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Zoonoses

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On the Cover

Rosa Bonheur (1822–1899)
Plowing in Nivernais (1850)
Oil on canvas
(133.4 cm × 259.1 cm)
SN433 Collection of the John and Mable Ringling Museum of Art, the State Art Museum of Florida, a Division of Florida State University

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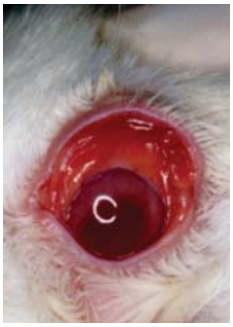

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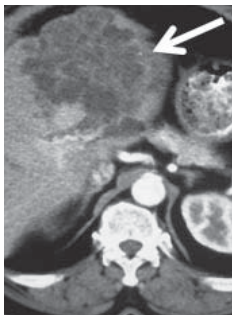

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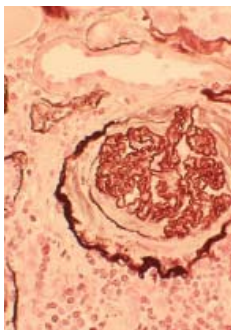

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Towards Control of *Streptococcus iniae*

Justice C.F. Baiano and Andrew C. Barnes

Streptococcus iniae is an emerging zoonotic pathogen; such infections generally occur through injuries associated with preparing whole fresh fish for cooking. Those infected to date have been of Asian descent, are usually elderly (average age 68 years), and have had ≥1 underlying conditions that may predispose them to infection. Studies of the foundations of growth characteristics of *S. iniae* and its interactions with piscine host cells have recently been complemented by molecular studies. Advances in molecular biology have allowed research groups to identify numerous virulence factors and to explore their roles in the progression of *S. iniae* infection. Many of these virulence factors are homologous to those found in the major human pathogen *S. pyogenes.* An increased understanding of the properties of these factors and their effect on the success of infection is leading to novel approaches to control *S. iniae* infection; in particular, vaccination programs at fish farms have reduced the reservoir of infection for additional clinical cases.

Streptococcus iniae is a major fish pathogen in many regions of the world. These bacteria are also zoonotic with infections in humans associated with the handling and preparation of infected fish. The first human infections were reported in 1996 (*1*), and *S. iniae* was noted as an emerging zoonotic disease transmitted by food animals at the International Conference on Emerging Infectious Diseases in 2000 (*2*). Human infections with *S. iniae* have been sporadic but continue to be reported with new cases arising in 2009 (*3*). Reports of these cases are likely to increase because of enhanced awareness, more reliable detection and identification methods, and the global expansion of finfish aquaculture. Most cases of human *S. iniae* infections have been in persons of Asian descent, who are elderly and commonly have ≥1 underlying conditions such as diabetes mellitus, chronic rheumatic heart disease, cirrhosis, or other conditions (*1*,*3*–*7*).

Carrier fish have been implicated in fish-to-fish transmission of *S. iniae* (*8*), and these carriers may be responsible for human infection because fish with overt signs of disease are unmarketable. Soft tissue injuries that occur during the preparation of fresh fish from wet markets usually result in bacteremic cellulitis of the hand, followed by ≥1 of these conditions: endocarditis, meningitis, arthritis, sepsis, pneumonia, osteomyelitis, and toxic shock (*7*). Infections are treated with a course of antimicrobial drugs such as penicillin, ampicillin, amoxicillin, cloxacillin, cefazolin, and/or gentamicin, doxycycline, and trimethoprim/sulfamethoxazole over a period of 1 to several weeks, depending on the nature of the infection (*3*–*5*,*9*). *S. iniae* is not currently assigned to any Lancefield group and is β-hemolytic on blood agar, with some clinical strains isolated from Asia being more mucoid than others (*6*).

