinants for antimicrobial drug resistance (*1*). The need for systematic epidemiologic studies of the role of integrons in antimicrobial drug resistance in bacteria has recently been emphasized (*2*). The prevalence of integrons is high among gram-negative isolates from patients in Europe (*3*,*4*), and some carry multiple integrons (*3*). Reports from Asian countries also have noted a high prevalence of class I integrons in gram-negative clinical isolates (*5*). Most of the resistance integrons found to date in clinical isolates of *Enterobacteriaceae* are class 1 integrons, which are highly associated with resistance to antimicrobial agents (*2*).

These data suggest that integrons are relatively common, especially among the *Enterobacteriaceae*, and that they contribute to the spread of antimicrobial drug resistance in healthcare settings. However, few studies from the United States have assessed the association between integron carriage and antimicrobial susceptibility patterns.

This study analyzes the association between class 1 integrons and resistance to selected antimicrobial agents in a convenience sample of *Escherichia coli* and *Klebsiella* isolates. Multivariate analysis was used to determine whether apparent associations were affected by interactions between variables. Isolates were submitted from hospitals participating in Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) (*6*). The protocol has been approved as exempt by the institutional review board at Emory University.

The Study

Clinical isolates of *E. coli*, *Klebsiella pneumoniae*, and *K. oxytoca* were obtained from 19 US hospitals during phase IV (2002–2004) of Project ICARE, which focused on nonoutbreak isolates of *Enterobacteriaceae* with decreased susceptibility to extended-spectrum cephalosporins, fluoroquinolones, or carbapenems. The 19 hospitals were located in 13 states throughout the country and had an approximately equal mix of teaching and nonteaching medical centers. Duplicate isolates from the same patient were excluded.

Isolates were tested for susceptibility to the antimicrobial drugs listed in Table 1 by using the broth microdilution reference method (BMD) described by the Clinical and Laboratory Standards Institute (*7*). Isolates were stored at −70°C and were subcultured to trypticase soy agar plates containing 5% defibrinated sheep blood (BD BioSciences, Sparks, MD, USA) ≥2 times before testing. For each organism, BMD tests were inoculated by using a cell suspension equivalent to a 0.5 McFarland standard. *Enterobacteriaceae* isolate identifications provided by participating laboratories were confirmed by colony shape, spot tests (*8*), and Vitek GNI+ cards (bioMérieux, Durham, NC, USA). Differences were resolved by using reference biochemical tests (*9*).

Isolates were analyzed by polymerase chain reaction (PCR) amplification techniques to determine whether a class 1 integron was present. Integrons were detected by PCR amplification of a class 1 integrase–specific fragment of the *IntI1* gene as previously described (*1*). The primer sequences used were IntI1-F: 5′-TCT CGG GTA ACA TCA AGG-3′ and IntI1-R: 5′-AGG AGA TCC GAA GAC CTC-3′.

Amplifications were performed in 10 μL of Taq PCR Master Mix (Qiagen, Valencia, CA, USA), 1.5 mmol/L $MgCl_2$, 5 pmol/L each primer, and 1 μg template DNA (*1*). DNA was extracted with a Genelite Bacterial Genomic DNA Kit (Sigma, Saint Louis, MO, USA). Amplification specifications were as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. PCR products were analyzed by gel electrophoresis with 1.5% agarose gels. All PCRs included positive and negative controls.

A statistical comparison of the frequencies of integron presence in *E. coli* and *Klebsiella* spp. was conducted by using odds ratios and 95% confidence intervals. Intermediate and resistant isolates were pooled as nonsusceptible for analysis. Multivariate logistic regression analysis was conducted with 1 representative from each

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Table 1. Crude association between nonsusceptibility to various antimicrobial drugs and positive test result for class 1 integron in *Escherichia coli* and *Klebsiella* spp. (*Klebsiella pneumoniae* and *K. oxytoca*)

Class/agent	*E. coli* (n = 209), OR (95% CI)*	*Klebsiella* spp. (n = 111), OR (95% CI)*
Aminoglycosides		
Amikacin	3.15 (0.32–30.79)	4.0 (0.86–18.51)
Gentamicin	**5.04 (2.69–9.47)**	**4.49 (1.80–11.22)**
Tobramycin	**5.55 (3.01–10.23)**	**5.0 (2.0–12.52)**
Fluoroquinolone		
Ciprofloxacin	**3.00 (1.40–6.42)**	1.33 (0.59–3.04)
3rd-generation cephalosporin		
Cefotaxime	1.14 (0.63–2.07)	2.26 (0.99–5.19)
Cefpodoxime	1.02 (0.59–1.75)	**2.61 (1.05–6.52)**
Ceftazidime	1.30 (0.73–2.31)	**2.54 (1.10–5.88)**
4th-generation cephalosporin		
Cefepime	0.35 (0.11–1.13)	1.87 (0.57–6.10)
Monobactam		
Aztreonam	1.58 (0.87–2.89)	1.10 (0.48–2.53)
β-Lactamase inhibitor		
Piperacillin-tazobactam	1.40 (0.75–2.62)	1.82 (0.80–4.14)
Others		
Chloramphenicol	**2.33 (1.30–4.17)**	2.29 (0.97–5.42)
Minocycline	1.57 (0.90–2.76)	1.20 (0.53–2.71)
Trimethoprim-sulfamethoxazole	**12.24 (6.28–23.86)**	**3.68 (1.57–8.62)**

*OR, odds ratio; CI, confidence interval. Variables with p values ≤0.05, in **boldface**, are considered significant.

major class of drugs tested. Data validation and analysis were performed by SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). Fit of the data to this model was evaluated by the Hosmer-Lemeshow χ^2 test.

A positive test result for class 1 integrons was found for 181 (57%) of the 320 bacterial isolates screened, including 103 (49%) of 209 *E. coli* isolates and 78 (70%) of 111 *Klebsiella* spp. isolates. A positive test result in *E. coli* isolates was significantly associated with nonsusceptibity to gentamicin, tobramycin, ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole (Table 1). A positive test result in *Klebsiella* isolates was significantly associated with nonsusceptibility to gentamicin, tobramycin, cefpodoxime, ceftazidime, and trimethoprim-sulfamethoxazole.

Multivariate analysis showed that the only drug variables associated with a positive test result for class 1 integrons in *E. coli* were nonsusceptibility to gentamicin and trimethoprim-sulfamethoxazole (with a strong interaction between the 2 variables), while an inverse association was present between a positive integron test result and nonsusceptibility to cefepime (Table 2). For *Klebsiella* spp., a positive association existed between a positive integron test result and nonsusceptibility to gentamicin and trimethoprim-sulfamethoxazole, as well as an inverse association between a positive integron test result and nonsusceptibility to aztreonam.

Conclusions

More than half of the selected isolates from US hospitals that we tested were positive for class 1 integrons. The prevalence of class 1 integrons in *E. coli* was 49% in selected non-outbreak isolates from hospitalized patients from 2002 to 2004 in our study, 52% in consecutive urinary tract isolates in 2001 in Sweden (*10*), and 15% in isolates from blood in hospitals in Sweden in 1998 and 1999 (*11*). The prevalence of integrons was 70% in *Klebsiella* isolates from hospitalized patients in our study and 73% in extended-spectrum β-lactamase (ESBL)–producing *Klebsiella* spp. collected from 3 hospitals in Australia from 1989 to 1999 (*12*). These data cannot be directly compared because of differences in selection criteria and testing procedures, but as a whole they suggest that prevalence of integrons in these bacteria in the United States is high, as it is in other areas of the world. Carbapenem resistance was infrequent in the isolates we tested, so we could not address the association of integron carriage and metallo-β-lactamases that has been observed in other parts of the world (*2*).

Our study found an inverse association between integron presence and nonsusceptibility to cefepime in *E. coli* and to aztreonam in *Klebsiella* spp., which indicates that resistance determinants for these drugs are not frequently carried by integrons. One possible reason for the lack of an association between nonsusceptibility to cefepime and integron carriage is the relatively recent approval of cefepime for use in the United States. CTX-M-type β-lactamases are the most common resistance determinants in cefepime-resistant isolates worldwide, but these enzymes are just beginning to appear in the United States (*13*).

In phase IV of Project ICARE, participating sites were asked to submit isolates that showed decreased susceptibility to fluoroquinolones, extended-spectrum cephalosporins, or carbapenems. We did not track whether isolates

Table 2. Association of nonsusceptibility to various antimicrobial agents and presence of integrons in *Escherichia coli* and *Klebsiella* spp. (*Klebsiella pneumoniae* and *K. oxytoca*)*

Antimicrobial drug	Estimate	OR (95% CI)	p value†
E. coli			
Gentamicin‡	1.16	3.19 (1.40–7.30)	0.006
Ciprofloxacin	0.31	1.37 (0.46–4.03)	0.573
Ceftazidime	–0.81	0.45 (0.14–1.45)	0.180
Cefepime	–1.77	0.17 (0.04–0.82)	0.027
Piperacillin-tazobactam	–0.38	0.68 (0.28–1.66)	0.407
Aztreonam	1.19	3.29 (0.90–12.0)	0.071
Chloramphenicol	0.29	1.34 (0.60–2.99)	0.476
Minocycline	–0.11	0.89 (0.39–2.06)	0.791
Trimethoprim-sulfamethoxazole‡	2.56	12.9 (5.73–29.0)	<0.001
Source§	1.10	3.02 (1.23–7.45)	0.02
Intercept	–2.43		<0.0001
Klebsiella spp.			
Gentamicin	1.44	4.23 (1.48–12.03)	0.007
Ciprofloxacin	0.34	1.40 (0.43–4.56)	0.578
Ceftazidime	0.92	2.51 (0.49–12.80)	0.270
Cefepime	0.05	1.05 (0.27–4.14)	0.942
Piperacillin-tazobactam	0.02	1.02 (0.26–4.07)	0.979
Aztreonam	–1.64	0.19 (0.04–0.99)	0.048
Chloramphenicol	–0.57	0.57 (0.13–2.44)	0.447
Minocycline	0.13	1.14 (0.35–3.73)	0.835
Trimethoprim-sulfamethoxazole	1.52	4.61 (1.39–15.29)	0.012
Source§	0.56	1.75 (0.64–4.78)	0.275
Intercept	–0.28		0.591

*Multivariate logistic regression with variables included for 1 drug of each major class that was tested. Hosmer and Lemeshow goodness-of-fit test statistic: *E. coli* total χ^2 = 10.852, *df* = 8, p = 0.21; *Klebsiella* spp. total χ^2 = 4.538, *df* = 8, and p = 0.81. OR, odds ratio; CI, confidence interval.
†By χ^2 approximation.
‡Significant interaction between these 2 variables (p<0.001).
§Nonurine source vs. urine source (reference).

were from patients in intensive care units or not. Thus, our isolates made up a convenience sample strongly associated with drug resistance mechanisms carried as integron gene cassettes and otherwise, and they are not necessarily representative of isolates from the United States in general. We did not examine carriage of >1 integron, as documented in earlier studies (*10*).

In these selected isolates, the crude association between a positive test result for class 1 integrons and nonsusceptibility to a fluoroquinolone (ciprofloxacin) in *E. coli*, and nonsusceptibility to a β-lactam (ceftazidime) in *Klebsiella* spp. failed to remain as independent variables in the multivariate model. This finding suggests that the mechanisms of resistance to fluoroquinolones and β-lactams are associated with mechanisms of resistance to the drugs that remained independently associated with class 1 integrons (aminoglycosides and trimethoprim-sulfamethoxazole), but for reasons unrelated to integron carriage. A previous study also found that ciprofloxacin resistance no longer predicted integron presence in *Enterobacteriaceae* when multivariate analysis was conducted (*1*). A Spanish study (*14*) found no association between integron carriage and β-lactam resistance in ESBL-producing *E. coli* strains unless strains contained metallo-β-lactamases, which were infrequently encountered in our study population. A biologic mechanism for association of resistance to fluoroquinolones and aminoglycosides (i.e., the inactivation of ciprofloxacin by the *aac(6′)-Ib-cr* aminoglycoside acetylating gene) has been recently reported. (*15*).

In our study, both crude and multivariate associations between integron positivity and nonsusceptibility varied for the *E. coli* and *Klebsiella* groups. This finding emphasizes that studies of this type must assess whether analysis should combine results for organisms of different genus and species into larger groups (e.g., *Enterobacteriaceae*).

When determinants encoding resistance to a variety of antimicrobial classes are contained within an integron, use of any of these agents may select for and enhance expression of the other determinants (*2*). Thus, in the example of our studied isolates, use of aminoglycosides may lead to spread of a multidrug-resistant bacterial strain such as *K. pneumoniae*. The differential association of class 1 integrons with resistance in the isolates we studied suggests that integrons facilitate the spread of antimicrobial drug resistance in the United States.

Acknowledgments

We thank the microbiology personnel at participating hospitals for sending the isolates included in this study.

Phase 5 of Project ICARE is supported in part by unrestricted grants to the Rollins School of Public Health of Emory University by Astra-Zeneca Pharmaceuticals, bioMérieux Incorporated, Elan Pharmaceuticals, and Pfizer Incorporated.

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References

1. Leverstein-van Hall MA, Blok HEM, Donders ART, Paauw A, Fluit AC, Verhoef J. Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species or isolate origin. J Infect Dis. 2003;187:251–9.
2. Norrby SR. Integrons: adding another threat to the use of antibiotic therapy. Clin Infect Dis. 2005;41:10–1.
3. Sallen B, Rajoharison A, Desvarenne S, Mabilat C. Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of *Enterobacteriaceae*. Microb Drug Resist. 1995;1:195–202.
4. Martinez-Freijo P, Fluit AC, Schmitz FJ, Grek VS, Verhoef J, Jones ME. Class I integrons in gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J Antimicrob Chemother. 1998;42:689–96.
5. Yu HS, Lee JC, Kang HY, Ro DW, Chung JY, Jeong YS, et al. Changes in gene cassettes of class 1 integrons among *Escherichia coli* isolates from urine specimens collected in Korea during the last two decades. J Clin Microbiol. 2003;41:5429–33.
6. McGowan JE Jr, Hill HA, Volkova NV, Lawton RM, Haber MJ, Tenover FC, et al. Does antimicrobial resistance cluster in individual hospitals? J Infect Dis. 2002;186:1362–5.
7. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 6th ed. NCCLS document M7–A6. Wayne (PA): The Committee; 2003.
8. Forbes B, Sahm D, Weissfeld A. *Enterobacteriaceae*. Saint Louis (MO): Mosby; 1998.
9. Farmer JJ III. *Enterobacteriaceae*: introduction and identification. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology. 8th ed. Washington: American Society for Microbiology; 2003. p. 636–53.
10. Grape M, Farra A, Kronvall G, Sundstrom L. Integrons and gene cassettes in clinical isolates of co-trimoxazole–resistant gram-negative bacteria. Clin Microbiol Infect. 2005;11:185–92.
11. Heir E, Lindstedt BA, Leegaard TM, Gjernes E, Kapperud G. Prevalence and characterization of integrons in blood culture *Enterobacteriaceae* and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. Ann Clin Microbiol Antimicrob. 2004;3:12.
12. Jones LA, McIver CJ, Kim MJ, Rawlinson WD, White PA. The aadB gene cassette is associated with blaSHV genes in *Klebsiella* species producing extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 2005;49:794–7.
13. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother. 2004;48:1–14.
14. Machado E, Canton R, Baquero F, Galan JC, Rollan A, Peixe L, et al. Integron content of extended-spectrum-beta-lactamase-producing *Escherichia coli* strains over 12 years in a single hospital in Madrid, Spain. Antimicrob Agents Chemother. 2005;49:1823–9.
15. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, et al. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med. 2006;12:83–8.

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Hantaviruses in Serbia and Montenegro

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Hantaviruses are endemic in the Balkan Peninsula. An outbreak of hemorrhagic fever with renal syndrome occurred in 2002 in Serbia and Montenegro. The epidemiologic characteristics and genetic relatedness of Dobrava/Belgrade virus strains responsible for most cases are described.

Hantaviruses (*Bunyaviridae*) are enveloped, single-stranded, negative-sense RNA viruses with a tripartite genome consisting of a small (S), a medium (M), and a large (L) segment, which encode the nucleocapsid protein, the glycoprotein precursor and the putative RNA polymerase, respectively (*1*). Hantaviruses are transmitted to humans through aerosols of excreta from small mammals, mainly rodents, that have had silent lifelong-infections. More than 30 different hantaviruses have been distinguished so far, at least half are related to disease in humans. These viruses cause hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and hantavirus pulmonary syndrome (HPS) in America. HFRS is caused by Hantaan (HTNV), Dobrava/Belgrade (DOBV), Seoul (SEOV), and Puumala (PUUV) hantaviruses, while HPS is caused by Sin Nombre (SNV) and related viruses. Each hantavirus is associated with a specific primary rodent reservoir of the *Muridae* family; these relationships have coevolved over a long period, probably >50 million years (*1*).

HFRS is endemic in the Balkan Peninsula, where sporadic cases or outbreaks have been reported. The disease is seen during the summer and affects mainly adults (*2*,*3*), although infections in children, some fatal (*4*), have been reported. Hantaviruses associated with disease in humans in Balkans are DOBV, carried by the yellow-necked mouse (*Apodemus flavicollis*), which causes severe HFRS with a fatality rate up to 10%, and PUUV, carried by the red bank vole (*Clethrionomys glareolus*). PUUV causes nephropathia epidemica, a milder form of HFRS, with a fatality rate <1% (*3*,*5*–*8*). Recently, *A. agrarius* was found to be an additional host of DOBV, causing a milder disease than that associated with *A. flavicollis* (*9*). Additionally, Tula virus RNA was amplified from lung tissues of a European pine vole (*Pitymys subterraneus*) in Serbia (*10*).

The first probable HFRS case was reported in former Yugoslavia in 1952 (*11*,*12*); the first identified epidemic of HFRS occurred in 1961 (*13*). Some years (namely, 1961, 1967, 1979, 1986, 1989, and 1995 [*2*]) are characterized by increased HFRS cases. Different factors, such as weather and food abundance, could influence the dynamics of rodent populations.

The more recent large epidemic in Serbia and Montenegro occurred in 2002 with 128 laboratory-confirmed cases. The number of confirmed cases was lower in the following years. In 2003, 16 cases occurred in Serbia and 18 in Montenegro (1 fatal). In 2004, 20 cases (1 fatal) occurred in Serbia and 11 in Montenegro.

During 2002, a total of 376 serum samples from patients with suspected HFRS cases were tested in Torlak Institute, Belgrade, by indirect immunofluorescent assay (IFA) for the presence of hantavirus antibodies. IFA was performed on spot slides containing Vero E6 cells infected with HTNV, SEOV, PUUV, and DOBV. For 128 cases (77 from Serbia, 51 from Montenegro), a laboratory diagnosis of HFRS was made. Most patients (77.3%) were infected with DOBV-like viruses; the rest were infected with PUUV-like viruses. Briefly, 53 (69%) of 77 samples from Serbia and 46 (90%) of 51 from Montenegro had higher antibody titers to HTNV and DOBV than to PUUV; the other samples had higher titers to PUUV. Two Serbian patients who lived in Leskovac died. Most DOBV-like infections from Serbia occurred in the south (Leskovac, Vranje, Nis, Surdulica, Vlasina), while the PUUV-like infections occurred in the north (Vojvodina and area near the River Drina) (map of Serbia and Montenegro available from http://www.un.org/Depts/Cartographic/map/profile/yugoslav.pdf).