Underreporting of human cases is likely because identification of *S. iniae* is based on biochemical testing of isolates with commercial kits; the use of kits is associated with problems because *S. iniae* is not listed in commercial or clinical databases, and many atypical strains are assigned low matches (*1*,*4*). According to the Australian Institute of Health and Welfare (www.aihw.gov.au), between 1999–2000 and 2006–2007, a total of 2,824 cases of "other" or "unspecified" streptococcal sepsis required hospitalization in Australia that were attributed to nongroup A, B, or C streptococci, or *S. pneumoniae,* and 2,026 cases were in persons ≥50 years of age. During the same period, the trend in the number of cases per year attributable to other or unspecified streptococci has been upward, rising from a total of 278 cases in 1999–2000 to 430 in 2006–2007 (155% increase). In the ≥50 years age group, this upward trend is more pronounced, with a 168% increase in cases requiring hospitalization. It is therefore probable that some cases of *S. iniae* infection in Australia in the at-risk age group have

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been misidentified. Misidentification of *S. iniae* infection is likely to be the main reason for low levels of detection because most cases of this emerging pathogen are detected during retrospective studies specifically targeting *S. iniae*. This finding is likely to be the case in countries around the world that have reported outbreaks in fish farms, but no human cases to date. Molecular-based detection and identification methods have recently been developed (information on this aspect of identification can be found in a recent review by Agnew and Barnes) and these will lead to improved reporting in future years (*10*).

Observations on the epidemiology and pathogenesis of *S. iniae* infections are still ongoing; however, valuable information on the differentiation of strains (as being either commensal or pathogenic) has benefitted research. Because of the lack of potential virulence factors or phenotypic differences between commensal and pathogenic strains, pulsed-field gel electrophoresis (PFGE) analysis showed that differences existed between human clinical isolates and those from fish surfaces (*1*). The human clinical isolates showed little variation between one another, while considerable differences were found between the 2 American Type Culture Collection (Manassas, VA, USA) dolphin strains and 32 other fish isolates. It was determined that some unknown factors important to pathogenicity were not present in all strains (*1*). Little variation has also been found between clinical strains from the United States and Canada, although 1 strain from Pennsylvania had a PFGE pattern similar to the type strain (*11*), and 2 clinical isolates from Hong Kong were unrelated to a strain from Canada (*4*). In a PFGE study of isolates from Australia, similarities between fish pathogenic isolates and human clinical isolates from North America, in addition to multiple genotypes between and within different fish farms, were reported (*12*). Another PFGE study of strains from a variety of species of diseased fish in the People's Republic of China found that there were 17 genotypes from 27 strains clustering into 5 major groups (*13*). As with the study from Australia, multiple genotypes were found between and within different farms.

Clinical isolates from the United States were able to multiply by 2 to 5 generations in 3 hours in fresh human blood; however, 2 isolates from Canada were able to survive, but not multiply, in human blood (*11*). Resistance to phagocytic killing in whole blood by pathogenic strains of *S. iniae* contrasted with isolates identified as commensal strains that were susceptible (*9*). The pathogenic strain 9117 (a human clinical isolate causing cellulitis) caused weight loss in experimentally infected mice and was highly cellulytic to human brain microvessel endothelial cell (BMEC) monolayers and invasive of Hep-2 cells. However, adherence to, and invasion of, BMEC cells by strain 9117 was lower than that for commensal strain 9066 (obtained in a swab sample from a healthy fish) (*9*). In a similar study, pathogenic strains were more resistant to oxidative burst activity in macrophages (*14*).

The mode of invasion of *S. iniae* was studied in skin epithelial cell monolayers of rainbow trout viewed under polarized light (*15*). *S. iniae* adhered to, then invaded epithelial cells, but its persistence and replication inside the cells was short-lived. However, transcytosis from epithelial cells occurred within 30 minutes of contact without damaging the cells or cellular junctions (*15*). Once the epithelial cell layer had been breached, dissemination throughout the fish through internalization in macrophages can occur. By inducing macrophage death, this process is one of the most efficient ways of transporting the infection into the brain (*15*,*16*).

Recent molecular research into factors that contribute to the virulence of *S. iniae* has identified several candidates, including surface proteins, capsular polysaccharides, and extracellular secreted products (Figure). Moreover, the recent sequencing of the complete genome of *S. iniae* will accelerate the discovery of additional virulence factors and lead to identification of targets for effective vaccines for farmed fish, thus reducing the potential for zoonotic infection. In light of the recent rapid increase in our knowledge of this emerging pathogen, we will present a synopsis of the processes involved in infection that have been elucidated to date.