Thirty-one serum samples from the IFA-positive patients were sent to Aristotle University for additional testing. Samples were taken from 21 HFRS patients with a mean age of 40.3 years (21–68 years); 1 sample was obtained from a 5-month-old male infant, whose mother had HFRS at the time of delivery. Two of 21 patients died. Enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin G (IgG) and IgM antibodies to HTNV and PUUV was performed with kits by Progen (Biotechnik GmbH, Heidelberg, Germany). IgM antibodies to HTNV were detected in 18 of 21 patients; 9 also carried IgM antibodies to PUUV, although in lower titers than to HTNV (Table 1). IgG antibodies to HTNV were present in 17 of 21 patients; in 3 patients low titers of IgM antibodies to PUUV were also detected. The infant had IgG antibodies to HTNV. In 1 sample (DR) no antibodies to HTNV or

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Table 1. ELISA and PCR results from 31 serum samples tested in this study*

			ELISA (indexes)					PCR		
Patient	Sex	Year of birth	Collection date (day of illness)	IgM HTNV	IgG HTNV	IgM PUUV	IgG PUUV	MS	MM-G1	PPT
GD	M	1979	Oct 17	2.5	1.8	Cutoff	Cutoff	Neg		
CS	M	1972	Oct 17	2.9	Neg	Cutoff	Neg			
PM	M	1958	Oct 9	Neg	1.8	Neg	1.4			
DZ	F	1951	Sep 20	3.2	2.0	Cutoff	Neg	Neg		
RD	M	1968	Aug 19	3.3	2.3	Cutoff	Cutoff	Neg		
DR	M	1958	Aug 9	Cutoff	Neg	Cutoff	Cutoff			
SS	F	1973	Aug 1	2.9	2.1	Neg	Neg			
DO	M	1956	Jul 26	1.8	2.5	1.5	1.5	Neg		Neg
RM	M	UNK	Jul 12	2.65	2.13	Cutoff	Cutoff			
VM	M	1962	Jun 14	2.7	Cutoff	1.61	Cutoff	Neg		Neg
MM	M	1936	Jun 10	2.7	2.4	Neg	Neg			
GM	M	1937	May 13	3.2	2.8	Cutoff	Cutoff	Neg		
TV	F	1964	Apr 23 (day 11)	1.6	3.3	Neg	1.7	Neg		
			Apr 24 (day 12)	1.7	3.5	Neg	2.0	DOBV	Neg	
			May 8 (day 26)	Neg	4.7	Neg	1.9			
IR	F	1981	May 17 (day 8)	6.9	2.0	1.6	Cutoff	Neg		Neg
			May 24 (day 15)	6.6	2.9	1.5	Cutoff			
CJ	M	1957	May 24 (day 11)	5.0	2.5	1.5	Neg	Neg		Neg
			May 27 (day 14)	4.2	2.7	1.8	Neg			
			Oct 23 (day 5)	Neg	4.2	Neg	Neg			
TD	M	1958	Jun 11 (day 12)	4.0	1.7	1.9	2.2	Neg		Neg
			Jun 13 (day 14)	5.0	2.5	1.8	2.2			
			Jun 28 (day 29)	4.4	3.4	2.0	2.2			
GA	M	2002	Jun 19	Neg	1.8	Neg	Cutoff	Neg		Neg
SM	M	1961	Jul 3 (day 7)	3.1	1.1	Neg	Neg	Neg		Neg
			Jul 7 (day 11)	4.6	2.4	Neg	Neg			
PV	F	1972	Jun 20 (day 5)	4.3	2.0	Neg	Neg	DOBV	Neg	
MD	M	1981	Sep 18 (day 8)	4.3	1.2	Neg	Neg	DOBV	DOBV	
			Sep 30 (day 20)	4.1	5.0	Neg	Neg	Neg		

*ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; HTNV, Hantaan virus; PUUV, Puumala virus; DOBV, Dobrava/Belgrade virus; MS, primers for partial N coding regions of hantaviruses associated with rodents of the *Murinae* subfamily; MM-G1, primers for partial G1 coding regions of hantaviruses associated with rodents of the *Murinae* subfamily; PPT, primers for partial N coding regions of hantaviruses associated with rodents of the *Arvicolinae* subfamily; Unk, unknown; neg, negative; DOBV, a positive PCR band yielding Dobrava/Belgrade virus nucleotide sequences.

PUUV were detected, although it was positive by IFA. ELISA results suggested that all 21 patients had an HTNV-like infection.

Viral RNA was extracted from IgM-positive samples (a sample from the neonate was also included) by using the viral RNA extraction kit (Qiagen GmbH, Hilden, Germany). Reverse transcription and nested amplification were performed with primers previously designed to detect partial S and M segment sequences from hantaviruses associated with rodents of the *Murinae* and *Arvicolinae* subfamilies (*14,15).* Three samples (M.D., T.V., P.V.) gave a PCR product of the expected size of 599 bp, when primers specific for the S segment of hantaviruses associated with *Murinae* rodents were used; 1 sample (MD) gave a product of 317 bp with the primers for the M segment of the same hantaviruses. No product was obtained when primers specific for the S segment of hantaviruses associated with *Arvicolinae* rodents were used. Nucleotide sequences were aligned with respective hantavirus sequences retrieved from GenBank; genetic distances were measured by the neighbor-joining method, and phylogenetic trees were constructed by using PHYLIP (Phylogeny Inference Package by J. Felsenstein [http://evolution.genetics.washington.edu/phylip.html]). The nucleotide sequences were assigned the accession numbers DQ305279-DQ305282.

Two phylogenetic trees were constructed, one for the S segment (Figure 1) and another for the M segment (Figure 2). In both trees, hantavirus strains from Serbia and Montenegro cluster with other DOBV sequences and were associated with the rodent *A. flavicollis.* In the S segment tree, sequences of this study comprise the Serbian clade in the DOBV–*A. flavicollis* cluster. In the same cluster are the Slovenian, Slovakian, and Greek clades. Sequences of this study differ by 0.3%–2.6% at the nucleotide level, with identical deduced amino acid sequences. Genetic distances with other DOBV sequences are seen in Table 2. Concerning the M segment, a fragment of the G1-coding region of patient MD differed by 5.7% at the nucleotide level from the Slovenian DOBV strain isolated from *A.*

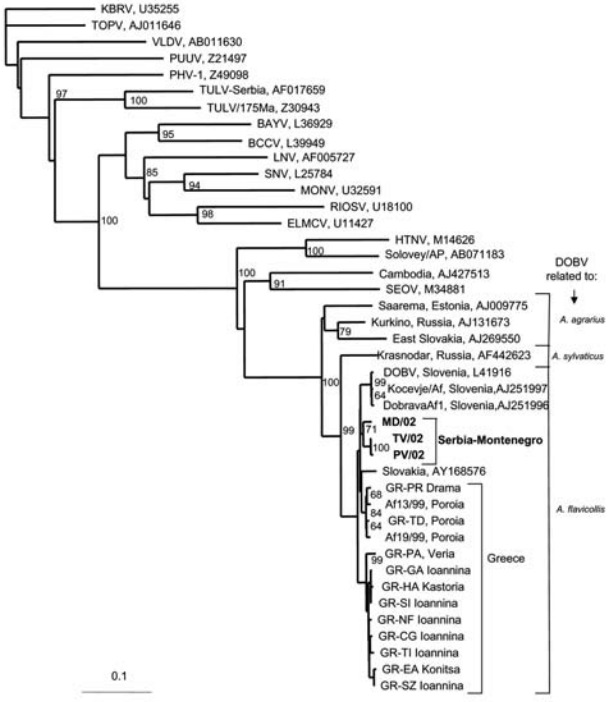

Figure 1. Phylogenetic tree based on partial S segment fragment showing the clustering of the sequence obtained from this study and respective representative hantavirus strains from GenBank database. The numbers indicate percentage bootstrap replicates (of 100); values <60% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Vertical distances are for clarity only. BAYV, Bayou virus; BCCV, Black Creek Canal virus; ELMCV, El Moro Canyon virus; HTNV, Hantaan virus; KBRV, Khabarovsk virus; LNV, Laguna Negra; MONV, Monongahela virus; NYV, New York virus; PHV, Prospect Hill virus; PUUV, Puumala virus; RIOSV, Rio Segundo virus; SEOV, Seoul virus; SNV, Sin Nombre virus; TOPV, Topografov virus; TULV, Tula virus; VLDV, Vladivostok virus. Accession numbers of Greek DOBV strains are AF060014–AF060024 for sequences from human cases and AJ410615 and AJ410619 from *Apodemus flavicollis* (Afl) sequences. Sequences in this study are indicated in **boldface**.

Figure 2. Phylogenetic tree based on partial M segment fragment showing the clustering of the sequence obtained from this study and respective representative hantavirus strains from GenBank database. The numbers indicate percentage bootstrap replicates (of 100); values <60% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Vertical distances are for clarity only. Sequences in this study are indicated in **boldface**. Abbreviations of the viruses are as in Figure 1.

flavicollis, with identical deduced amino acid sequences. The differences from DOBV strains from northwestern Greece were 8.5%–9.4% and 1% at nucleotide and amino acid levels, respectively.

Patient TV was a 38-year-old woman who lived in Vranje. Patient PV was a 29-year-old woman who lived in Leskovac. Both of these locations are in southeastern Serbia. PV died on day 6 of illness. Patient MD was living in Beograd. However, his sequences were similar to those of patients TV and PV. His travel history showed that 18 days before the onset of illness, he was on vacation in Kolasin Mountain in Montenegro, where he was probably infected. Thus, all sequences of this study were from the southern region of the country and clustered with other DOBV strains associated with *A. flavicollis* rodents.

Table 2. Genetic distances (%) in partial S segment fragment (364–963 nucleotides) of hantaviruses associated with *Murinae* rodents among Yugoslavian DOBV strains and representative DOBV strains related with different *Apodemus* spp. rodents*

	Yugoslavian DOBV strains			*A. flavicollis* related					*A. agrarius* related		*A. sylvaticus* related, Krasnodar Russia
Strain	7937/02 (PV)	5157/02 (TV)	9744/02 (MD)	GR-EA NW Greece	GR-PA NC Greece	GR-TD NE Greece	AP-Af9 NE Greece	DOBV-1 Slovenia	862 East Slovakia	Saarema Estonia	
PV	–	0.3	2.6	4.9	4.9	3.0	3.2	4.0	17.5	15.6	9.5
TV		–	2.2	4.5	4.5	2.8	3.0	3.6	17.0	15.0	9.0
MD			–	4.5	4.5	2.8	3.4	3.6	16.1	15.3	9.4

*DOBV, Dobrava/Belgrade virus; –, 0.0.

However, the involvement of other hantaviruses in the outbreak cannot be excluded.

Although the number of samples tested was limited, this study gives the first genetic information on DOBV strains circulating in Serbia and Montenegro. Further studies of both patients and small mammals in the region are needed to find out the exact epidemiology of HFRS in the country.

Acknowledgments

We thank Vassiliki Pavlidou and Panagiota Papadopoulou for excellent technical assistance.

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References

1. Nichol ST, Ksiazek TG, Rollin PE, Peters CJ. Hantavirus pulmonary syndrome and newly described hantaviruses in the United States. In: Elliott RM, editor. The Bunyaviridae. New York: Plenum Press; 1996. p. 269–80.
2. Avšic-Zupanc T. Hantaviruses and hemorrhagic fever with renal syndrome in the Balkans. In: Saluzzo JF, Dodet B, editors. Factors in the emergence and control of rodent-borne viral diseases. Paris: Elsevier SAS; 1999. p. 93–8.
3. Antoniadis A, Stylianakis A, Papa A, Alexiou-Daniel S, Lampropoulos A, Nichol ST, et al. Direct genetic detection of Dobrava virus in Greek and Albanian patients with hemorrhagic fever with renal syndrome. J Infect Dis. 1996;174:407–10.
4. Peco-Antic A, Popovic-Rolovic M, Gligic A, Popovic D, Jovanovic O, Kostic M. Clinical characteristics of haemorrhagic fever with renal syndrome in children. Pediatr Nephrol. 1992;6:335–8.
5. Glicic A, Dimkovic N, Xiao SY, Buckle GJ, Jovanovic D, Velimirovic D, et al. Belgrade virus: a new hantavirus causing severe hemorrhagic fever with renal syndrome in Yugoslavia. J Infect Dis. 1992;166:113–20.
6. Avšic-Zupanc T, Xiao SY, Stojanovic R, Gligic A, van der Groen G, LeDuc JW. Characterization of Dobrava virus: a Hantavirus from Slovenia, Yugoslavia. J Med Virol. 1992;38:132–7.
7. Lundkvist A, Hukic M, Hörling J, Gilljam M, Nichol S, Niklasson B. Puumala and Dobrava viruses cause haemorrhagic fever with renal syndrome (HFRS) in Bosnia-Herzegovina: evidence of highly cross-neutralizing antibody responses in early patient sera. J Med Virol. 1997;53:51–9.
8. Markotic A, Nichol ST, Kuzman I, Sanchez AJ, Ksiazek TG, Gagro A, et al. Characteristics of Puumala and Dobrava infections in Croatia. J Med Virol. 2002;66:542–51.
9. Avšic-Zupanc T, Nemirov K, Petrovec M, Trilar T, Poljak M, Vaheri A, et al. Genetic analysis of wild-type Dobrava hantavirus in Slovenia: co-existence of two distinct genetic lineages within the same natural focus. J Gen Virol. 2000;81:1747–55.
10. Song JW, Gligic A, Yanagihara R. Genetic analysis of Tula hantaviral sequences amplified from tissues of *Pitymys subterraneus* captured in the Cacak region of Serbia-Yugoslavia. Int J Infect Dis. 2002;6:31–6.
11. Simic M, Miric V. Successful application of peritoneal dialysis in a case of renal insufficiency [in Serbian]. Vojnosanit Pregl. 1952;9:285–90
12. Radosevic Z, Mohacek I. The problem of nephropathia epidemica Myhrman-Zetterholm in relation to acute interstitial nephritis. Acta Med Scand. 1954;149:221–8.
13. Heneberg D, Vuksic L. Epidemic of hemorrhagic fever in certain workplaces in Fruska Gora [in Serbian]. Zbornik Vojnomedicinske Akademije. 1962;4:263–71.
14. Bowen MD, Gelbmann W, Ksiasek TG, Nichol ST, Nowotny N. Puumala virus and two genetic variants of Tula virus are present in Austrian rodents. J Med Virol. 1997;53:174–81.
15. Papa A, Johnson AM, Stockton PC, Bowen MD, Spiropoulou CF, Alexiou-Daniel S, et al. Retrospective serological and genetic study of the distribution of hantaviruses in Greece. J Med Virol. 1998;55:321–7.

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Raccoons and Skunks as Sentinels for Enzootic Tularemia

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We analyzed sera from diverse mammals of Martha's Vineyard, Massachusetts, for evidence of *Francisella tularensis* exposure. Skunks and raccoons were frequently seroreactive, whereas white-footed mice, cottontail rabbits, deer, rats, and dogs were not. Tularemia surveillance may be facilitated by focusing on skunks and raccoons.

Martha's Vineyard, an island located off the coast of Cape Cod, Massachusetts, has been the location of the only 2 outbreaks of primary pneumonic tularemia reported in the United States (*1*). The first outbreak occurred in 1978, with 15 tularemia cases reported, 12 of which were considered pneumonic. The cases were believed to be linked to a common source, and exposure was presumed to be inhalational because of the absence of ulcers or lymphadenopathy associated with vector bites (*2*). The second outbreak, which began in 2000, has yielded 50 confirmed or probable cases by 2005, of which more than half have resulted from pneumonic exposure. Landscaping activities were identified as a risk factor (*3*), but fomites remain undescribed. As the first step in identifying the biologic basis for risk as a function of landscaping activities, we determined whether a tularemia epizootic had occurred on Martha's Vineyard.

Common mammals were collected during Lyme disease surveillance and specifically for the purposes of this study. White-footed mice (*Peromyscus leucopus fusus*) were trapped from Chilmark and Edgartown sites during 1994–2004. Skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), and squirrels (*Sciurus carolinensis*) were live-trapped during 2001–2004 from sites in Chilmark, West Tisbury, and Edgartown, where landscapers had indicated that they had worked intensively. Rabbits (*Sylvilagus floridanus*) were sampled from West Tisbury and Chilmark during 2000–2004. Rats (*Rattus norvegicus*) were trapped at Edgartown and Chilmark and also were obtained from a local exterminator during 2000–2004. Deer (*Odocoileus virginianus*) serum specimens were obtained from animals killed by hunters during the 2003–2004 shotgun seasons. During 2000–2001, canine (*Canis familiaris*) serum samples were obtained from a veterinary diagnostic laboratory, and these samples were analyzed and compared to those from wild animals. Serum specimens or plasma from all animals were stored at –20°C until analysis.

We used the microagglutionation test (MAT) for detecting specific antibody to *F. tularensis* as described (*4*). An *F. tularensis tularensis* strain, isolated from a Martha's Vineyard rabbit, was used to make microagglutination antigen. A sample was considered seropositive for *F. tularensis* if the agglutination titer was ≥128. Known positive and negative control sera were used with each assay. Because high-titered *F. tularensis* antisera will cross-react in a MAT that uses *Brucella* spp. as antigen, we analyzed all *F. tularensis* MAT reactive results for possible cross-reactivity to this agent by using *Brucella abortus* slide antigen (Difco, Detroit, MI, USA) and the MAT.

No reactivity was detected in the rabbit, white-footed mouse, and squirrel serum samples (Table 1). However, half of the raccoon and skunk samples were considered positive (Table 2). In addition, a few samples from rats, dogs, and deer were considered reactive. None of these were reactive for *Brucella* agglutinins, except for 1 skunk sample, which was positive at a titer of 1,024. This sample had an extremely high *F. tularensis* MAT titer of 8,192. Skunks and raccoons appear to have been frequently exposed to *F. tularensis*; few rats, dogs, or deer had been; and no other rodents or lagomorphs apparently had been exposed. Accordingly, of the diverse animals that we sampled, only skunks and raccoons were commonly exposed.

Thirty skunks (including 3 pups) were MAT negative, demonstrating that the great seroprevalence that we observed is not attributable to a nonspecific agglutinin inherently associated with serum from this host. In addition, 9 adult raccoon and 2 adult skunk samples from nearby Great Island (South Yarmouth, MA) collected in 1988 were nonreactive, despite being trapped from a site where dog ticks, rabbits, and deer flies are as common as they are on Martha's Vineyard (unpub. data). Of 72 deer serum specimens sampled from mainland Massachusetts sites, none were reactive. The reactivity that we have observed thus reflects exposure and not innate nonspecific reactivity.

The great seroprevalence in skunks and raccoons trapped from Martha's Vineyard demonstrates an ongoing tularemia epizootic. In other sites, *F. tularensis* seroreactivity in skunks or raccoons ranged from 3.2% to 25.7% (*5*); a seropositive raccoon was found among the few animals surveyed during investigation of the Martha's Vineyard outbreak by the Centers for Disease Control and Prevention (*1*). We suggest that skunks and raccoons may serve as sensitive indicators for enzootic tularemia activity: both animals are scavengers and may prey on infected

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Table 1. Seroreactivity among diverse mammals sampled from Martha's Vineyard, 2001–2004

Animal	No. examined (% positive)	95% CI*
Deer	44 (2.3)	0.06–12.0
Dog	58 (6.9)	1.9–16.7
Mice	319 (0)	–
Rabbit	21 (0)	–
Raccoon	21 (52.4)	29.8–74.3
Rat	7 (4.3)	0.4–57.9
Skunk	61 (49.2)	36.1–62.3
Squirrel	4 (0)	–

*CI, confidence interval, by exact binomial method.

animals that are sick or dying of tularemia. In addition, both are definitive hosts for dog ticks (*Dermacentor variabilis*), a known tularemia vector (*6*,*7*). Of the raccoons and skunks sampled during tick season for which tick infestations were determined, all were infested with a range of 6 to 102 dog ticks per animal (mean 43.4 ± 26.8 SD, n = 31). Whether the serologic evidence implies reservoir capacity is not clear. None of the skunks or raccoons appeared to be actively infected based on polymerase chain reaction (PCR) of whole blood samples (unpub. data). Larval and nymphal dog ticks do not feed on medium-sized mammals, and thus skunks and raccoons would not contribute to producing infected adult dog ticks. Skunks and raccoons might facilitate transovarial transmission of *F. tularensis* (*8*) by infecting adult female dog ticks that will eventually oviposit. Dog ticks removed from them, however, were not more frequently infected than those from vegetation (*7*), but this analysis may have missed finding sparse bacteria that had been ingested and remained within the tick gut.

Skunks and raccoons on Martha's Vineyard are frequently seropositive, whereas other animals that we examined were not. This finding may reflect differential survival of hosts that are infected by *F. tularensis*. Rodents and rabbits generally die rapidly after exposure (*9*), likely before they mount an antibody response. Indeed, of the 21 rabbits that were examined, 3 were moribund and yielded evidence of active infection by *F. tularensis* (by PCR, direct fluorescent antibody test, or isolation), but none were seroreactive. Thus, serosurveys of healthy rodents and rabbits may comprise only those that were never exposed or at most were only recently exposed; dead rodents or rabbits, of course, would not be captured (but see [*10*]).

Table 2. Skunk and raccoon seroreactivity by year of sampling*

Year	No. skunks examined (% positive)	GMT	No. raccoons examined (% positive)	GMT
2001	10 (60)	1,290	–	–
2002	32 (47)	446	10 (60)	456
2003	12 (50)	323	6 (33)	1,448
2004	7 (43)	1,024	5 (60)	813

*GMT, geometric mean titer.

The seroreactivity of 1 rat suggests that these animals should be examined more carefully as potential sentinels; rats are known to be relatively resistant to challenge with virulent type A organisms (*11*). That a deer was seroreactive is puzzling. Dog ticks do not feed on deer, and deer ticks sampled from Martha's Vineyard sites have not been found to be infected (*6*). Possibly, tabanid flies, which may feed on deer, are involved in perpetuating *F. tularensis* on Martha's Vineyard as they are in the western United States (*12*). Dogs, on the other hand, are well-described as sensitive sentinels for tickborne infections such as Lyme disease (*13*) and may be good candidates for detecting tularemia activity as well.

The peridomestic behavior of skunks and raccoons implies the possibility of direct risk of human exposure. Indeed, human infection has been associated with skinning skunks (*9*). If exposed skunks and raccoons foraging around people's homes leave infectious excreta, these may serve as the fomites for the presumably aerogenic tularemia outbreak. Testing such a hypothesis requires detecting viable *F. tularensis* in skunk or raccoon feces collected from sites of active transmission. Regardless of whether raccoons or skunks may serve as reservoirs, focusing serosurveys on these hosts as opposed to other species such as mice may quickly demonstrate tularemia transmission within American sites. In addition, raccoon and skunk sentinel surveillance could potentially assist in discriminating between natural transmission to humans and illegitimate introduction events.

Acknowledgments

We thank the residents and agencies of Martha's Vineyard who have provided invaluable assistance to our work, particularly John Varkonda, William Searle, Cathy and William Glover; and Connie and Walter Ashley. Bela Matyas kept us updated on the ongoing outbreak, reviewed early versions of the manuscript, and made helpful suggestions.

Our work is supported by grants from the National Institutes of Health (R21 AI 053411, NO1 AI30050, and RO1 AI 39002).

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References

1. Feldman KA, Enscore RE, Lathrop SL, Matyas BT, McGuill M, Schriefer ME, et al. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N Engl J Med. 2001;345:1601–6.
2. Teutsch SM, Martone WJ, Brink EW, Potter ME, Eliot G, Hoxsie R, et al. Pneumonic tularemia on Martha's Vineyard. N Engl J Med. 1979;301:826–8.

3. Feldman KA, Stiles-Enos D, Julian K, Matyas BT, Telford SR III, Chu MC, et al. Tularemia on Martha's Vineyard: seroprevalence and occupational risk. Emerg Infect Dis. 2003;9:350–4.
4. Brown SL, McKinney FT, Klein GC, Jones WL. Evaluation of a safranin-O stained antigen microagglutination test for *Francisella tularensis* antibodies. J Clin Microbiol. 1980;11:146–8.
5. Bigler WJ, Jenkins JH, Cumbie PM, Hoff GL, Prather EC. Wildlife and environmental health: raccoons as indicators of zoonoses and pollutants in southeastern United States. J Am Vet Med Assoc. 1975;167:592–7.
6. Goethert HK, Shani I, Telford SR III. Genotypic diversity of *Francisella tularensis* infecting *Dermacentor variabilis* ticks on Martha's Vineyard, Massachusetts. J Clin Microbiol. 2004;42:4968–73.
7. Hopla CE. The ecology of tularemia. Adv Vet Sci Comp Med. 1974;18:25–53.
8. Parker RR, Spencer RR. Hereditary transmission of tularemia infection by the wood tick, *Dermacentor andersoni* Stiles. Public Health Rep. 1926;41:1403–7.
9. Jellison WL. Tularemia in North America. Missoula (MT): University of Montana Press; 1974.
10. Shoemaker D, Woolf A, Kirkpatrick R, Cooper M. Humoral immune response of cottontail rabbits naturally infected with *Francisella tularensis* in southern Illinois. J Wildl Dis. 1997;33:733–7.
11. Downs CM, Buchele L, Edgar EP. Studies on pathogenesis and immunity in tularemia. I. The pathogenesis of tularemia in the white rat. J Immunol. 1949;63:117–33.
12. Francis E, Mayne B. Tularemia Francis 1921: II. Experimental transmission by the fly, *Chrysops discalis*. Public Health Rep. 1921;36:1738–46.
13. Eng TR, Wilson ML, Spielman A, Lastavica CC. Greater risk of *Borrelia burgdorferi* infection in dogs than in people. J Infect Dis. 1988;158:1410–1.

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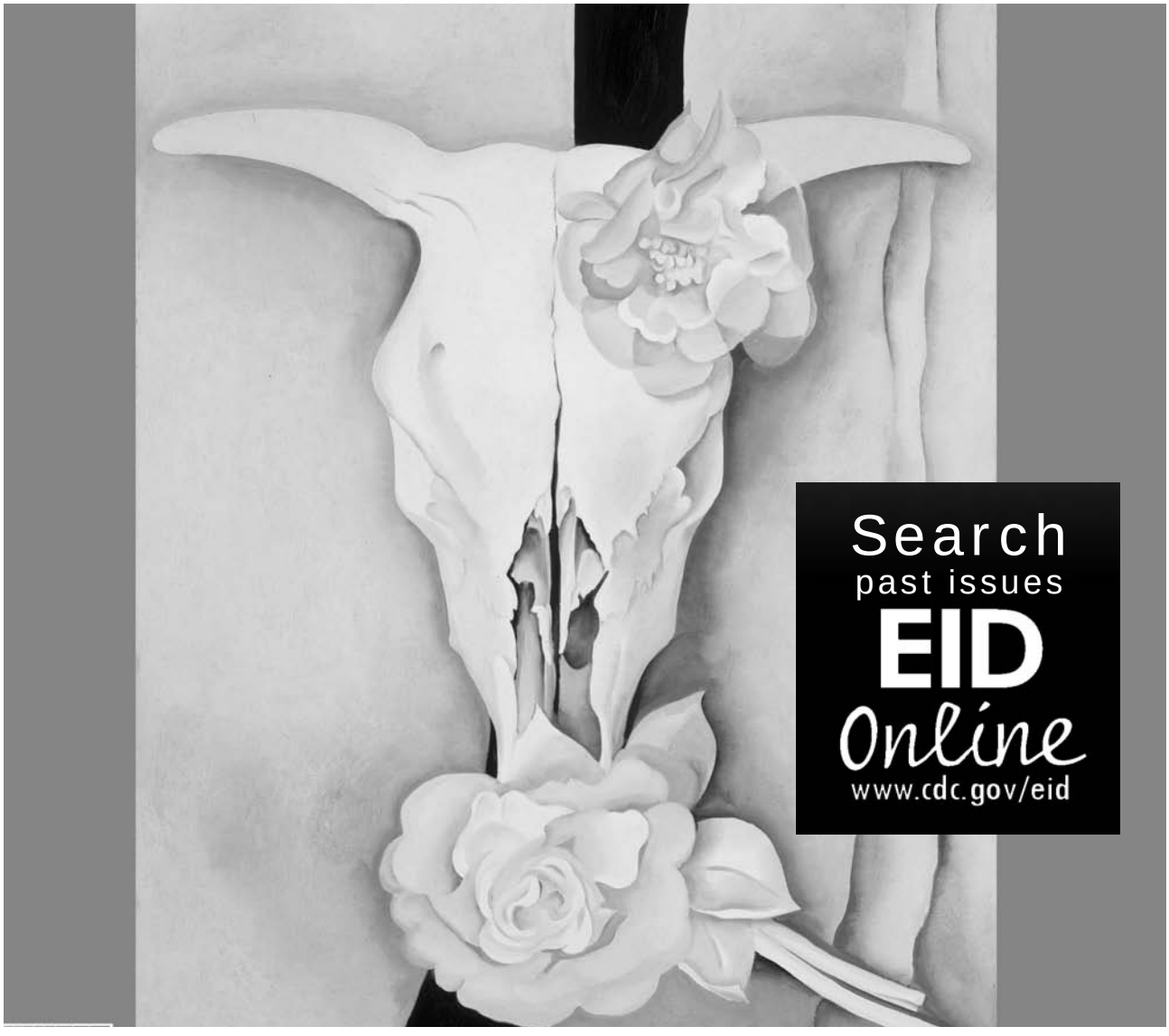

West Nile Virus Infection in Humans and Horses, Cuba

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A surveillance system to detect West Nile virus (WNV) was established in Cuba in 2002. WNV infection was confirmed by serologic assays in 4 asymptomatic horses and 3 humans with encephalitis in 2003 and 2004. These results are the first reported evidence of WNV activity in Cuba.

West Nile virus was first detected in the Western Hemisphere during an outbreak of encephalitis in New York State in 1999 (*1*). Genetic analyses showed that the virus responsible for the 1999 outbreak was nearly identical to a WNV strain circulating in Israel in 1998 (*2*). Recent outbreaks of WNV disease in the United States and Canada have been accompanied by a high proportion of deaths in birds (*3*,*4*), substantial illness in equines (*4*,*5*), and thousands of cases of severe neurologic disease in humans (*6*). The range of WNV has rapidly expanded across the continental United States and Canada (*7*). WNV infection in humans, equines, and birds in Mexico (*8*), the Caribbean (*9*), and South and Central America (*10*,*11*) shows southward movement of the virus. Because Cuba is close to areas of the United States where WNV is endemic and because of recent evidence that suggests spread of WNV into the Caribbean, surveillance was established to monitor for WNV in Cuba. Beginning in 2002, the Medical Services and Ministry of Agriculture and Veterinarian Services of Cuba established a national surveillance program by using birds, horses, and humans to detect WNV activity. In this report, we summarize the key findings of surveillance activities.

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The Study

The Ministry of Agriculture and Veterinarian Services coordinated the collection of dead birds. A total of 1,217 dead birds were shipped at 4°C to the Tropical Medicine Institute "Pedro Kouri" and identified by ornithology experts. Brain, heart, and kidneys were removed and tested for WNV by using reverse transcription–polymerase chain reaction (RT-PCR) (*12*). Briefly, RNA was extracted by using the QIAmp viral RNA kit (Qiagen, Inc., Valencia, CA, USA). Primers WN212 (5′-TTGTGTTGGCTCTCTTGGCGTTCTT-3′) and WN619c (5′-CAGCCGACAGCACTGGACATTCATA-3′) were used to detect viral RNA. A second RT-PCR with primers WN9483 (5′-CACCTACGCCCTAAACACTTTCACC-3′) and WN9794 (5′-GGAACCTGCTGCCAATCATACCATC-3′) was performed on the same RNA preparation.

Serum specimens from horses in Havana and Havana Province were tested for antibodies to WNV by using a competitive enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies 3.1112G and 6B6C-1 as described by Blitvich et al. (*13*). We tested 210 serum specimens from horses collected as part of an infectious anemia study. The immunoglobulin M (IgM) test was not performed because horses were never suspected of having WNV and did not have any history of suspected viral encephalitis or other illness or symptoms. An inhibition value >30% was used as the diagnostic criterion to identify flavivirus antibody (Table 1).

The Cuban Health Ministry and Medical Services division conducted surveillance for encephalitis of unknown origin in patients >30 years of age. Serum and cerebrospinal fluid specimens were shipped at 4°C to the Tropical Medicine Institute "Pedro Kouri." Human sera were screened for WNV IgM and IgG by using commercial IgM and IgG ELISA kits (Focus Technologies, Cypress, CA, USA) according to manufacturer's instructions. Hemagglutination-inhibition (HI) tests were also undertaken with WNV and Saint Louis encephalitis virus (SLEV) antigen (*14*).

Reactive serum samples were further tested by a plaque reduction neutralization test (PRNT) with WNV (NY99, Ontario, Canada, 2001 isolate), SLEV (Parton strain, American Type Culture Collection catalog no. VR-1265), and dengue virus (dengue 2, NG-C strain). PRNT was performed to confirm WNV-specific antibody and was carried out as described previously (*15*) by using a neutral red double-overlay procedure. Horses or human patients were considered seropositive for a particular flavivirus if the 90% PRNT titer for that virus was ≥4-fold greater than the neutralization titers determined for other viruses used in the assay. Endpoint titrations were defined as the highest dilution of serum that reduced plaque formation by ≥90%.

Most (58%) of the 1,217 birds tested were resident

Table 1. Summary of horse sera tested for evidence of WNV infection by ELISA and PRNT*

Sample no.	% inhibition (ELISA)		90% PRNT titer		Interpretation
	Anti-WNV	Antiflavivirus	WNV	SLEV	
C-7	65.3	65.2	160	20	WNV
C-13	58.9	60.4	320	–	WNV
C-19	63.2	63.3	320	–	WNV
C-20	74.6	62.2	640	–	WNV
C-2	44.4	60.4	–	20	SLEV
C-4	11.6	40.1	–	20	SLEV
C-5	67.5	67.6	–	20	SLEV
C-11	10.5	26.7	–	40	SLEV
C-12	32.2	68.0	–	20	SLEV
C-15	27.4	68.9	–	40	SLEV
C-16	64.6	65.7	–	40	SLEV
C-18	59.1	68.2	–	40	SLEV
C-10	62.7	66.3	80	40	Unidentified flavivirus
C-1	22.3	74.4	–	–	Unidentified flavivirus
C-3	40.5	54.1	–	–	Unidentified flavivirus
C-6	28.5	74.8	–	–	Unidentified flavivirus
C-9	40.9	73.6	–	–	Unidentified flavivirus
C-14	34.8	41.0	–	–	Unidentified flavivirus
C-21	9.9	68.4	–	–	Unidentified flavivirus

*WNV, West Nile virus; ELISA, enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; SLEV, Saint Louis encephalitis virus.

species of Cuba, primarily chestnut manakins (*Lonchura malaccas*), blue jays (*Cyanocitta cristata*), herring gulls (*Larus argentatus*), yellow-faced grassquits/olive finches (*Tiaris olivacea*), and northern parulas (*Parula americana*). None of the birds were positive for WNV RNA by RT-PCR. We attempted to isolate WNV from most samples, but virus was not cultured. Most dead birds were received in good condition for testing.

Nineteen (9.0%) of the 210 horses had serum specimens with antibodies to flaviviruses (Table 1). Four and 8 animals had WNV- and SLEV-specific antibodies, respectively, in the PRNT. Two horses seropositive for WNV came from Havana City, and 2 others came from Havana Province. Seven serum samples had antibodies to undetermined flaviviruses on the basis of results of neutralization assays, and further virus characterization was not performed. None of the horses, including those that were positive for flavivirus antibodies, showed any signs of illness at the time of serum collection.

Serum specimens from 13 patients with encephalitis were tested for possible WNV infection. Two of these patients (M.M. and K.R.) were identified as part of WNV surveillance in 2003. Both acute-phase and convalescent-phase serum specimens from these patients were positive for flavivirus antibody by IgM and IgG ELISAs (Table 2). Cerebrospinal fluid from patient K.R. was also positive for IgM by WNV ELISA. Acute-phase and convalescent-phase serum samples from M.M. and K.R. were also positive by HI assay and showed seroconversion to WNV. Convalescent-phase serum samples were tested for WNV-specific antibody by PRNT, and the neutralization titers were 320 and 160, respectively. Neutralizing antibodies

Table 2. Summary of human sera tested for evidence of WNV infection by ELISA and PRNT*

Patient code	ELISA result†		HI titer†	90% PRNT titers			Interpretation
	IgM	IgG		WNV	SLEV	DENV	
M.M.	+/+	+/+	40/160	320	–	–	WNV
K.R.	+/+	+/+	20/80	160	–	–	WNV
O.G.	–/–	+/+	20/20	40	–	–	WNV
M.L.	–/–	+/+	20/20	–	640	80	SLEV
A.M.L.	–/–	+/+	20/40	–	320	–	SLEV
R.A.	ND/–	ND/+	1,280	–	80	–	SLEV
P.Y.	ND/–	ND/+	<20	–	40	–	SLEV
M.V.	–/–	+/+	20/40	–	80	–	SLEV
J.A.R.	–/–	+/+	20/40	40	80	80	Unidentified flavivirus
R.C.	–/–	+/+	20/20	–	–	80	DENV
A.P.	–/–	+/+	20/20	–	–	–	Unidentified flavivirus
L.V.	–/–	+/+	20/40	–	–	–	Unidentified flavivirus
D.B.	ND/–	ND/+	ND	–	–	–	Unidentified flavivirus

*WNV, West Nile virus; ELISA, enzyme-linked immunosorbent assay; PRNT, plaque reduction neutralization test; Ig, immunoglobulin; HI, hemagglutination inhibition; SLEV, Saint Louis encephalitis virus; DENV, dengue virus; ND, not done;
†Acute-phase/convalescent-phase serum samples.

against SLEV or dengue virus were not detected in the serum specimens of either patient. These persons are the first to have confirmed cases of WNV-associated illness in Cuba. Both patients had histories of febrile illness, muscle weakness, and encephalitis, and both were hospitalized. These persons had jobs that required them to spend large amounts of time outdoors, and they lived in communities in Santi Spiritus and Villa Clara in central Cuba. Another person (O.G., Table 2), who also resided in Santi Spiritus, had a low WNV titer by HI assay but had neutralizing antibodies to WNV, which suggests a past WNV infection. This patient was identified during surveillance in 2004 but may have been exposed to WNV in 2003.