Virulence Factors

SiM Protein

M proteins are one of the major virulence factors in group A streptococci (*S. pyogenes*; GAS). The high level of diversity of *emm* gene types (a hypervariable gene encoding the M protein) has contributed to the success of GAS in causing infections in humans. The M-like protein from *S. iniae*, SiM, is also a prime candidate in virulence (*17*,*18*). The SiM protein is a coiled-coil protein that has a molecular mass of ≈53 kDa, although 2 other variants, one with a 1-aa insertion between the coils and another with a much larger mass of ≈59 kDa, have also been described (*17*). An additional variant had a natural frameshift/premature termination of the SiMA1 type in a strain from an infected tilapia (*18*). All SiM proteins possess the classical gram-positive membrane anchor motif LPXTG, and although they have several repeat motifs in the coil regions, they are not in tandem as in M proteins from other species. In common with M proteins from *S. pyogenes*, *S. equi,* and *S. dysgalactiae*, SiM protein is a surface protein that binds human fibrinogen to protect the bacterium from phagocytic activity (*17*). SiM proteins may also bind trout immunoglobulin by the Fc region (*19*). An allelic exchange study showed that SiM protein is a major virulence factor of *S. iniae*, contributes to adherence to fish epithelial cells (*18*), and also confirmed

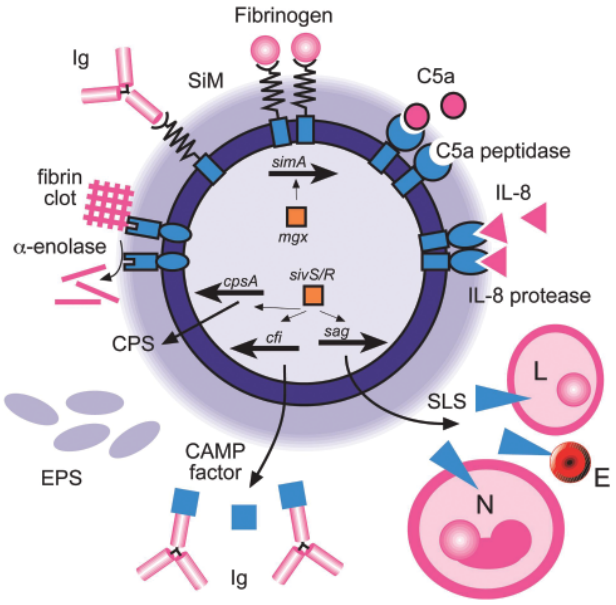

Figure. Virulence factors of *Streptococcus iniae*. A diagrammatic representation of a cell of *S. iniae* showing the regulatory genes involved in virulence factor expression (inside cell) and the virulence factors on the outside of the cell. In a clockwise direction, SiM protein (*simA*) expression is likely to be regulated by *mgx*. SiM protein binds immunoglobulin (Ig) and fibrinogen. C5a peptidase and interleukin-8 (IL-8) protease degrade their respective chemokines to impair phagocyte signaling. Production of the cytolysin streptolysin S (*sag*; SLS) is regulated by the *sivS/R* system. SLS lyses lymphocytes (L), erythrocytes (E), and neutrophils (N). The CAMP factor gene, *cfi,* is also regulated by *sivS/R* and is known to bind immunoglobulin by the Fc region. Capsular polysaccharide (*cpsA*; CPS) synthesis is controlled by *sivS/R* and is represented by a haze around the cell. Exopolysaccharide (EPS) is produced in excess and contributes to highly viscous growth. α–enolase degrades fibrin clots and promotes dissemination.

earlier observations that SiM contributes to macrophage resistance (*17*).

The SiM protein genes, *simA* and *simB*, are likely to be regulated by a multigene regulatory protein, Mgx, that is homologous to the Mga protein found in GAS (*17*). There are sequence elements upstream of the *simA* and *simB* genes that are similar to DNA binding sites described for Mga. The finding of a second *mga*-like element (*mgx2*) immediately downstream of *mgx* in a tilapia brain abscess isolate may represent part of an alternative virulence strategy (*18*).