Serum specimens from the 10 remaining patients were negative for WNV IgM but were positive for flavivirus IgG by ELISA; most of these were also positive by HI assay (Table 2). One person appeared to have been exposed to dengue virus, and 4 had antibodies to unidentified flaviviruses. Five patients had serum with SLEV-specific antibodies. Seroconversions were not demonstrated in any of these persons, so we cannot say whether their illnesses were associated with SLEV or dengue virus infections.

Conclusions

We report the first evidence of antibodies to WNV in horses and humans in Cuba. The fact that human and horse infections have been detected strongly suggests that a local amplification cycle has been established in Cuba. The mode of entry of the virus into Cuba is unknown.

In North America, avian death from WNV infection has been well documented (*3*–*5*). However, none of the dead birds collected during this study showed evidence of viral infection. Although 1,217 animals were tested, a more intensive dead bird surveillance program may be needed to identify animals that die from WNV infection. Resident bird species in Cuba may be less susceptible to WNV infection, and death rates among birds in the Caribbean may be lower than those observed in Canada and the United States.

This study also provides evidence that suggests WNV and SLEV may co-circulate in Cuba. Further studies are required to confirm these observations and to characterize the transmission cycles involved. Finally, expansion of existing mosquito control programs in Cuba, which currently focus on *Aedes aegypti* and dengue prevention, may be required to respond to this new public health threat.

Acknowledgments

We thank staff of the Hygiene and Epidemiology Province Centers for collecting sera from patients; Adriana Sánchez, Sheila Cabezas, Lester Gonzalez, Mayling Alvarez, Odaysa Valdés, and Yaime Vázquez for technical assistance; Susana Vazquez for her comments; Ernie Gould, Thomas Briese, Ian Lipkin, Ildefonso Salas, and J.F. Contreras for materials they donated to this study; and Susan Shead and Antonia Dibernardo for expert technical assistance.

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References

1. Petersen LR, Roehrig JT. West Nile virus: a reemerging global pathogen. Emerg Infect Dis. 2001;7:611–4.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science. 1999;286: 2333–7.
3. Drebot MA, Lindsay R, Barker IK, Buck PA, Fearon M, Hunter F, et al. West Nile virus surveillance and diagnostics: a Canadian perspective. Canadian Journal of Infectious Diseases. 2003;14:105–14.
4. Gould LH, Fikrig E. West Nile virus: a growing concern? J Clin Invest. 2004;113:1102–7.
5. US Geological Survey. West Nile virus maps. 2006 Feb 14 [cited 2006 Apr 13]. Available from http://westnilemaps.usgs.gov./index.html
6. Petersen LR, Hayes EB. Westward ho?—The spread of West Nile virus. N Engl J Med. 2004;351:2257–9.
7. Granwehr BP, Lillibridge KM, Higgs S, Mason PW, Aronson JF, Campbell GA, et al. West Nile virus: where are we now? Lancet Infect Dis. 2004;4:547–56.
8. Blitvich BJ, Fernandez-Salas I, Contreras-Cordero JF, Marlenee NL, Gonzalez-Rojas JI, Komar N, et al. Serologic evidence of West Nile virus infection in horses, Coahuila State, Mexico. Emerg Infect Dis. 2003;9:853–6.
9. Komar O, Robbins MB, Klenk K, Blitvich BJ, Marlenee NL, Burkhalter KL, et al. West Nile virus transmission in resident birds, Dominican Republic. Emerg Infect Dis. 2003;9:1299–302.
10. Mattar S, Edwards E, Laguado J, González M, Alvarez J. West Nile virus antibodies in Colombian horses. Emerg Infect Dis. 2005;11:1497–8.
11. Cruz L, Cardenas VM, Abarca M, Rodriguez T, Reyna RF, Serpas MV, et al. Short report: serological evidence of West Nile virus activity in El Salvador. Am J Trop Med Hyg. 2005;72:612–5.
12. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol. 2000;38:4066–71.
13. 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.
14. Clarke DH, Casals J. Techniques for hemagglutination inhibition with arthropod-borne viruses. Am J Trop Med Hyg. 1958;7:561–73.
15. Beaty BJ, Calisher CH, Shope RS. Arboviruses. In: Schmidt NJ, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. 6th ed. Washington: American Public Health Association; 1989. p. 797–856.

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Mixed *Cryptosporidium* Infections and HIV

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Mixed *Cryptosporidium* infections were detected in 7 of 21 patients with a diagnosis of rare *Cryptosporidium canis* or *C. felis* infections; 6 patients were infected with 2 *Cryptosporidium* spp. and 1 patient with 3 species. Mixed infections may occur more frequently than previously believed and should be considered when assessing cryptosporidiosis.

Cryptosporidium spp. infect humans and other vertebrate animals. Persons with compromised immune systems can suffer life-threatening chronic diarrhea, especially when their CD4+ lymphocyte counts fall <200 cells/μL. At least 7 *Cryptosporidium* spp. have been detected in immunocompromised patients (*1*). Nonetheless, the role of concurrent or mixed infections in the pathogenesis and transmission of *Cryptosporidium* spp. is unclear. Mixed infections of *Cryptosporidium hominis* and *C. parvum* have been reported in several patients from Switzerland and England (*2*,*3*). Additional studies from the United Kingdom reported simultaneous infections with these 2 species: 4 cases in 2 waterborne outbreaks and 2 cases of sporadic infections from 1995 to 1999 (*4*). In a more recent study, 12% of 135 clinical specimens from Aberdeenshire, Scotland, had concurrent *C. parvum* and *C. hominis* infections (*5*). Mixed *C. hominis–C. parvum* infections were also seen in 2 of 38 archived human specimens in a study conducted in the United States (*6*). These observations suggest that mixed *Cryptosporidium* infections are not uncommon.

Mixed infections may not be readily identified by commonly used molecular diagnostic tools because of preferential polymerase chain reaction (PCR) amplification of the predominant genotypes or the specificity of molecular tools (*6*). For example, PCR–restriction fragment length polymorphism (RFLP) tools based on the small subunit (SSU) rRNA gene are frequently used in genotyping *Cryptosporidium* spp. because they have higher sensitivity and detect more species than PCR-RFLP tools based on other genes (*7*).

Two previous studies in Peru used an SSU-rRNA–based PCR-RFLP tool to genotype *Cryptosporidium* specimens from children (*8*) and AIDS patients (*1*). A variety of *Cryptosporidium* spp. were found in both patient populations; *C. hominis* was the predominant species, followed by *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis*, but mixed infections were rarely detected (*1*,*8*). However, a recent study of some of the specimens that used PCR tools that selectively amplify DNA of *C. parvum* and closely related species identified concurrent infections of *C. hominis* in specimens previously diagnosed as having only *C. canis*, *C. muris*, or *C. suis* (*7*). Another recent study has shown that an SSU rRNA–based PCR-RFLP tool had only a 31%–74% success rate in detecting concurrent infections with *C. parvum* and *C. hominis* (*9*).

The Study

We addressed the question of whether Peruvian HIV-positive patients infected with the usual *C. canis* or *C. felis* parasites were co-infected with *C. hominis*, *C. parvum*, or *C. meleagridis* (*7*). The study protocol was approved by the participating institutional review boards. All participants gave written informed consent.

Mixed infections were identified by using 2 PCR-RFLP tools that only amplify *C. hominis*, *C. parvum*, or *C. meleagridis* (*7*). One tool was based on the dihydrofolate reductase (DHFR) gene (*10*) and the other on the *Cryptosporidium* oocyst wall protein (COWP) gene (*11*). Fifty-six stool specimens from 21 HIV-infected persons with previous diagnoses of *C. canis* or *C. felis* with an SSU rRNA–based PCR-RFLP tool were re-analyzed with these 2 molecular tools. DNA was extracted by using the QIAamp stool DNA extraction kit (Qiagen Inc., Valencia, CA, USA), and 1 μL DNA was used in nested PCR analyses of the DHFR and COWP genes. Secondary PCR products positive for *Cryptosporidium* were digested with restriction enzymes *BpuA* I for the DHFR tool or *Rsa* I for the COWP tool (*10*,*11*). Results of RFLP diagnosis were confirmed by DNA sequence analysis. All secondary PCR products were sequenced with a 3100 ABIPrism Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were aligned with reference sequences from GenBank by using BioEdit version 7.0.5 (Isis Pharmaceuticals, Carlsbad, CA, USA).

The PCR analysis of both DHFR and COWP genes showed that 17 specimens from 7 patients yielded products of the expected size for *Cryptosporidium* spp. (Figure, panel A, and Table). Restriction analysis of DHFR products with *BpuA* I showed that 4 patients had banding patterns indicative of *C. hominis*, 1 patient had the pattern of *C. parvum*, 1 patient had the pattern of *C. meleagridis*, and

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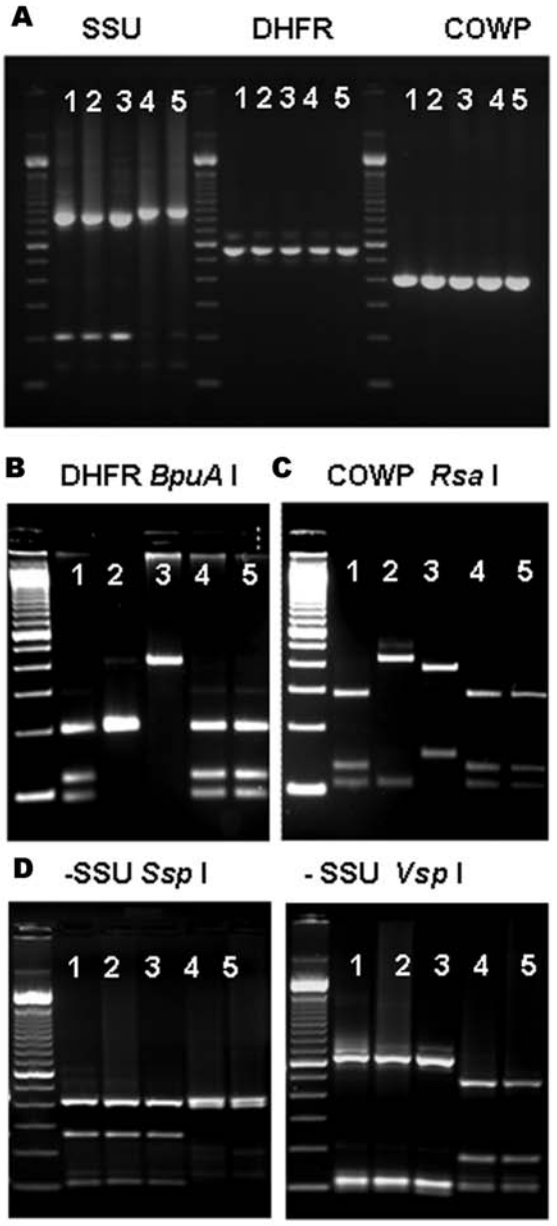

Figure. Multilocus polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of specimens previously identified as *Cryptosporidium canis* and *C. felis*. A) Agarose gel electrophoresis of PCR-amplified products of specimens previously identified as *C. canis* (lanes 1–3) and *C. felis* (lanes 4 and 5) with molecular tools based on the small subunit (SSU) rRNA, dihydrofolate reductase (DHFR), and *Cryptosporidium* oocyst wall protein (COWP). Molecular markers in all photos are 100-bp ladders. B) RFLP analysis of DHFR-based PCR amplification products using *BpuA* I restriction enzyme; lanes 1, 4, and 5 are *C. hominis*; lane 2 is *C. parvum*; and lane 3 is *C. meleagridis*. C) RFLP analysis of COWP-based PCR amplification products using *Rsa* I restriction enzyme; lanes 1, 4, and 5 are *C. hominis*; lane 2 is *C. parvum*; and lane 3 is *C. meleagridis*. D) RFLP analysis of the SSU-based PCR products using restriction enzymes *Ssp* I (left) and *Vsp* I (right); the combined patterns for lanes 1 to 3 correspond to *C. canis* and lanes 4 and 5 to *C. felis*.

1 patient had the patterns of *C. hominis* and *C. meleagridis* (Figure panel B). Likewise, RFLP analysis of the COWP PCR products digested with *Rsa* I showed 3 banding patterns that were in complete agreement with the results obtained for the DHFR PCR-RFLP tool (Figure panel C). Therefore, 2 of the 12 *C. canis*–infected patients had *C. hominis*, 1 had *C. parvum* and 1 had both *C. hominis* and *C. meleagridis*; of the 9 *C. felis*-infected patients, 2 had *C. hominis* and 1 had *C. meleagridis* (Table).

All DHFR and COWP PCR products were sequenced, which confirmed the results of the RFLP diagnosis. Altogether, 8, 2, and 3 DHFR sequences were obtained for *C. hominis*, *C. parvum*, and *C. meleagridis*, respectively. The *C. hominis* and *C. meleagridis* DHFR sequences were identical to XM_660774 and AY391725, respectively. The *C. parvum* DHFR sequences were homologous to XM_625460, with an insertion at position 37 and 4 bp substitutions at positions 66, 69, 364, and 367. Likewise, 10, 2, and 3 COWP sequences were obtained for *C. hominis*, *C. parvum*, and *C. meleagridis*, respectively, and were identical to AF481960, AF266273, and AY166840, respectively, in GenBank. The *C. parvum* DHFR nucleotide sequence obtained from this study is deposited in GenBank under accession no. DQ352814.

To confirm the original diagnosis of *C. canis* and *C. felis* infection, we reanalyzed all DNA preparations of these specimens with the SSU rRNA genotyping tool (*7*). Results were in complete agreement with those obtained previously (*7*): 19 specimens from 12 patients had *C. canis*, 15 specimens from 9 patients had *C. felis*, and no specimens had mixed *Cryptosporidium* spp., as indicated by RFLP patterns (Table and Figure panel D).

Data on diarrhea at study enrollment were available for 4 of the 7 patients with mixed infections and all 14 patients without mixed infections. Among persons with mixed infections, 1 did not have diarrhea, 2 had diarrhea lasting ≤30 days, and 1 had diarrhea ≥5 months. Seven of 14 patients without mixed infections had diarrhea: 5 had acute diarrhea lasting ≤30 days, and 2 had chronic diarrhea lasting >5 months (difference in prevalence of diarrhea for mixed versus single infections was not significant by the Fisher exact test). The average CD4+ lymphocyte count among the patients with mixed infections was 130 cells/μL. Of the 7 patients with mixed infections, 3 had specimens collected >30 days after the first detection, and mixed infections with the same species were still identified. The persistence of 2 species for >1 month is in contrast to a report that 1 *Cryptosporidium* genotype rapidly displaces the other during experimental infections of animals (*6*).

Conclusions

Concurrent infection with multiple *Cryptosporidium* spp. may affect clinical manifestations since *C. hominis*

Table. Results of multilocus genotyping of *Cryptosporidium* specimens originally diagnosed as *Cryptosporidium canis* and *C. felis* by an SSU rRNA–based PCR-RFLP tool*

Participant	No. specimens tested	No. days between first and last specimen	*Cryptosporidium* genotype by locus (no. specimens)			Mixed infection
			SSU rRNA	COWP	DHFR	
0043D	7	29	*C. canis*	–	–	No
0214D	2	5	*C. canis*	–	–	No
0448D	4	45	*C. canis*	*C. hominis* (1) and *C. meleagridis* (2)	*C. hominis* (2) and *C. meleagridis* (2)	Yes
1083D	1	–	*C. canis*	–	–	No
1322D	2	2	*C. canis*	–	–	No
0002D	1	–	*C. canis*	–	–	No
0034D	7	56	*C. canis*	*C. parvum* (2)	*C. parvum* (2)	Yes
0482D	1	–	*C. canis*	*C. hominis* (1)	*C. hominis* (1)	Yes
0500D	1	–	*C. canis*	–	–	No
0533D	3	3	*C. canis*	–	–	No
0670D	4	414†	*C. canis*	*C. hominis* (4)	*C. hominis* (2)	Yes
0725D	1	–	*C. canis*	–	–	No
0044A	1	–	*C. felis*	*C. meleagridis* (1)	*C. meleagridis* (1)	Yes
0076A	4	31	*C. felis*	*C. hominis* (3)	*C. hominis* (1)	Yes
0668A	3	3	*C. felis*	*C. hominis* (1)	*C. hominis* (2)	Yes
0673A	5	31	*C. felis*	–	–	No
0817A	2	2	*C. felis*	–	–	No
0891A	1	–	*C. felis*	–	–	No
1344A	3	3	*C. felis*	–	–	No
0569D	2	2	*C. felis*	–	–	No
0776D	1	–	*C. felis*	–	–	No

*PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; SSU, small subunit; COWP, *Cryptosporidium* oocyst wall protein; DHFR, dihydrofolate reductase gene.
†Specimens correspond to 2 visits 14 months apart.

and *C. parvum* induce different sequelae in humans (*12*). The frequent finding of *C. hominis* in *C. canis*– and *C. felis*–infected persons also raises the question of infection sources. Although these 2 species are traditionally associated with animals, anthroponotic transmission may play a role in their acquisition in humans. Recent analyses demonstrate that a large proportion of human infections with *C. parvum*, another traditional zoonotic species, are actually due to anthroponotic transmission (*13,14*).

Our results also suggest that although the SSU rRNA–based PCR-RFLP tool or similar PCR techniques can detect and differentiate a wide range of *Cryptosporidium* species or genotypes, their usefulness in detecting mixed infections was compromised by preferential PCR amplification of the dominant species or genotype in specimens. This problem is likely inherited with most PCR tools. Thus, the use of PCR tools with broad specificity in combination with species-specific tools is needed to address the issue of mixed *Cryptosporidium* infections.

Our findings demonstrate that mixed infections are more frequent and persist longer in HIV-infected patients than previously believed. The clinical importance of these findings is not clear because of the study's cross-sectional nature. Future studies should employ tools that can detect mixed *Cryptosporidium* infections in longitudinal studies, evaluate the frequency of mixed infections of *C. hominis* and *C. parvum*, and assess their clinical and epidemiologic implications in both immunocompetent and immunocompromised persons.

This work was supported in part by funds from the Opportunistic Infections Working Group of the Centers for Disease Control and Prevention. Dr Gilman is supported by National Institutes of Health–National Institute of Allergy and Infectious Diseases grants Peru-TMRC 5P01AI051976-04 and R21 AI 059661-01.

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References

1. Cama VA, Bern C, Sulaiman IM, Gilman RH, Ticona E, Vivar A, et al. *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. J Eukaryot Microbiol. 2003;50(Suppl):531–3.
2. Fretz R, Svoboda P, Ryan UM, Thompson RC, Tanners M, Baumgartner A. Genotyping of *Cryptosporidium* spp. isolated from human stool specimens in Switzerland. Epidemiol Infect. 2003;131:663–7.

3. Gile M, Warhurst DC, Webster KA, West DM, Marshall JA. A multiplex allele specific polymerase chain reaction (MAS-PCR) on the dihydrofolate reductase gene for the detection of *Cryptosporidium* parvum genotypes 1 and 2. Parasitology. 2002;125:35–44.
4. McLauchlin J, Amar C, Pedraza-Diaz S, Nichols GL. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal specimens from humans and 105 fecal specimens from livestock animals. J Clin Microbiol. 2000;38:3984–90.
5. Mallon M, MacLeod A, Wastling J, Smith H, Reilly B, Tait A. Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium parvum*. J Mol Evol. 2003;56:407–17.
6. Tanriverdi S, Arslan MO, Akiyoshi DE, Tzipori S, Widmer G. Identification of genotypically mixed *Cryptosporidium parvum* populations in humans and calves. Mol Biochem Parasitol. 2003;130:13–22.
7. Jiang J, Xiao L. An evaluation of molecular diagnostic tools for the detection and differentiation of human-pathogenic *Cryptosporidium* spp. J Eukaryot Microbiol. 2003;50(Suppl):542–7.
8. Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, et al. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J Infect Dis. 2001;183:492–7.
9. Reed C, Sturbaum GD, Hoover PJ, Sterling CR. *Cryptosporidium parvum* mixed genotypes detected by PCR-restriction fragment length polymorphism analysis. Appl Environ Microbiol. 2002;68:427–9.
10. Gibbons CL, Gazzard BG, Ibrahim MA, Morris-Jones S, Ong CS, Awad-El-Kareim FM. Correlation between markers of strain variation in *Cryptosporidium parvum*: evidence of clonality. Parasitol Int. 1998;47:139–47.
11. Pedraza-Diaz S, Amar C, Nichols GL, McLauchlin J. Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. Emerg Infect Dis. 2001;7:49–56.
12. Hunter PR, Hughes S, Woodhouse S, Raj N, Syed Q, Chalmers RM, et al. Health sequelae of human cryptosporidiosis in immunocompetent patients. Clin Infect Dis. 2004;39:504–10.
13. Alves M, Xiao L, Sulaiman I, Lal AA, Matos O, Antunes F. Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J Clin Microbiol. 2003;41:2744–7.
14. Mallon ME, MacLeod A, Wastling JM, Smith H, Tait A. Multilocus genotyping of *Cryptosporidium parvum* type 2: population genetics and sub-structuring. Infect Genet Evol. 2003;3:207–18.

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Pets in Voluntary Household Quarantine

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Outbreaks of severe acute respiratory syndrome (SARS) have resulted in increased discussion about community-based infection control measures, including voluntary quarantine. In the 2003 SARS outbreak in Toronto, Canada, at least 23,000 persons participated in voluntary quarantine in their homes because of possible exposure (*1*). Quarantined persons were told to remain at home, not allow anyone to visit, wear a mask when in the same room as other members of the household, and sleep in a separate room (*2*). These protocols were developed to decrease the risk of transmitting the SARS coronavirus to persons in the household. This situation highlights 1 aspect of community-based quarantine that has been overlooked: the potential role of household pets in disease transmission.

When SARS was first identified, potential host animal species were unknown, as was the risk of transmission between animals and humans. Despite the severity of SARS, the lack of information on the potential for interspecies transmission, and the potential implications of animals acting as reservoirs of infection, we are unaware of quarantine protocols that consider household pets. No specific data are available on pet ownership by quarantined persons; however, based on the prevalence of pet ownership in Canada, we assume that thousands of quarantined persons had household pets. Whether any precautions were taken to reduce the risk of SARS transmission to pets is unclear. Presumably, household pets had prolonged close contact with many quarantined persons. Additionally, many of these pets may have had close contact with other persons, both inside and outside the home, and contact with other animals. We now know that domestic cats and ferrets are susceptible to experimental infection by the SARS coronavirus and that they can transmit this virus to other cats and ferrets (*3*). What would have happened if cats were naturally infected in households and could transmit infection to humans or other animals? Were measures in place to reduce the risk for this transmission and detect it had it occurred? If SARS had established itself in the feral cat population in affected cities, would it have been controllable?

Although SARS is the most recent example of an emerging disease for which quarantine was implemented, the potential for household transmission through pets should be considered in any new disease when information is incomplete regarding potential hosts and the risk for interspecies transmission. If one considers that an estimated 75% of transmissible emerging diseases are zoonoses (*4*), the relevance becomes clear.

While most of the discussion of zoonoses has focused on food-producing animals and wildlife, companion animals require closer scrutiny because of the number of persons exposed to pets and the nature of human-animal interaction. Pets are present in ≈58.3% of households in the United States; the pet population includes ≈62 million dogs, ≈69 million cats, ≈10 million birds, and ≈3 million reptiles (*5*). Also included are smaller numbers of ferrets, rabbits, rats, hamsters, hedgehogs, and other small mammals and exotic species. Many, if not most, owners of household pets likely have more prolonged and close contact with their pets than with most other persons. Ample reports exist regarding transmission of bacterial, viral, and fungal pathogens between humans and pets (in both directions) in the household (*6*–*11*). In addition to SARS, some pathogens that have recently been identified as of concern include methicillin-resistant *Staphylococcus aureus* (*12*), monkeypox (*6*), and H5N1 influenza (*13*). Although transmission of pathogens from domestic pets often focuses on the household, many other persons also have regular or sporadic contact with household pets owned by friends or family or through animal visitation programs.

Development of community-based quarantine protocols that consider the role of domestic animals in transmission of disease remains a gap in current preparedness planning activities. We believe that the potential role of household pets should be considered in transmission of all

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emerging infectious diseases. This would include promptly and thoroughly evaluating the susceptibility of pets of various species to clinical disease and subclinical infection and assessing the possibility of transmission of pathogens between humans and pets, in both directions. Community-based quarantine measures may need to address contingency protocols for placing household pets in quarantine as well as human family members. Among the factors that need to be evaluated are the following: when pets should be quarantined, what type of unprotected animal-human contact should be allowed, what types of outdoor access by pets should be allowed (if any), what infection control measures should be implemented in the household to decrease the risk of pathogen transmission, how pet fecal material should be handled in the household and outdoors and in community settings, and what measures should be taken when and if veterinary care is required. Additionally, clinical and epidemiologic studies involving household pets may be indicated during the emergence of infectious diseases to evaluate the potential role of pets in disease transmission, to help manage disease in pets, and to determine whether pets may act as sentinel species.

We recommend that a coordinated effort between the human and veterinary medical fields and public health authorities be undertaken to address these issues. Relevant groups would involve national or regional regulatory bodies, public health agencies, infection control specialists in the human and veterinary fields, veterinary organizations, primary care veterinarians, laboratory animal veterinarians, comparative medicine specialists, and humane society personnel. Because of the number of groups that should be included and the potential complexity of the situation, proactive planning is needed.

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References

1. DiGiovanni C, Conley J, Chiu D, Zaborski J. Factors influencing compliance with quarantine in Toronto during the 2003 SARS outbreak. Biosecur Bioterror. 2004;2:265–72.
2. Ontario Ministry of Health and Long-term Care. SARS: questions and answers, 2002. [cited 2005 Nov 16]. Available from http://www.health.gov.on.ca/english/public/pub/disease/sars_4.html
3. Martina BE, Haagmans BL, Kuiken T, Fouchier RA, Rimmelzwaan GF, van Amerongen G, et al. Virology: SARS virus infection of cats and ferrets. Nature. 2003;425:915.
4. Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci. 2001;356:983–9.
5. American Veterinary Medical Association. U.S. pet ownership and demographics Sourcebook. Schaumburg (IL): American Veterinary Medical Association; 2002.
6. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, et al. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med. 2004;350:342–50.
7. Chomel BB, Boulouis HJ, Breitschwerdt EB. Cat scratch disease and other zoonotic *Bartonella* infections. J Am Vet Med Assoc. 2004;224:1270–9.
8. Marinella MA. Community-acquired pneumonia due to *Pasteurella multocida*. Respir Care. 2004;49:1528–9.
9. Walker DH, Barbour AG, Oliver JH, Lane RS, Dumler JS, Dennis DT, et al. Emerging bacterial zoonotic and vector-borne diseases. Ecological and epidemiological factors. JAMA. 1996;275:463–9.
10. Rosen T. Hazardous hedgehogs. South Med J. 2000;93:936–8.
11. Cefai C, Ashurst S, Owens C. Human carriage of methicillin-resistant *Staphylococcus aureus* linked with pet dog. Lancet. 1994;344:539–40.
12. Manian FA. Asymptomatic nasal carriage of mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MRSA) in a pet dog associated with MRSA infection in household contacts. Clin Infect Dis. 2003;36:e26–8.
13. 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.

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Weeds

Ronald O. Valdiserri*

The even mead, that erst brought sweetly forth
The freckled cowslip, burnet, and green clover,
Wanting the scythe, all uncorrected, rank,
Conceives by idleness, and nothing teems
But hateful docks, rough thistles, kecksies, burs,
Losing both beauty and utility.

– William Shakespeare
Henry V

Sometimes in the spring, when I'm on my hands and knees among the azaleas, pulling out purslane and dandelion, I think how wonderful gardening would be if only it weren't for weeds. Most of the time I dislike weeding, especially when I see it as a battle. Given the natural guile of the enemy, I become pessimistic, thinking that the weeds will always be one step ahead of me, that I can never defeat them. I can create huge mounds of compost from the honeysuckle vine, chickweed, and pokeberry that I remove from my woodland garden beds and still hundreds of malignant survivors will have escaped my surveillance. When my frustration grows past endurance, my war escalates to chemical weapons. I don't spray herbicides very often, though, for I'm not proud of resorting to excessive force. Weeds, it seems, are truly like troubles: prodigious, vexing, and capable of bringing out the worst in people. But like troubles, they can also coax out the best.

Sometimes, when I'm weeding, the simple action of plunging the fork into the earth, uprooting the plant, and shaking loose the soil that clings to its roots is very soothing. Plunge, pull, shake—repeated over and over, just like a mantra. On these occasions, my thoughts turn philosophical. Maybe the strong smell of the earth loosens memories from an earlier, less careworn time. Or maybe this contemplative sensibility is a byproduct of repetitious action. In either case, when this mood takes over, I lose the notion that the weeds are my enemy, that their leaves and roots are acting malevolently. The weeds, I realize, are neither good nor bad; they simply exist. Gardens do not grow without weeds, and life does not unfold without misfortune. And then I think about the way my life has been touched by AIDS.

I can still remember the first time I came across a description of an unusual immune disorder in a medical journal—long before the disease even had a name. Because of my training in pathology, I was curious about this mysterious new disease. I remember feeling a ghoulish interest in an illness that could cause such tremendous, irreparable damage. Even then, before any of us knew its pathogenesis, its destructive potential was clear, and I felt, perhaps, much the same as the physicists who first glimpsed the horrendous possibilities of the atomic bomb. My scientific detachment didn't live long; AIDS soon became more than a medical curiosity for me. It began to take people I knew, friends who I had hoped would surround me all my life. Sources of comfort and understanding dried up. I started feeling like an uprooted, disenfranchised farmer of Dust Bowl days, driven from once green and fertile land and forced to migrate to unknown places.

Over that first horrible decade of its debut, AIDS managed to dissolve my sense of permanence and distort my perception of time. Before AIDS, I didn't know much about misfortune. I thought about the future primarily in terms of its potential to bring me more gratification and greater achievements. After AIDS, I understood that the future also has the power to disappoint. That knowledge has made me supremely impatient. Now it seems that nothing is quick enough for me; everything takes too long. I feel as if my internal clock has been wound so tightly that

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at any moment the hands might go spinning clean off the face, so rapidly are they going around and around.

AIDS changed my professional life as well. I had been content to study disease from a distance, behind a microscope. As a pathologist I thought about illness primarily as a disruption in normal physiology, and I was satisfied with the contributions I could make to patient care by diagnosing unusual tumors and peculiar lesions. But after the epidemic took hold, my interest in abnormal physiology waned. The accurate diagnosis of diseases, including AIDS, though it is an essential step in patient care, had become too passive for me, seemed to imply acceptance of the inevitability of illness. No longer satisfied with identifying disease, I wanted to learn how to prevent it from occurring. I was determined to join those who were already trying to stop the spread of the epidemic. And so, after I finished my public health degree, I left my job as a university-based pathologist to work full-time on AIDS prevention at the Centers for Disease Control and Prevention.

It would be easy to assume that the AIDS epidemic, so often associated with loss, generates nothing but sorrow in those it touches, that the personal adjustments we make are all negative. I find, on the contrary, that AIDS has helped me to clarify just what does and does not matter during our brief time in the world. The epidemic has not taken hope away from me, but it has taught me the inadequacy of looking toward the future as a means of rescue from the present. AIDS has shown me that hope is strongest in us when we seek our fulfillment in the circumstances of the present, when we refuse to defer our dreams or to accept defeat.

Sometimes when I'm working in my garden, I think about the invasiveness of weeds and disease, how they appear in the garden, in the human population, uninvited, unwelcome, capable of causing tremendous destruction if left unattended. Yes, it would be wonderful if some spectacular act of God or nature would banish AIDS from our existence. Gardeners dream that they'll awake some morning to find that the crabgrass has magically departed from the perennial beds. Weeds, though, whether real or metaphorical, will not be banished. Pain and loss are intrinsic to our existence in the temporal world. The best way to keep weeds from engulfing the garden is not to rail at the injustice of fate but to get down on our hands and knees and begin clearing them away.

Author's Note

I wrote "Weeds" in the early 1990s; the essay appeared in a collection on AIDS, Gardening in Clay (*1*). Part memoir, part social commentary, the book sprung from my desire to honor and eventually memorialize my twin brother Edwin—who sickened and died of AIDS during the course of my writing the book.

In 1992, the year Edwin died, tennis great Arthur Ashe announced he had AIDS; the Centers for Disease Control and Prevention estimated 140,000–168,000 persons were living with AIDS in the United States (*2*); the International AIDS Society moved its 8th International Conference from Boston to Amsterdam in protest of US policy on HIV-infected travelers; and the Food and Drug Administration was some 3 years off approving the first protease inhibitor, saquinavir.

How far we've come in the 25 years since the first AIDS cases were reported in the United States is difficult to ignore (*3*). Gone is the bleak pessimism that accompanied diagnosis of infection with human T-cell lymphotropic virus type III or lymphadenopathy-associated virus, as the virus was formerly known (*4*). Today, persons who learn they are infected with HIV can enjoy longer and healthier lives as a result of highly active combination antiretroviral therapy. Gone, too, are the urgency and importance Americans once placed on AIDS, despite its continued and substantial national toll, especially in communities of color (*5*).

As "Weeds" suggests, in the continuing chronicles of humans versus retroviruses, we are far from ridding our garden of weeds.

References

1. Valdiserri RO. Gardening in clay: reflections on AIDS. Ithaca (NY): Cornell University Press; 1994.
2. Centers for Disease Control and Prevention. Projections of the number of persons diagnosed with AIDS and the number of immunosuppressed HIV-infected persons—United States, 1992–1994. MMWR Recomm Rep. 1992;41(RR-18):1–29.
3. Centers for Disease Control and Prevention. Pneumocystis pneumonia—Los Angeles. MMWR Morb Mortal Wkly Rep. 1981;30:250–2.
4. Valdiserri RO. HIV/AIDS in historical profile. In: Valdiserri RO, editor. Dawning answers: how the HIV/AIDS epidemic has helped to strengthen public health. Oxford: Oxford University Press; 2003. p. 3–32.
5. Centers for Disease Control and Prevention. Racial/ethnic disparities in diagnoses of HIV/AIDS—33 states, 2001–2004. MMWR Morb Mortal Wkly Rep. 2006;55:121–5.

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Simple Respiratory Mask

To the Editor: The US Department of Labor recommends air-purifying respirators (e.g., N95, N99, or N100) as part of a comprehensive respiratory protection program for workers directly involved with avian influenza–infected birds or patients (*1*). N95 respirators have 2 advantages over simple cloth or surgical masks; they are ≥95% efficient at filtering 0.3-μm particles (smaller than the 5-μm size of large droplets—created during talking, coughing, and sneezing—which usually transmit influenza) and are fit tested to ensure that infectious droplets and particles do not leak around the mask (*2–4*). Even if N95 filtration is unnecessary for avian influenza, N95 fit offers advantages over a loose-fitting surgical mask by eliminating leakage around the mask.

The World Health Organization recommends protective equipment including masks (if they not available, a cloth to cover the mouth is recommended) for persons who must handle dead or ill chickens in regions affected by H5N1 (*5*). Quality commercial masks are not always accessible, but anecdotal evidence has showed that handmade masks of cotton gauze were protective in military barracks and in healthcare workers during the Manchurian epidemic (*6,7*). A simple, locally made, washable mask may be a solution if commercial masks are not available. We describe the test results of 1 handmade, reusable, cotton mask.

For material, we choose heavyweight T-shirts similar to the 2-ply battle dress uniform T-shirts used for protective masks against ricin and saxitoxin in mouse experiments (*8*). Designs and T-shirts were initially screened with a short version of a qualitative Bitrex fit test (*9*) (Allegro Industries, Garden Grove, CA, USA). The best were tested by using a standard quantitative fit test, the Portacount Plus Respirator Fit Tester with N95-Companion (TSI, Shoreview, MN, USA) (*10*). Poor results from the initial quantitative fit testing on early prototypes resulted in the addition of 4 layers of material to the simplest mask design. This mask is referred to as the prototype mask (Figure).

A Hanes Heavyweight 100% preshrunk cotton T-shirt (made in Honduras) was boiled for 10 minutes and air-dried to maximize shrinkage and sterilize the material in a manner available in developing countries. A scissor, marker, and ruler were used to cut out 1 outer layer (≈37 × 72 cm) and 8 inner layers (≤18 cm^2). The mask was assembled and fitted as shown in the Figure.

A fit factor is the number generated during quantitative fit testing by simulating workplace activities (a series of exercises, each 1 minute in duration). The Portacount Plus Respirator Fit Tester with N95-Companion used for the test is an ambient aerosol instrument that measures aerosol concentration outside and inside the prototype mask. The challenge agent used is the ambient microscopic dust and other aerosols that are present in the air.

A commercially available N95 respirator requires a fit factor of 100 to be considered adequate in the workplace. The prototype mask achieved a fit factor of 67 for 1 author with a Los Alamos National Laboratory (LANL) panel face size of 4, a common size. Although insufficient for the workplace, this mask offered substantial protection from the challenge aerosol and showed good fit with minimal leakage. The other 2 authors with LANL panel face size 10, the largest size, achieved fit factors of 13 and 17 by making the prototype mask inner layers slightly larger (22 cm^2).

We do not advocate use of this respirator in place of a properly fitted commercial respirator. Although subjectively we did not find the work of breathing required with the prototype mask to be different from that required with a standard N95 filtering facepiece, persons with respiratory compromise of any type should not use this mask. While testers wore the mask for an hour without difficulty, we cannot comment on its utility during strenuous work or adverse environmental conditions.