C5a Peptidase

C5a peptidase hydrolyses the neutrophil chemoattractant complement factor C5a (*18*) and thus impairs the ability of the infected host to fight the infection. C5a peptidase is a surface protein with a LPXTN gram-positive anchor motif (*18*). In GAS, C5a peptidase is found in culture supernatants; however, this observation has not been made for *S. iniae* (*18*). In *S. iniae*, C5a peptidase is a 123-kDa protein, encoded by *scpI*, with similar structural features and conserved residue positions to the GAS counterpart (*18*). C5a peptidase may have arisen in *S. iniae* by horizontal gene transfer, given its close proximity to a transposase and similar genetic organization found in GAS (*18*). Notably, allelic exchange has shown that this protein by itself is not required for virulence in fish and its role in pathogenesis is likely a minor one (*18*).

Interleukin-8 Protease

Interleukin-8 (IL-8) is produced in the host in response to stimuli such as lipopolysaccharides, viruses, and other cytokines. IL-8 protease is a cell envelope protease that is able to degrade the chemokine IL-8 and results in increased neutrophil resistance and disease dissemination (*20*). It is encoded by the *cepI* gene, resulting in a 1,631-aa protein with a C-terminal LPXTG gram-positive anchor motif and is homologous to the *cepA* gene in GAS (*20*).

Streptolysin S

The ability of *S. iniae* to hemolyse erythrocytes and damage host cell membranes results from the activity of cytolysins (*21*). The cytolysin possessed by *S. iniae* is homologous to streptolysin S (SLS) from GAS (*21*) and affects erythrocytes, neutrophils, lymphocytes, and some tissue types in tissue culture (*22*), but does not have roles in phagocytic resistance nor epithelial cell adherence and invasion (*23*). Nine genes in the *sag* operon are involved in SLS formation; these share 73% homology with GAS SLS genes (*21*). The number of genes and their genetic order are identical in both microorganisms (*21*). The *sagA* gene encodes a peptide that is 73% identical to the sagA protein from GAS, and the *sagB* gene encodes a protein with 77% identity to the sagB protein from GAS, which is predicted to use flavin mononucleotide as a cofactor (*21*). The *sagC-F* genes are similar to their counterpart genes in GAS, and the *sagG-I* genes encode ATP binding cassette-type (ABC) transport systems (*21*). Other sequence features, such as inverted repeats between the *sagA* and *sagB* genes and after the *sagI* gene, have similarities with GAS *sag* operon genes (*21*). All genes in the operon are required to produce SLS as knockout of *sagB* in *S. iniae* caused loss of hemolytic activity (*21*). Likewise, when the *S. iniae sagA* gene was transformed into a nonhemolytic allelic mutant strain of GAS (NZ131 *sagAΔcat*), the *S. iniae* version of the *sagA* gene restored hemolytic activity (*21*,*23*). The cytotoxic properties of SLS toward fish cells and the likely promotion of cerebrovascular trauma represent a major virulence factor in the pathogenesis of *S. iniae* (*23*).

The *sagA* gene in *S. iniae* is regulated by a 2-component signal transduction system called *sivS/R* (*24*). *sivS/R* regulates virulence in vivo because no deaths occurred in

a mouse infection model when mice were infected with a deletion mutant of pathogenic strain 9117 (9117Δ*siv*) compared with 75% deaths when mice were infected with the wild type strain (*24*). In the *siv* deletion mutant, *sagA* expression was decreased by 3-fold. *sivS/R* also regulates the expression of surface proteins, including a lipoprotein/ABC transporter homologous to Spy1228, pyruvate kinase, and a hyaluronate-associated protein homologous to that from *S. equi* or an ABC transporter from *S. agalactiae* (*24*). However, the role for these proteins in a virulence setting is hitherto unknown in *S. iniae*.

CAMP Factor

The pore-forming toxin CAMP factor synergistically acts with sphingomylinase-producing *Staphylococcus aureus* to produce a distinct arrow-shaped area of complete lysis of erythrocytes on sheep blood agar (*24*). CAMP factor has also been shown to bind immunoglobulin by the Fc region and therefore contributes to virulence (*24*). *S. iniae* harbors a CAMP factor–like gene, *cfi*, (*24*) that encodes a peptide of ≈27 kDa and shares 62% identity with *cfa* from GAS that is regulated by the *sivS/R* system. Knockout mutants of the *sivS/R* system resulted in a reduced lytic reaction, and real-time PCR analysis of *cfi* gene expression showed it was expressed at only 10% of wild-type levels (*24*).