We showed that a hand-fashioned mask can provide a good fit and a measurable level of protection from a challenge aerosol. Problems remain. When made by naive users, this mask may be less effective because of variations in material, assembly, facial structure, cultural practices, and

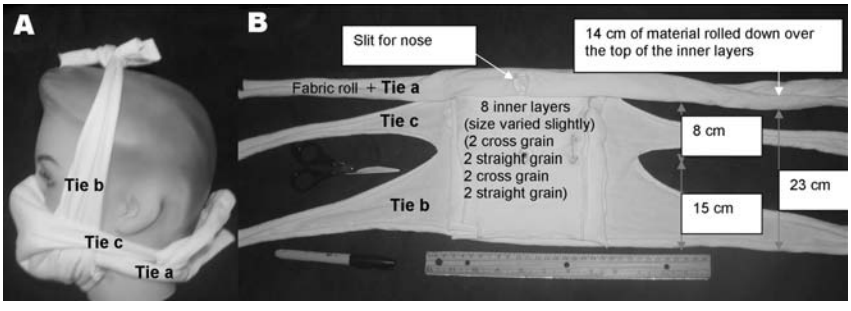

Figure. Prototype mask: A) Side view, B) Face side. This mask consisted of 1 outer layer (≈37cm × 72 cm) rolled and cut as in panel B with 8 inner layers (≤18 cm^2) placed inside (against the face). The nose slit was first placed over the bridge of the nose, and the roll was tied below the back of the neck. The area around the nose was adjusted to eliminate any leakage. If the seal was not tight, it was adjusted by adding extra material under the roll between the cheek and nose and/or pushing the rolled fabric above or below the cheekbone. Tie b was tied over the head. A cloth extension was added if tie b was too short. Finally tie c was tied behind the head. The mask was then fit tested.

handling. No easy, definitive, and affordable test can demonstrate effectiveness before each use. Wearers may find the mask uncomfortable.

We encourage innovation to improve respiratory protection options. Future studies must be conducted to determine levels of protection achieved when naive users, following instructions, produce a similar mask from identical or similar raw materials. Research is needed to determine the minimal level of protection needed when resources are not available for N95 air-purifying respirators since the pandemic threat from H5N1 and other possible influenza strains will exist for the foreseeable future.

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References

1. Occupational Safety and Health Administration. Guidance for protecting workers against avian flu. [cited 2005 Oct 23]. Available from http://www.osha.gov/dsg/guidance/avian-flu.html
2. National Institute for Occupational Safety and Health. 42 CFR Part 84 Respiratory protective devices. 1995 [cited 2005 Oct 23]. Available from http://www.cdc.gov/niosh/pt84abs2.html
3. Garner, JS. Guideline for isolation precautions in hospitals. The Hospital Infection Control Practices Advisory Committee. Infect Control Hosp Epidemiol. 1996;17:53–80.
4. Centers for Disease Control and Prevention. Laboratory performance evaluation of N95 filtering facepiece respirators, 1996. MMWR Morb Mortal Wkly Rep. 1998.47:1045–9.
5. World Health Organization Regional Office for the Western Pacific. Advice for people living in areas affected by bird flu or avian influenza. 2004 Nov 8 [cited 2005 Oct 22]. Available from http://www.wpro.who.int/NR/rdonlyres/04FA6993-8CD1-4B72-ACB9-EB0EBD3D0CB1/0/Advice10022004rev08112004.pdf
6. Capps JA. Measures for the prevention and control of respiratory infections in military camps. JAMA. 1918;71:448–50.
7. Kool, JL. Risk of person-to-person transmission of pneumonic plague. Clin Infect Dis. 2005;40:1166–72.
8. Darling RG. Biological warfare and bioterrorism. Slides 47 and 48. [cited 2006 Mar 19]. Available from http://www.regionsem.org/~trjoing/papers/123456/clr/Slides%20with%20Notes/Biological%20Warfare%20&%20Bioterrorism.pdf
9. Occupational Safety and Health Administration. Fit testing procedures (mandatory)–1910.134 App A. [cited 2006 Jan 21]. Available from http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9780&p_text_version=FALSE#Appendix%20A
10. TSI incorporated. How to quantitatively fit test filtering-face piece respirators using a TSI Portacount Plus and N95-Companion (ITI-054) c2006. [cited 2006 Jan 21]. Available from http://www.tsi.com/AppNotes/appnotes.aspx?Pid=33&lid=445&file=iti_054

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Linguatuliasis in Germany

To the Editor: Pentastomids or tongue worms are a unique group of vermiform parasites, phylogenetically related to arthropods (*1*). Of the many pentastomid species, only a few, including *Linguatula serrata*, infect humans. The adult parasites are long, flat, or annulated and have 4 hooks surrounding a central mouth. Adult *L. serrata* inhabit the nasal passages and paranasal sinuses of wild and domestic canids, which serve as definitive hosts. Infective eggs containing larvae are discharged into the environment by nasopharyngeal secretions and are ingested by herbivores, the natural intermediate hosts. Humans can become dead-end intermediate hosts; visceral linguatuliasis then develops (*2*) if infective eggs are ingested. The liver is the organ most often involved (*3–5*), but the lung (*4,6,7*) or other organs (*4,8*) may be affected. Parasites may also be found in lymph nodes. In the viscera, the primary 4-legged larva molts several times and eventually forms the legless nymph. Lesions due to *Linguatula* may be confused with malignancy, particularly in the lung (*6*).

We describe a recent infection with *L. serrata* in Germany in a patient who had pulmonary symptoms and in whom malignancy was suspected. The patient was a 39-year-old man of Russian origin who had been living in Germany since 1999. He was admitted to the hospital with weight loss, night sweats, chest pain, and coughing. He had been a smoker for 20 years, and his past medical history included pneumonia and sinusitis in 1989 during his military service at Lake Baikal, Russia. The patient had been living in a farmhouse in Karaganda, Kazakhstan, until he immigrated to Germany.

A chest radiograph and computed tomographic scan showed multiple, small lesions in both lungs. Malignancy was suspected, and a bronchoscopy was performed. Numerous granulomatous nodules were discovered. Thoracotomy was performed, and stringlike nodules on the pleural surface were resected. Except for a mild eosinophilia (7%, 500 cells/μL), the leukocyte count was normal. All other parameters, including C-reactive protein levels, angiotensin-converting enzyme, and tumor markers were normal. Histologic examination of the nodules showed a targetoid appearance with a sharp demarcation from the surrounding lung tissue by a thick fibrocollagenous capsule. In the center of the nodules, a transverse section (Figure, right inset) and a longitudinal section (Figure, main panel) of a parasite were visible. The parasite had a chitinous cuticle 2.5 μm thick and cuticular

spines 20–30 μm long. The spines and the serrated aspect are characteristic for *L. serrata*, a pentastome. Ringlike structures in the body wall were interpreted as sclerotized openings, a key feature of pentastomes. In close contact to host tissue, a shed cuticle was visible and assigned to the previous instar larva. The biometric data of the parasite were comparable to those measured by others (*6*,*9*). Hooks, typical for the oral armature of pentastomes, were found by serial sectioning (Figure, left inset). Except for some subcuticular glands, the parasite's inner organs were no longer distinguishable. The patient was initially treated with albendazole before the histologic diagnosis of linguatuliasis was established. Findings from magnetic resonance imaging of the abdomen were unremarkable, and no further lesions appeared during 12 months of followup. Intermittent cough and chest pain remained, possibly due to scar tissue and the remains of the nymphs.

At the beginning of the last century, visceral linguatuliasis of humans occurred frequently in Germany. In 1904 and 1905, among 400 autopsies in Berlin, 47 (11.8%) remains were infected with *L. serrata* (*7*). In contrast, reports of human infections are now rare. Our report is the first recent case description in Germany. Where the patient acquired the infection is unknown. *L. serrata* has a worldwide distribution. Recent cases have been reported from China (*4*) and Italy (*6*). An increasing number of infections can be suspected in the Western Hemisphere because of incremental travel to linguatuliasis-endemic areas. Humans are usually tolerant to nymphal pentastomid infections, and most patients are asymptomatic (*4*). The living nymph provokes little inflammation, whereas the death of the parasite leads to a prominent host response (*2*). Most findings of visceral linguatuliasis are made at autopsy (*4*,*6*), and the parasites are mainly located in the liver (*3*–*5*). Infection of the lung is rare (*6*,*7*). The nymphs in human granulomas are typically degenerated at the time of examination (*3*,*6*,*9*), but the cuticle with its associated structures remains visible for some time (*2*).

Histopathologic diagnosis is guided by the presence of remnants of the cuticle with sclerotized openings and by calcified hooks. Among pentastomids observed in humans, only *L. serrata* has prominent spines (*2*–*4*). In contrast to trematodes, the spines protrude from the cuticle and do not end in the body wall of the parasite. Diagnosis should be made etiopathologically, subetiopathologically, or presumptively on the basis of whether entire nymphs, cuticle-associated structures, or pearly lesions ("*Linguatula* nodules" [*10*]) with targetoid appearance are found (*4*). The differential diagnosis includes malignancies and tuberculosis because of the radiologic coinlike appearance. On histologic examination, one must distinguish between tissue-inhabiting diptera larvae, infections with metacestodes, trematodes, tissue filariids, and gnathostomiasis. Once diagnosis is established, no treatment is necessary (*3*) for the parasites will degenerate after some time, and no effective antiparasitic therapy exists. Avoiding contact with canine saliva and drinking water used by dogs or wild canids prevents this infection.

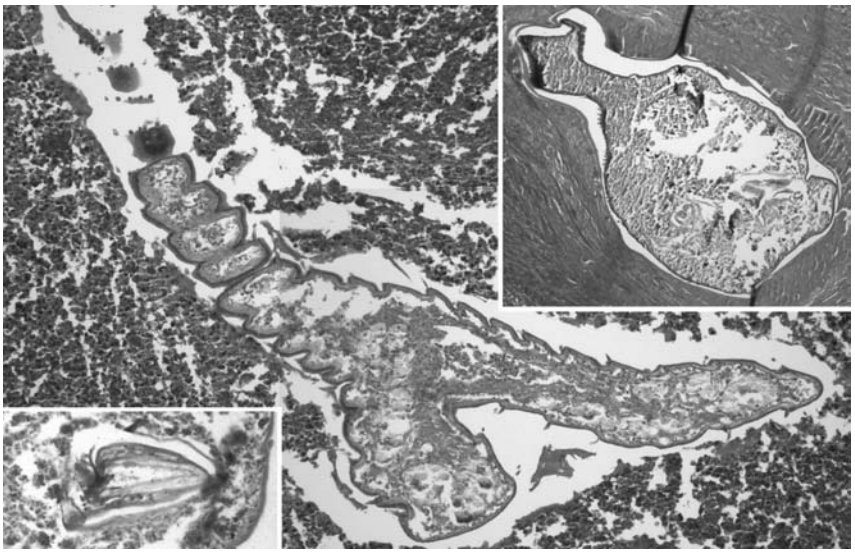

Figure. *Linguatula serrata* nymphs in lung tissue. Main panel shows the parasite's serrated nature and the cuticular spines (magnification ×200, Masson trichrome stain). Right upper inset, pulmonary nodule with prominent fibrotic reaction and shed cuticle around 1 nymph (magnification ×200, Masson trichrome stain). Left lower inset, detailed view of 1 parasite hook (magnification ×630, hematoxylin and eosin stain).

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References

1. Phylogenetic position of the Pentastomida and (pan) crustacean relationships. Proc Biol Sci. 2004;271:537–44.
2. Baird JK, Carey JC. Pentastomiasis. In: Connor DH, Chandler FW, editors. Pathology of infectious diseases. Stamford (CT): Appleton & Lange; 1997. p. 1671–4.
3. Hepatic granuloma in a man from North America caused by a nymph of *Linguatula serrata*. Pathology. 1988;20:198–9.
4. Pathological differentiation of suspected cases of pentastomiasis in China. Trop Med Int Health. 2002;7:166–77.

5. Hepatic granuloma due to a nymph of *Linguatula serrata* in a woman from Michigan: a case report and review of the literature. Am J Trop Med Hyg. 1984;33:187–9.
6. A nodular pulmonary lesion due to *Linguatula serrata* in an HIV-positive man. Parassitologia. 2001;43:105–8.
7. Koch M. Zur Kenntnis des Parasitismus der Pentastomen. Verh Dtsch Ges Pathol. 1906;10:265–79.
8. Intraocular pentastomiasis causing unilateral glaucoma. Br J Ophthalmol. 1987;71: 391–5.
9. Ocular linguatuliasis in Ecuador: case report and morphometric study of the larva of *Linguatula serrata*. Am J Trop Med Hyg. 1999;60:405–9.
10. St. Symmers WC, Valteris K. Two cases of human infestation by larvae of *Linguatula serrata*. J Clin Pathol. 1950;3:212–9.

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Epidemic *Clostridium difficile* Strain in Hospital Visitation Dog

To the Editor: Rates of illness and death from *Clostridium difficile*–associated disease (CDAD) and reports of CDAD in persons without traditional risk factors (*1*) have been increasing. One particular strain of *C. difficile* has been implicated in outbreaks of CDAD in hospitals in North America and Europe and appears to be spreading internationally at an alarming rate. This strain is classified as ribotype 027, toxinotype III, and possesses genes encoding toxins A, B, and CDT (binary toxin) as well as a deletion in the *tcdC* gene, which is believed to increase virulence (*2*).

We report this toxin-variant strain of *C. difficile* in a healthy, 4-year-old toy poodle that visits persons in hospitals and long-term care facilities in Ontario on a weekly basis. *C. difficile* was isolated from a fecal sample collected in the summer of 2004 as part of a cross-sectional study evaluating pathogen carriage by visitation dogs (*3*). The isolate was subsequently characterized by ribotyping (*4*) and by polymerase chain reaction (PCR) detection of genes that encode production of toxins A and B (*5*). Toxin CDT was confirmed by amplifying the portion of the gene (*cdtB*) that encodes for the receptor-binding component of the toxin, according to a previously reported protocol (*6*). As a result, the isolate was classified as ribotype 027, toxinotype III (*7*), and was found to possess all 3 toxin genes. The *tcdC* gene deletion was also confirmed with PCR (*8*).

These results indicate that this canine isolate is indistinguishable from the major strain implicated in outbreaks of highly virulent CDAD around the world. According to the infection control practitioner at the hospital the dog visited, CDAD cases were occurring at increased frequency in the facility around the time the dog's fecal specimen was collected. However, patient diagnosis was made solely through fecal toxin testing, and strains were not characterized. The facility has reported only sporadic cases of CDAD in the past few years.

This is the first report of this human, epidemic strain of *C. difficile* in a dog. Many *C. difficile* strains isolated from animals, including dogs, are indistinguishable from strains associated with disease in humans (*9*). To date, no study, including this one, has shown that interspecies transmission occurs; however, that possibility exists, as is becoming apparent with other pathogens, such as methicillin-resistant *Staphylococcus aureus*. The recurrent exposure of this dog to human healthcare settings suggests that the animal acquired this strain during visits to the hospital or long-term care facility, either from the healthcare environment or contaminated hands of human contacts. We recommend that future studies evaluating the dissemination of this strain and investigations of the movement of *C. difficile* into the community consider the role of animals.

Acknowledgments

We thank Joyce Rousseau for her assistance with culturing and identifying strains of *C. difficile*.

This work was supported by the Pet Trust Foundation of the Ontario Veterinary College.

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References

1. Centers for Disease Control and Prevention. Severe *Clostridium difficile*–associated disease in populations previously at low risk—four states, 2005. MMWR Morb Mortal Wkly Rep. 2005;54:1201–5.
2. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366:1079–84.
3. Lefebvre SL, Waltner-Toews D, Peregrine AS, Reid-Smith R, Hodge L, Arroyo LG, et al. Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. J Hosp Infect. Epub 2006 Feb 5.
4. Barbut F, Lalande V, Burghoffer B, Thien HV, Grimprel E, Petit JC. Prevalence and genetic characterization of toxin A variant strains of *Clostridium difficile* among adults and children with diarrhea in France. J Clin Microbiol. 2002;40:2079–83.
5. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A–negative, toxin B–positive *Clostridium difficile* by PCR. J Clin Microbiol. 1998;36:2178–82.
6. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol Lett. 2000;186:307–12.

7. Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmee M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J Clin Microbiol. 1998;36:2240–7.
8. Cohen SH, Tang YJ, Silva J Jr. Analysis of the pathogenicity locus in *Clostridium difficile* strains. J Infect Dis. 2000;181: 659–63.
9. Arroyo LG, Kruth SA, Willey BM, Staempfli HR, Low DE, Weese JS. PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. J Med Microbiol. 2005;54:163–6.

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Streptobacillus moniliformis Endocarditis

To the Editor: *Streptobacillus moniliformis* is a facultatively anaerobic, pleomorphic, gram-variable bacillus often seen in chains and as long unbranched filaments. It is found in the nasopharynx and oropharynx of wild and laboratory rats. Human infections result either from rodent bites (rat bite fever) or contaminated milk or other foods (Haverhill fever). The most common manifestations of infection are arthralgia, fever, and rash; endocarditis occurs as a rare complication (*1*). We report a case of *S. moniliformis* endocarditis in India in a patient with congenital heart disease.

An 18-year-old man was admitted to the Department of Cardiology at the Government General Hospital in Chennai, India, in November 2005, with a fever of 2 months' duration with cough, epistaxis, palpitations, and persistent joint pain. His medical history indicated congenital heart disease with a ventricular septal defect. On physical examination, his blood pressure was 100/70 mm Hg, pulse rate was 100 beats/min, and temperature was 38.5°C. Laboratory tests showed a leukocyte count of 7,600/μL, a platelet count of 127,000/μL, and an erythrocyte sedimentation rate of 70 mm/h. An electrocardiogram showed normal sinus rhythm. A transthoracic echocardiogram demonstrated a ventricular septal defect and vegetations on the septal leaflet of the tricuspid valve.

Three blood cultures were prepared, and treatment with antimicrobial drugs (intravenous penicillin G, 3×10^6 U every 6 h, and gentamicin, 50 mg every 8 h for 4 weeks) was initiated. The blood cultures were incubated at 37°C in an atmosphere of 5%–10% CO_2. Characteristic white, downy, crumblike granules were observed on the surface of the erythrocytes in all 3 cultures within 18–24 h of incubation. Characteristic puff balls were seen after 48 h of incubation. Gram-stained smears showed gram-negative bacilli in long chains. Cultures were subcultured onto 5% sheep blood agar plates and MacConkey agar plates. The plates were incubated at 37°C in an atmosphere of 5%–10% CO_2. After 18–24 h of incubation, growth was seen on the sheep blood agar plates. Colonies were 1–2 mm in diameter, gray, smooth, and butyrous. A Gram stain of these colonies identified gram-variable, pleomorphic coccobacilli that were negative for catalase, oxidase, urease, and citrate, and did not produce indole or reduce nitrate.

Antimicrobial susceptibility testing was performed by using the Kirby-Bauer disk diffusion method according to recommendations of the National Committee for Clinical laboratory Standards (*2*). The isolate was sensitive to penicillin G, ceftriaxone, cephalexin, amoxicillin, gentamicin, and erythromycin. The patient responded well to treatment and became afebrile within 48 h after initiation of therapy. Treatment with antimicrobial drugs was continued for 4 weeks. The blood cultures were negative when repeated after 2 weeks. The patient had an uneventful recovery and was discharged from the hospital.