Immunoglobulin-Binding Proteins

A putative protein G-like protein, a cell wall associated protein first identified from group G streptococci (≈70 kDa), from *S. iniae* was capable of binding trout immunoglobulin only when grown in the presence of trout serum (*19*). Proteins of ≈35 kDa, ≈70 kDa, and >100 kDa were found to bind trout immunoglobulin. The size of one of the detected bands >100 kDa is similar in size to the tetramer formation of SiM proteins (*17*); however, experimental evidence is needed to confirm this. CAMP factor is also known to bind immunoglobulin (*24*).

Capsule

One of the most effective ways for a bacterium to avoid phagocytosis is by the production of capsular polysaccharide (CPS), and strains with CPS are more virulent than their unencapsulated counterparts (*25*–*27*). The presence of capsule is also involved in inhibiting complement C3 deposition (*27*). Miller and Neely (*27*) used signature tagged mutagenesis to identify virulence genes using a zebrafish (*Danio rerio*) model. Five attenuated mutants with unique insertions in polysaccharide synthesis genes with homology to those found in *S. thermophilus* plus 2 additional clones with insertions in a homologous gene near capsule synthesis genes from *Bacteroides thetaiotaomicron* were found. In contrast to the wild-type strain 9117, these mutants aggregated in broth culture with chain lengths up to 4× longer than the wild type. Differences in buoyancy due to degree of encapsulation showed that the production of excess capsule is likely to be as detrimental to survival as too little capsule (*27*).

The capsule operon of *S. iniae* is ≈21 kb in size and consists of 21 open reading frames (*28*). The genes have homology to genes found in other streptococci such as *S. pyogenes, S. agalactiae, S. suis,* and *S. thermophilus* (*28*). An insertion sequence, IS*981*, was found between *cpsL* and *cpsM* in strain 9117 (*28*). *cpsY* (78% identity to CpsY in GAS) precedes *cpsA* and is transcribed in the opposite direction and is thought to be the promoter of the capsule operon (as well as other virulence genes) because it has a high level of homology to the LysR transcriptional regulators (*28*). A major difference was found in the operons from the virulent strain 9117 and commensal strain 9066 with strain 9066 having an ≈10-kb deletion missing the genes *cpsF-L* and *orf276, orf193*, and *orf151*. In addition, the *cpsM* gene in the commensal strain 9066 was truncated with the first 154 nt being absent, which casts doubt on the functionality of the gene (*28*).

The genes *cpsA-E* are responsible for the length of the monosaccharide sugar chains and their export (*29*,*30*). The central region of the operon from *cpsF* to *cpsL* contains several genes encoding glycosyl transferases, which have a role in the polymerization of the capsule chain (*28*). This region is where most mutations in the virulent strain 9117 were found to play a role in pathogenesis (*27*) and is the same region where the deleted genes in the commensal strain 9066 occurred (*28*). The G + C content of the operon genes varies widely from 22% to 40% (*28*). The *cpsF-L* region has a G + C content of ≈27%, which is lower than that found for other *S. iniae* sequences and may indicate horizontal acquisition from other members of the *Firmicutes* (*28*).

Mutations in *cpsA* in strain 9117 resulted in long chain formation, aggregation of cells in broth culture and high buoyancy characteristic of reduced encapsulation (*28*). The *cpsA* mutant was attenuated in both brain and heart tissues. A *cpsY* mutant was slightly less encapsulated than the wild type and was attenuated only in heart tissue (*28*).

Allelic exchange of the *cpsD* gene resulted in reduced capsule, increased chain length, a marked decrease in all capsular monosaccharides, and a high degree of attenuation (*31*). *cpsD* encodes an autophosphorylating tyrosine kinase thought to be responsible for capsule polymerization and export (*31*). *cpsD* mutants were able to bind more effectively to host tissues, such as epithelial cells, due to loss of overall negative charge (*31*). Insertions in the *cpsH* and *cpsM* genes have resulted in underproduction of capsule and overproduction of capsule, respectively (*28*). In a flounder isolate obtained in Japan, insertions in *cpsH*, *cpsM*, *cpsI*,

and *orf276* of strain NUF631 resulted in attenuation, which was measured by increased chemiluminescence response of macrophages and loss of acidic polysaccharides (*26*). Like CAMP factor and streptolysin S, the 2-component system *sivS/R* is involved in transcriptional regulation of the capsular operon (*32*).