Rat bite fever is a zoonosis caused by either *Streptobacillus moniliformis* or *Spirillum minus* (*1,3*). *S. moniliformis* is found in the nasopharynx of small rodents, especially rats. Rats that are carriers have no symptoms but can effectively transmit the infection by bite or through infected body fluids such as urine.

This patient had a history of living in a rat-infested area, and admitted having been bitten by a rat several months before the onset of symptoms. However, we considered it unlikely that disease contracted by a rat bite would take months to be manifested. Thus, it is more likely that he contracted the infection from food or water contaminated with rat excreta. Endocarditis is a rare complication of *S. moniliformis* infection, and cardiac valvular abnormalities have been reported in 50% of cases (*4*). This patient, however, had only a small ventricular septal defect. This is the first report of *S. moniliformis* endocarditis from India.

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References

1. Mutters R. Actinobacillus, Capnocytophaga, Eikenella, Kingella, and other fastidious or rarely encountered gram-negative rods. In: Murray PR, Baron EJ, Pfaller MA, Tenover F, Yolken RH, editors. Manual of clinical microbiology. 7th ed. Vol. 1. Washington: American Society for Microbiology Press; 1999. p. 568–9.
2. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; NCCLS document M2A7. Wayne (PA): The Committee; 2004.

3. Ojukwu IC, Christy C. Rat-bite fever in children: case report and review. Scand J Infect Dis. 2002;34:474–7.
4. Rodorf T, Zuger C, Zbinden R, von Graevenitz A, Pirovino M. *Streptobacillus moniliformis* endocarditis in an HIV-positive patient. Infection. 2000;28:393–4.

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West Nile Virus in Horses, Guatemala

To the Editor: West Nile virus (WNV, *Flaviviridae*: *Flavivirus*) is emerging as a public health and veterinary concern. Since its introduction into North America in 1999, it has spread rapidly, reaching the Caribbean Basin in 2001, Mexico in 2002, El Salvador in 2003, and Colombia in 2004 (*1*). However, reports of equine illness and deaths in Latin America are inconclusive. With the exception of viral isolates from a dead bird, a human, and a mosquito pool in Mexico (*2*,*3*), all reports of WNV presence in Latin America have relied on serologic evidence. WNV is a member of the Japanese encephalitis serocomplex, which in the Western Hemisphere includes St. Louis encephalitis virus (SLEV) (*4*). Serologic investigations for WNV in Latin America must use highly specific assays to differentiate WNV infection from potentially cross-reactive viruses such as SLEV or possibly additional unknown viruses. In particular, SLEV is of concern since it was previously isolated from Guatemalan mosquitoes (*5*).

Alerted by the findings of WNV transmission in the region (*1*), we collected serum samples from horses from 19 departments of Guatemala from September 2003 to March 2004, to initially estimate the extent of WNV spread and its potential public health risk. Because no animals exhibited signs of neurologic illness at the time of the survey, only healthy horses were sampled. Before 2005, equine WNV vaccines were prohibited and unavailable in Guatemala (Unidad de Normas y Regulaciones, Ministerio de Agricultura Ganadería y Alimentación, Guatemala, pers. comm.); as such, cross-reactivity due to prior vaccination is highly unlikely. Samples were initially tested for WNV-reactive antibodies by using an epitope-blocking enzyme-linked immunosorbent assay (blocking ELISA) (*6*). The ability of the test sera to block the binding of the monoclonal antibodies to WNV antigen was compared to the blocking ability of control horse serum without antibody to WNV. Data were expressed as relative percentages and inhibition values ≥30% were considered to indicate the presence of viral antibodies.

A subset of positive samples was further confirmed by plaque-reduction neutralization test (*7*). Of 352 samples, 149 (42.3%) tested positive with the 3.1112G WNV-specific monoclonal antibody. Of 70 blocking ELISA–positive samples, the neutralization tests indicated the infecting agent was WNV, SLEV, and undifferentiated flavivirus in 9, 33, and 21 samples, respectively. Titers were expressed as the reciprocal of serum dilutions yielding ≥90% reduction in the number of plaques in a plaque-reduction neutralization test ($PRNT_{90}$). $PRNT_{90}$ titers of horses seropositive for WNV ranged from 80 to 320. $PRNT_{90}$ titers of horses seropositive for SLEV ranged from 40 to 2,560. For the differential diagnosis of samples with neutralizing antibody titers against both WNV and SLEV in this test, a ≥4-fold titer difference was used to identify the etiologic agent. The undifferentiated flavivirus-reactive specimens had <4-fold difference in cross-neutralization titers. Likely possibilities for the inability to distinguish the infecting virus include previous infection with these or other flaviviruses (previously described or unknown) resulting in elevated cross-reactive titers. The remaining 10% of specimens that tested negative by PRNT probably represent nonneutralizing antibodies in the serum or false positivity in the blocking ELISA.

Our serologic results provide indirect evidence of past transmission of WNV, SLEV, and possibly other flaviviruses to horses in Guatemala. Although no confirmed cases of WNV-attributed disease have been reported in Central America to date, flavivirus transmission appears to be widely distributed in Guatemala (Figure). Efforts are under way to confirm WNV transmission by viral isolation and to evaluate the impact of WNV on human, horse, and wildlife populations. More information is needed to establish the public health threat of WNV and other zoonotic flaviviruses in the region.

Acknowledgments

We thank animal owners for permitting sampling, numerous veterinarians of the "Ministerio de Agricultura Ganadería y Alimentación," Alejandra Castillo for technical assistance, and Nicholas Komar for helpful discussions and reviewing the manuscript.

This work was supported in part by Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, "Organismo Internacional Regional de Sanidad Agropecuaria" and by grant U50 CCU820510 from the Centers for Disease Control and Prevention.

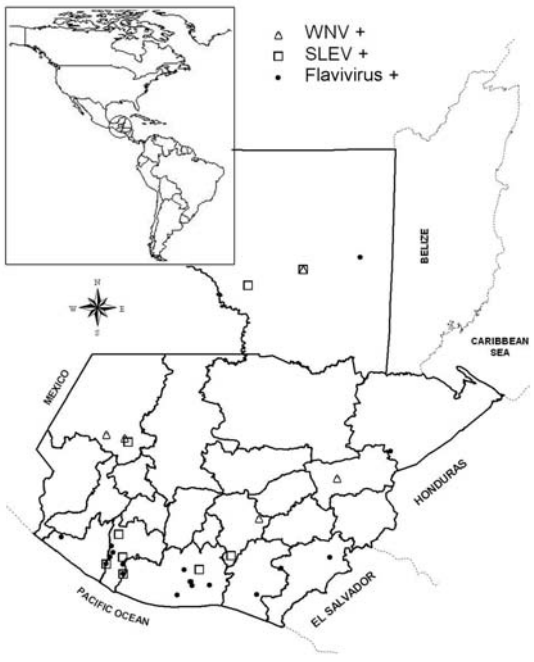

Figure. Geographic distribution in Guatemala of horses showing previous infections with West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), or undifferentiated flavivirus as confirmed by plaque reduction neutralization test. Each location may have multiple positive horses.

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References

1. Komar N, Clark G. West Nile virus activity in Latin America and the Caribbean. Rev Panam Salud Publica. 2006;19:112–7.
2. Estrada-Franco JG, Navarro-Lopez R, Beasley DW, Coffey L, Carrara AS, Travassos da Rosa A, et al. West Nile virus in Mexico: evidence of widespread circulation since July 2002. Emerg Infect Dis. 2003;9:1604–7.
3. Elizondo-Quiroga D, Davis TC, Fernandaz-Salas I, Escobar-Lopez R, Olmos DV, Gastalum LCS, et al. West Nile virus isolation in human and mosquitoes, Mexico. Emerg Infect Dis. 2005;11:1449–52.
4. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol. 1989;70:37–43.
5. Cupp EW, Scherer WF, Lok JB, Brenner RJ, Dziem GM, Ordonez JV. Entomological studies at an enzootic Venezuelan equine encephalitis virus focus in Guatemala, 1977–1980. Am J Trop Med Hyg. 1986;35:851–9.
6. Blitvich BJ, Bowen RA, Marlenee NL, Hall RA, Bunning ML, Beaty BJ. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. J Clin Microbiol. 2003;41:2676–9.
7. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Lennette EH, Lennette DA, Lennette ET, editors. Diagnostic procedures for viral, rickettsial, and chlamydial infections. 7th ed. Washington: American Public Health Association; 1995. p. 189–212.

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Borrelia burgdorferi in *Ixodes scapularis* Ticks, Chicago Area

To the Editor: Lyme disease is a multisystem disorder associated with skin, myocardial, musculoskeletal, and central and peripheral nervous system manifestations caused by infection with *Borrelia burgdorferi* sensu lato spirochetes (*1*). In the United States, the illness is caused by transmission of *B. burgdorferi* sensu stricto from the bite of infected *Ixodes scapularis* (deer) ticks found primarily in the northeastern and upper midwestern United States (*2*). *B. burgdorferi*–infected ticks have also been recovered in portions of northern Illinois but have not yet been reported in the Chicago region (*3*). In fact, a previous survey (*4*) of forested areas in the heavily populated regions immediately adjacent to Chicago confirmed a low risk of contracting Lyme disease. Researchers failed to recover deer ticks from 7 sampling sites and recovered only a single *Borrelia* isolate from well-described *B. burgdorferi* sensu stricto rodent reservoirs (mice, voles, chipmunks) captured from 5 sampling sites. A subsequent genetic analysis confirmed the isolate was *B. bissettii* (*5*), a genomic group that is likely nonpathogenic to humans in the United States.

However, the area of the Midwest where Lyme disease is endemic has continued to expand from its origin in northeastern Minnesota and northwestern Wisconsin (*2*,*6*). The Chicago metropolitan region has numerous parks and natural areas that are biologically similar to the known midwestern focus (*7*), and these areas support a large population of white-tailed deer. Moreover, we have seen anecdotal reports of persons with clinical signs and symptoms of Lyme disease and epidemiologic evidence that suggests local acquisition. Two ticks submitted by a resident who had hiked

in DuPage County near the east branch of the DuPage River were *I. scapularis*. We therefore began searching for questing ticks in this area and 9 other geographically diverse areas of DuPage County. The 10 sites were flagged by dragging white cotton sheets through the underbrush for 2- to 10-hour intervals during the spring of 2005. We timed these collections on the basis of information that adult deer ticks were questing in Wisconsin. Recovered ticks were placed in sealed vials and transported to the North Park University Laboratory where their identity was confirmed. The midguts were then removed aseptically, and each was placed into a separate vial that contained 2 mL modified Barbour-Stoenner-Kelly medium that could support the growth of small numbers of *B. burgdorferi* sensu stricto (*8*). Cultures were incubated for 6 weeks at 35°C and examined weekly for spirochetes by darkfield microscopy.

Deer ticks were not found at 8 of the DuPage County sample sites. However, 90 adult or nymphal *I. scapularis* were collected from the remaining 2 sites, and spirochetes were recovered from 3 (3%) of the tick midgut cultures. Because of these findings and anecdotal reports of deer ticks in Cook County, we also surveyed 3 sites in Cook County during the fall of 2005. Deer ticks were not found at 2 sites, but 37 adult *I. scapularis* ticks were collected from a site in the southwestern portion of Cook County, and spirochetes were recovered from 2 (5%) cultures.

We first examined the protein profiles to identify the spirochetes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analyses of 4 of the 5 isolates (1 isolate lost viability before analysis) showed that they were distinctly different from the *B. bissettii* isolate recovered previously from Cook County (*4*), but the isolates were strikingly similar to *B. burgdorferi* sensu stricto (Figure). We then amplified an intergenic spacer region of the *rrf-rrl* portion of the rRNA from 2 isolates by a previously described method (*9*) and sequenced the amplified products (sequencing by Laragen, Inc., Los Angeles, CA, USA). The sequences were identical to that of *B. burgdorferi* sensu stricto isolate B-31 (*9*).

The results confirmed that *I. scapularis* ticks infected with *B. burgdorferi* sensu stricto were recovered from forested areas surrounding Chicago. Additional studies to define the extent and severity of the risk are necessary, but clinicians and the public should be aware of the possibility of acquiring Lyme disease in the Chicago metropolitan region.

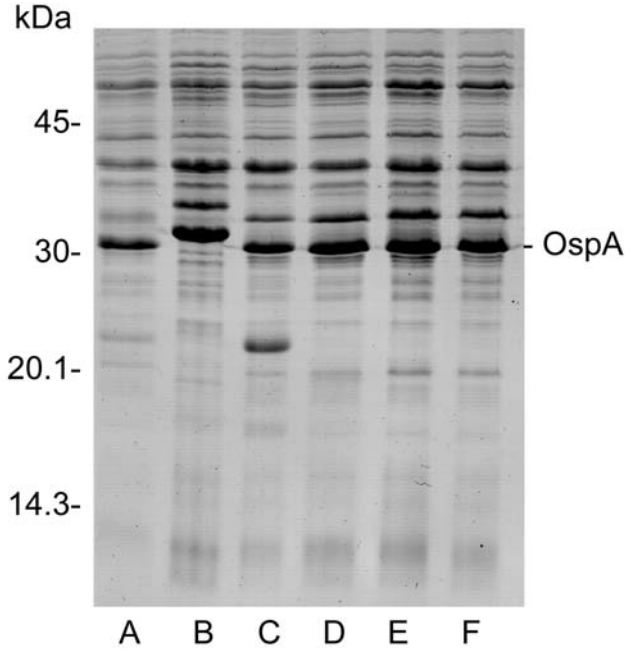

Figure. Protein profiles of *Borrelia burgdorferi* sensu stricto (lane A), *B. bissettii* (lane B), and spirochetes from *Ixodes scapularis* ticks collected from DuPage County (lanes C, D) or Cook County (lanes E, F), Illinois.

Acknowledgments

We thank Joan Bestudik and Linn D. Haramis for initiating the project; John and Tammie Bouseman, Mike O'Driscoll, Curt Colwell, Barb O'Meara, Peter Gianakis, Rachael Griffin, and our 16 student participants for fieldwork; John Bouseman for tick identification; and Steve Callister for guidance and manuscript review.

Partial funding for this work was received from the DuPage County Health Department (to J.A.N.).

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References

1. Auwaerter PG, Aucott J, Dumler JS. Lyme borreliosis (Lyme disease): molecular and cellular pathobiology and prospects for prevention, diagnosis, and treatment. Expert Rev Mol Med. 2004;6:1–22.
2. Centers for Disease Control and Prevention. Lyme disease—United States, 2001–2002. MMWR Morb Mortal Wkly Rep. 2004;53:365–8.

3. Nelson JA, Bouseman JK, Kitron U, Callister SM, Harrison B, Bankowski MJ, et al. Isolation and characterization of *Borrelia burgdorferi* from Illinois *Ixodes dammini*. J Clin Microbiol. 1991;29: 1732–4.
4. Callister SM, Nelson JA, Schell RF, Jobe DA, Bautz R, Agger WA, et al. Survey for *Ixodes* spp. and *Borrelia burgdorferi* in southeastern Wisconsin and northeastern Illinois. J Clin Microbiol. 1991;29:403–6.
5. Nardelli DT, Cloute JP, Luk KHK, Torrealba J, Warner TF, Callister SM, et al. CD4+ CD25+ T cells prevent arthritis associated with *Borrelia* vaccination and infection. Clin Diagn Lab Immunol. 2005;12:786–92.
6. Jackson CA, Lovrich SD, Agger WA, Callister SM. Reassessment of a midwestern Lyme disease focus for *Borrelia burgdorferi* and the human granulocytic ehrlichiosis agent. J Clin Microbiol. 2002;40:2070–3.
7. Guerra M, Walker E, Jones C, Paskewitz S, Cortinas MR, Stancil A, et al. Predicting the risk of Lyme disease: habitat suitability for *Ixodes scapularis* in the north central United States. Emerg Infect Dis. 2002;8:289–97.
8. Callister SM, Case KL, Agger WA, Schell RF, Johnson RC, Ellingson JL. Effects of bovine serum albumin on the ability of Barbour-Stoenner-Kelly medium to detect *Borrelia burgdorferi*. J Clin Microbiol. 1990;28:363–5.
9. Postic D, Ras NM, Lane RS, Henderson M, Baranton G. Expanded diversity among Californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN 127). J Clin Microbiol. 1998;36:3497–504.

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H5N1 Influenza A Virus and Infected Human Plasma

To the Editor: Since January 2004, a total of 22 persons have been confirmed infected with avian influenza A virus (H5N1) in Thailand; 14 of these patients died. Three waves of outbreaks occurred during the past 2 years. The last patient of the third wave was a 5-year-old boy whose symptoms developed on November 28, 2005; he was hospitalized on December 5 and died 2 days later. The child resided in the Ongkharak District, Nakhon Nayok Province, 70 km northeast of Bangkok. Villagers informed the Department of Livestock after the patient's illness was diagnosed. Five dead chickens had been reported in this area from November 28 to December 1, 2005. Samples from these chickens could not be obtained, thus, no H5N1 testing was performed. The boy had fever, headache, and productive cough for 7 days before he was admitted to the Her Royal Highness Princess Maha Chakri Sirindhorn Medical Center. Clinical examination and chest radiograph showed evidence of lobar pneumonia. He was treated with antimicrobial drugs (midecamycin and penicillin G) and supportive care, including oxygen therapy. On December 7, the patient's condition worsened, and severe pneumonia with adult respiratory distress syndrome developed. Laboratory tests showed leukopenia (2,300 cells/mm^3), acidosis, and low blood oxygen saturation by cutaneous pulse oximetry (81.6%). Oseltamivir was administered after his parents informed hospital staff about the boy's contact with the dead chicken. However, the boy died the same day; no autopsy was performed. On December 9, the cause of death was declared by the Ministry of Public Health to be H5N1 influenza virus.

A blood sample was collected from the patient on December 7; anticoagulation was accomplished with ethylenediaminetetraacetic acid (EDTA) for repeated biochemistry analysis and complete blood count. The plasma from the EDTA blood sample was separated 2 days later and stored at –20°C for 12 days. The sample was subsequently given to the Center of Excellence in Viral Hepatitis, Faculty of Medicine, Chulalongkorn University, for molecular diagnosis and then stored at –70°C, where specific precautions implemented for handling highly infectious disease specimens such as H5N1 influenza virus were observed. Plasma was examined by multiplex reverse transcription–polymerase chain reaction (RT-PCR) (*1*) and multiplex real-time RT-PCR (*2*), both of which showed positive results for H5N1 virus. The virus titer obtained from the plasma was 3.08×10^3 copies/mL. The plasma specimen was processed for virus isolation by embryonated egg injection, according to the standard protocol described by Harmon (*3*). Briefly, 100 μL 1:2 diluted plasma was injected into the allantoic cavity of a 9-day-old embryonated egg and incubated at 37°C. The infected embryo died within 48 hours, and the allantoic fluid was shown to contain 2,048 hemagglutinin (HA) units; also, subtype H5N1 was confirmed (*1*,*2*). Whole genome sequencing was performed and submitted to the GenBank database under the strain A/Thailand/NK165/05 accession no. DQ 372591-8. The phylogenetic trees of the HA and neuraminidase (NA) genes were constructed by using MEGA 3 (*4*) for comparison with H5N1 viruses isolated from humans, tigers, and chickens from previous outbreaks in 2004 and 2005 (Figure). The sequence analyses of the viruses showed that the HA cleavage site contained SPQRERRKKR, which differed from the 2004 H5N1 virus by an arginine-to-lysine substitution at posi-

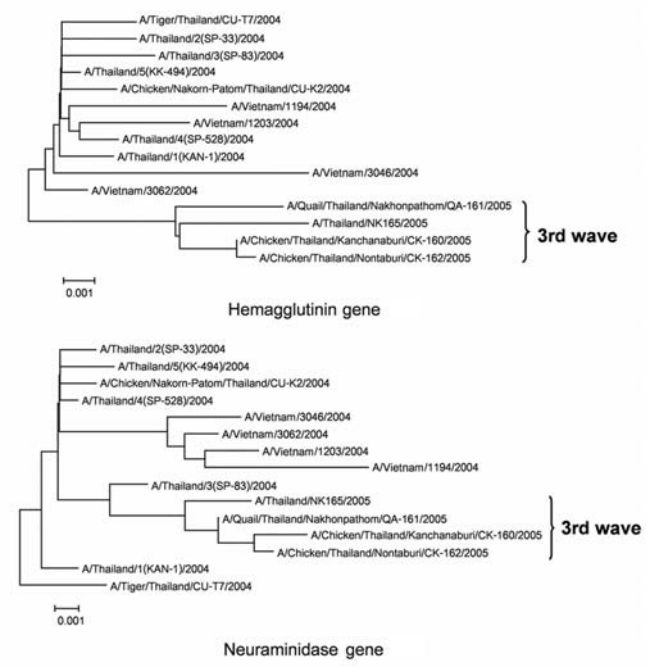

Figure. Phylogenetic analysis of the hemagglutinin and neuraminidase genes of H5N1 from study patient compared with sequences from previous outbreaks (2004–2005).

tion 341. That finding had also been observed in wild bird species during earlier outbreaks in Thailand in 2004 (*5*). Similar to the 2004–2005 H5N1 isolates from Thailand, a 20–amino acid deletion at the NA stalk region was observed. Moreover, the amino acid residues (E119, H274, R292, and N294) of the NA active site were conserved, which suggests that the virus was sensitive to oseltamivir. In addition, a single amino acid substitution from glutamic acid to lysine at position 627 of PB2 showed increased virus replication efficiency in mammals (*6*).

Observing live influenza virus in human serum or plasma is unusual. However, in 1963, low quantities of virus were isolated from blood of a patient on day 4 of illness (*7*), and in 1970, the virus was cultivated from blood specimens from 2 patients (*8*). Recently, a fatal case of avian influenza A (H5N1) in a Vietnamese child was reported. The diagnosis was determined by isolating the virus from cerebrospinal fluid, fecal, throat, and serum specimens (*9*); viral RNA was found in 6 of 7 serum specimens 4–9 days after the onset of illness (*10*). In this case, the H5N1 virus could be isolated from plasma on day 10 after symptoms developed. This case showed the virus in the patient's blood, which raises concern about transmission among humans. Because probable H5N1 avian influenza transmission among humans has been reported (*11*), this case should be a reminder of the necessity to carefully handle and transport serum or plasma samples suspected to be infected with H5N1 avian influenza. Because viable virus has been detected in blood samples, handling, transportation, and testing of blood samples should be performed in a biosafety (category III) containment laboratory to prevent the spread of the virus to healthcare and laboratory workers.

We express our thanks to the Thailand Research Fund (Senior Research Scholar), Royal Golden Jubilee PhD Program and Center of Excellence in Viral Hepatitis Research, and Prasert Auewarakul for their generous support of our study.

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References

1. Payungporn S, Phakdeewirot P, Chutinimitkul S, Theamboonlers A, Keawcharoen J, Oraveerakul K, et al. Single-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol. 2004;17:588–93.
2. Payungporn S, Chutinimitkul S, Chaisingh A, Damrongwantanapokin S, Buranathai C, Amonsin A, et al. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J Virol Methods. 2005;131:143–7.
3. Harmon MW. Influenza virus. In: Lennette EH, Smith TF, editors. Laboratory diagnosis of viral infection. 3rd ed. New York: Marcel Dekker, Inc.; 1999. p. 587–601.
4. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 2004;5: 150–63.
5. Keawcharoen J, Amonsin A, Oraveerakul K, Wattanodorn S, Papravasit T, Karnda S, et al. Characterization of the hemagglutinin and neuraminidase genes of recent influenza virus isolates from different avian species in Thailand. Acta Virol. 2005;49:277–80.
6. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology. 2004;320: 258–66.
7. Naficy K. Human influenza infection with proved viremia: report of a case. N Engl J Med. 1963;269:964–6.

8. Lehmann NI, Gust ID. Viraemia in influenza. A report of two cases. Med J Aust. 1971;2:1166–9.
9. de Jong MD, Cam BV, Qui PT, Hien VM, Thanh TT, Hue NB, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med. 2005;352:686–91.
10. Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5. Avian influenza A (H5N1) infection in humans. N Engl J Med. 2005;353:1374–85.
11. Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med. 2005;352:333–40.

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Infectious Diseases: A Clinical Approach, 2nd Edition

Allen Yung, Malcolm McDonald, Denis Spelman, Alan Street, Paul Johnson, Tania Sorrell, and Joe McCormack, editors

IP Communications, East Hawthorn, Victoria, Australia, 2005

ISBN: 095786177X
Pages: 602; Price: Australian $130.00 (includes CD-ROM)

The recent award of the Nobel Prize to Barry Marshall and J. Robin Warren for their discovery of *Helicobacter pylori* and its role in gastritis and peptic ulcer disease has again put infectious diseases on to the front page of newspapers around the world. Notably, their initial work was performed in Perth, Australia. The clinical practice of infectious diseases, and associated research into microorganisms, has long been established in Australia. This book capitalizes on the expertise of infectious disease physicians and microbiologists in Australia and surrounding regions. Infectious Diseases: A Clinical Approach is written from an Australian perspective but has potential for a global audience.

The book's first 2 parts deal with a general, clinical approach to the patient with a possible infection. This section is full of clinical "pearls," particularly relating to the patient with undifferentiated fever. Some sections, such as "Pitfalls in the Diagnosis of Serious Bacterial Infection, Especially Meningococcemia" or "Reasons Not To Treat Fever," are a joy to read and could be posted on the walls of emergency departments or general medical wards in every hospital. These sections are of relevance to internists and infectious disease fellows, but for a seasoned infectious diseases physician, they may not offer anything new.

Experienced infectious disease physicians, however, are likely to gain new insights into infections occurring in Asia, Australia, and the southwest Pacific region. A patient vacationing one day in Fiji or Tahiti may be visiting the emergency department of a hospital in North America or Europe the next day. While most physicians might search the Centers for Disease Control and Prevention website to find what infections are endemic in the region in question, or whether outbreaks have recently been reported there, this book provides context from authors working in the regions they describe. The clinical evaluation of infections in returned travelers and immigrants is also particularly well covered.

As an infectious disease physician working in the United States, I would not purchase this book to find detailed information on specific infections or specific antimicrobial agents. This is a book I would purchase for a first-year fellow or a resident who has enjoyed rounding with the infectious diseases service. The book is a gold mine of clinical advice based on obtaining a detailed history and performing a thorough physical examination. It is full of advice on "traps for young players" and ends with 28 golden rules of infectious disease practice. The book is dedicated to Richard Kemp, an infectious disease physician, who tragically died at the age of 50. Kemp was renowned as a clinician and would have undoubtedly endorsed the practical approach to infectious disease management advocated in this book.

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Albrecht Dürer (1471–1528). Self-Portrait with Sea Holly (1493). Parchment mounted on canvas (56 cm × 44 cm) Photo: Arnaudet. Réunion des Musées Nationaux/Art Resource, NY. Louvre, Paris, France

"Il Faut Cultiver Notre Jardin"[1]

Polyxeni Potter*

"This I drew, using a mirror; it is my own likeness, in the year 1484, when I was still a child," wrote Albrecht Dürer about his first self-portrait, sketched with great confidence and skill when he was 13 years old (*1*). Fascinated by his own image, he continued to paint it throughout his life. His faithful dog once barked and wagged its tail at a newly completed self-portrait of his master, related his friend humanist scholar Konrad Celtis (1459–1508), repeating a story based on the anecdote from Roman writer Pliny (*1*).

Though born into the goldsmith trade on his father's side of the family, Dürer also apprenticed with painter and woodcut illustrator Michael Wolgemut, who introduced him to commercial bookmaking. At age 19, he left his native Nürnberg to wander the world and improve his skills, as was the custom after successful apprenticeships. In Basel, Strasbourg, Vienna, and Venice, he came to know and excel in diverse styles and techniques. A painter, as well as master printmaker and engraver, he was the first serious artist to work in watercolor. His landscapes, painted directly from nature, were matched in his day only by those of Leonardo da Vinci.

"The art of measurement being the foundation of all painting," he wrote, "I propose to give the elements thereof and to explain its principles to young people wishing to educate themselves in their art, so that they may confidently start measuring with a pair of compasses and ruler, thereby recognizing and having before their eyes the genuine truth…." (*2*). An artist working by the rule of thumb, without theoretical foundation, Dürer maintained, was "a wild, unpruned tree," in need of the objective and rational standards of the Renaissance (*3*).

These standards, acquired during his travels abroad, particularly Italy, and brought back to Germany, he also embraced in his personal life. A mathematician and humanist, he sought knowledge across diverse fields. An innovator and tradesman, he applied his artistic skills to new technologies and the production of high quality prints for the open market. His radical techniques caught the

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[1]We must cultivate our garden. From Voltaire's Candide

attention of the intellectual elite of his day. His closest friend and mentor was one of Nürnberg's most distinguished scholars, Willibald Pirckheimer, translator of Hellenic texts into Latin and German. Dürer's copper plate portrait of Pirckheimer, created from printed graphics, contained the inscription "Man lives through his intellect; all else will belong to death" (*4*).

"What beauty is I know not," Dürer pondered in his Book on Human Proportions. "In some things we consider that as beautiful which elsewhere would lack beauty." Beauty as the goal of art continued to excite his imagination and later featured in his treatises on measurement and fortification. His drawings after Andrea Mantegna and Antonio Pollaiuolo reflected his appreciation of Italian painting, which at its peak was guided by knowledge of geometry and proportion. Near the end of his life, Mantegna heard that Dürer was in Italy and sent for him, "in order to instruct Albrecht's facility and certainty of hand in his own understanding and skill" (*5*). But Mantegna died before Dürer could reach Mantua, "the saddest event in all my life" (*5*).

"There, where the yellow spot is located, and where I point my finger, there it hurts," Dürer wrote on a pen and watercolor self-portrait he sent to a physician for consultation (*6*). This half-length portrait, The Sick Dürer (1471–1528), likely described the painter's illness contracted during travel to the Netherlands in 1520. "In the third week after Easter I was seized by a hot fever, great weakness, nausea, and headache," he wrote in his diary, "And before, when I was in Zeeland, a strange sickness came over me, such as I have never heard of from any man, and I still have this sickness" (*7*). In between periods of good health, the fever periodically returned until his death at age 56.

Self-portrait with Sea Holly, on this month's cover, painted when Dürer was 22, is the earliest known free-standing self-portrait. Hands are difficult to paint from a mirror image. These (cover detail) are rough, a painter's hands, cracked and smudged. Goethe, when he saw a preliminary version of the painting, recognized that they held a sprig of sea holly, a thistle-like plant regarded an aphrodisiac, which appears in other Dürer works. The plant's German name means "fidelity of man," and its presence in this self-portrait could be alluding to the painter's engagement to be married around that time.

Dürer's devotion to knowledge as the means to beauty and truth guided his life. In illness, he enlisted his knowledge of the human body to describe the pain, anticipating pain mapping several centuries later. He grasped that knowledge advances human life, from understanding the nature of beauty to curtailing the scourge of disease.

"He looked like a bundle of dried straw," said Pirckheimer of his dying friend, supporting speculation today that Dürer had malaria, an ancient and continuing scourge (*8*). Newer scourges have now sprung, not the least of them HIV/AIDS, a "rough thistle." Despite substantial progress in prevention and control, emerging HIV/AIDS takes a global toll, particularly among the young, the old, and the underprivileged, "a weed" still requiring of us to "get on our hands and knees and begin clearing… away" (*9*).

References

1. Albrecht Dürer. [cited 2006 Apr]. Available from http://www.artchive.com/artchive/D/durer.html
2. Mettais V. German paintings. In: your visit to the Louvre. Versailles: Art Lys; 1997.
3. Janson HW, Janson AF. History of art. New York: Harry N. Abrams, Inc.; 2001.
4. Willibald Pirckheimer. [cited 2006 Apr]. Available from http://www.kfki.hu/~/arthp/html/d/durer/2/13/5/102.html
5. Doorly P. Dürer's melencolia I: Plato's abandoned search for the beautiful. 2004 Jun 1 [cited 2006 Apr]. Available from http://www.highbeam.com/library/docfreeprint.asp?docid=1G1:118234665&ctr1Info=Round19%3Mode19a%3ADocFree%3A
6. Schott GD. The sick Dürer—a Renaissance prototype pain map. 2004 Dec 18 [cited 2006 Apr]. Available from http://bmj.bmjjournals.com/cgi/content/full/329/7480/1492
7. Hutchison JC. Albrecht Dürer. A biography. Princeton: Princeton University Press; 1990.
8. Roller DHD, editor. Perspectives in the history of science and technology. Norman (OK): University of Oklahoma Press; 1971.
9. Valdiserri RO. Weeds. In: Gardening in clay: reflections on AIDS. Ithaca (NY): Cornell University Press; 1994.

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the July issue for the following topics:

Global Public Health Surveillance under New International Health Regulations

Infectious Causes of Chronic Diseases

Neisseria meningitidis Sequence Type and Risk for Death, Iceland

Tickborne Arbovirus Surveillance in Market Livestock, Nairobi, Kenya

Rodent *Bartonella*-associated Febrile Illness, Rural Southwestern United States

Human Tularemia Caused by Type A and Type B, United States, 1964–2004

Maternal Outcomes in Pregnancy with Smallpox

Triple Reassortant H3N2 Influenza A Viruses, Canada, 2005

First Molecular Identification of Tickborne Encephalitis Virus, Norway and Denmark

Detection of Infectious Poxvirus Particles

European Bat Lyssavirus Type 2 RNA in *Myotis daubentonii*

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

Upcoming Infectious Disease Activities

June 17–22, 2006
Negative Strand Viruses 2006: Thirteenth International Conference on Negative Strand Viruses
Salamanca, Spain
Contact: 404-728-0564 or meeting@nsv2006.org
http://www.nsv2006.org

June 25–29, 2006
ISHAM 2006 (International Society for Human and Animal Mycology)
Palais des Congrès
Paris, France
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

July 24–August 4, 2006
Diagnostic Parasitology Course
Uniformed Services University of the Health Sciences
Bethesda, MD, USA
Contact: 301-295-3139 or jcross@usuhs.mil
http://www.usuhs.mil/pmb/TPH/dpcourse.html

August 6–10, 2006
Advancing Global Health: Facing Disease Issues at the Wildlife, Human, and Livestock Interface
55th Annual Meeting, Wildlife Disease Association with American Association of Wildlife Veterinarians
University of Connecticut
Storrs, CT, USA
Contact: wda.2006@gmail.com
http://www.conferences.uconn.edu/wildlife/

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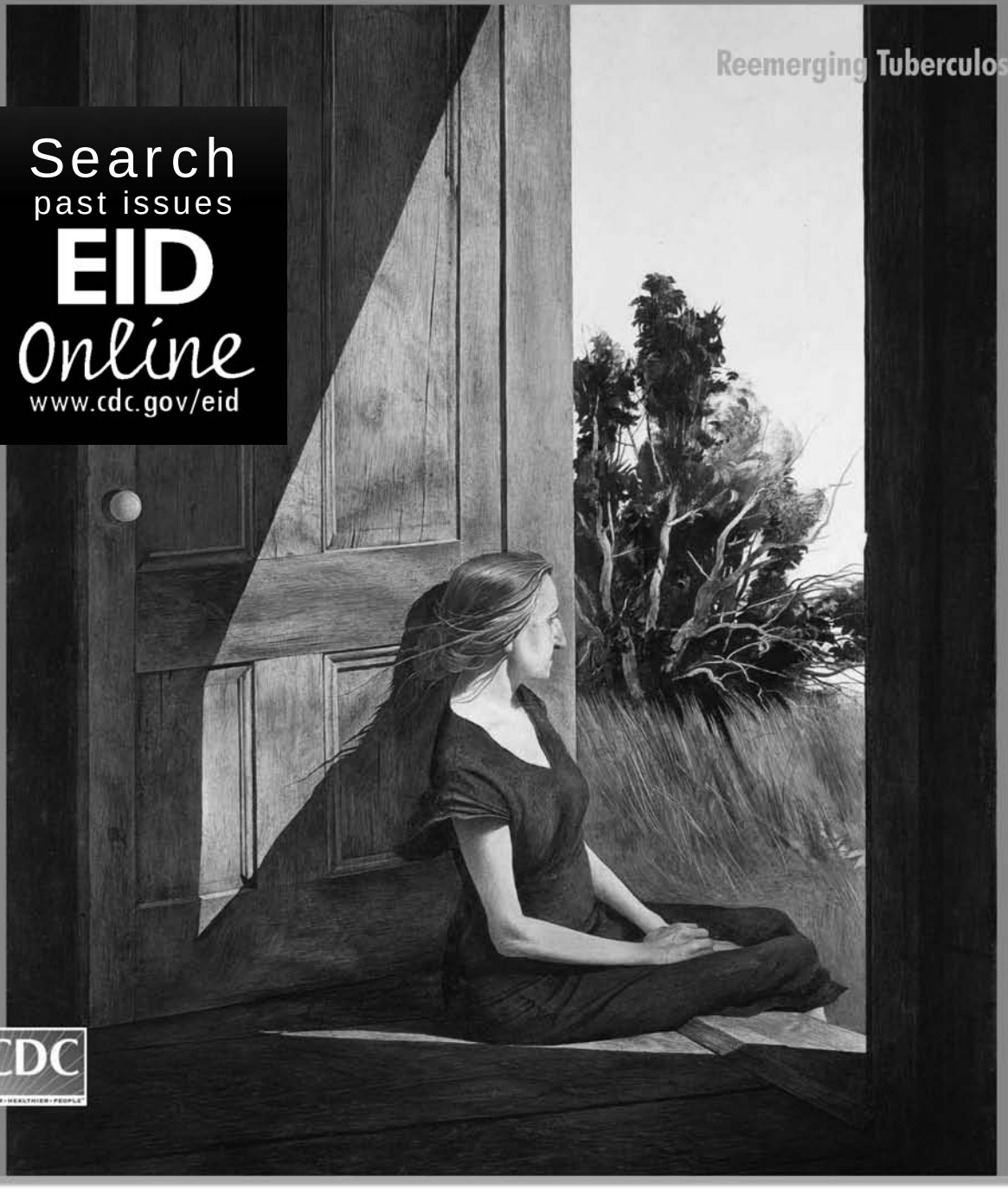

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Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

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

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

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

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

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

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

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

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

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.