Phosphoglucomutase

Also involved in capsular biosynthesis is phosphoglucomutase, a 571-aa protein encoded by *pgmA* that interconverts glucose 6-phosphate and glucose 1-phosphate (*33*). Transposon mutagenesis of the promoter region upstream of *pgmA* resulted in a highly attenuated mutant that was more susceptible to whole blood killing (*33*). This susceptibility was attributed to a decrease in the amount of exopolysaccharide capsule on the cell surface, decreased negative charge, and a larger cell volume 3–5× that of the wild type. Increased susceptibility to the pore-forming cationic antimicrobial peptide moronecidin was also reported, most likely due to changes in cell wall architecture because of increased cellular volume and a decrease in cell wall rigidity (*33*).

Exopolysaccharide

The quantitative composition of monosaccharides present in exopolysaccharide (EPS) is distinct from those found in CPS (*34*). The routine vaccination of fish in Israel has given rise to new strains of *S. iniae* responsible for mass fish deaths (*34*). These new strains were formed when an autogenous vaccine strain, KFP404, was succeeded by new strains KFP468, KFP477, and KFP523, which were characterized by a viscous broth culture similar to that observed with *S. thermophilus* used in yoghurt production (*34*). EPS production by the successor strains was 5× higher than the autogenous vaccine strain. Vaccination of fish with the EPS extracts elicited a survival rate of 78%, which was similar to the 72% survival rate when whole cells were used. Thus, EPS appears to be antigenic and excessive production may have been selected by vaccination (*34*).

α-Enolase

The ability of *S. iniae* to cross tissues through plasminogen activation is facilitated by α-enolase (*35*), which is also a known contributory factor to the virulence of GAS (*36*). The proteolytic activity of plasmin in dissolving fibrin clots enables pathogens to migrate faster through extracellular matrices (*37*), and α-enolase expedites invasion through the host tissues (*38*) and, ultimately, into the circulatory system. The α-enolase from *S. iniae* (≈50 kDa) is a plasmin/plasminogen binding enzyme that is 97% similar to the α-enolases of *S. agalactiae* and GAS (*35*). Immunoblot using antibodies raised against the purified recombinant protein showed cell wall association in *S. iniae*; however, it does not contain the classical gram-positive membrane anchor.

Conclusions

S. iniae opportunistically infects elderly persons with serious underlying conditions. The expression of a suite of virulence factors, many of them similar to those found in GAS, is responsible for successful entry, propagation, and evasion of immune defenses of the host by this bacterium. Another virulence factor, polysaccharide deacetylase, encoded by the *pdi* gene, has been recently described (*39*). With the global rise of aquaculture and the dependence on it to provide food in many areas of the world, the numbers of cases of *S. iniae* infection are likely to be much higher than currently reported and will increase in the future with the expansion of the industry. Understanding the pathogenic processes of *S. iniae* is already facilitating the development of vaccines for use in fish farms and represents the most sustainable and effective method of reducing the incidence of economically devastating outbreaks and clinical presentations in humans, especially in those most at risk.

Dr Baiano is a research officer in the Centre for Marine Studies at The University of Queensland. He is a microbial ecologist and has research interests in marine microbiology, aquatic animal health, and aquaculture.

Dr Barnes is associate professor in aquatic animal health at The University of Queensland, Centre for Marine Studies and School of Biological Sciences. His research interests include marine microbiology, comparative immunology, and host–pathogen interactions in marine animals.

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Genomic Signatures of Influenza A Pandemic (H1N1) 2009 Virus

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Adaptive mutations that have contributed to the emergence of influenza A pandemic (H1N1) 2009 virus, which can replicate and transmit among humans, remain unknown. We conducted a large-scale scanning of influenza protein sequences and identified amino acid–conserving positions that are specific to host species, called signatures. Of 47 signatures that separate avian viruses from human viruses by their nonglycoproteins, 8 were human-like in the pandemic (H1N1) 2009 virus. Close examination of their amino acid residues in the recent ancestral swine viruses of pandemic (H1N1) 2009 virus showed that 7 had already transitioned to human-like residues and only PA 356 retained an avian-like K; in pandemic (H1N1) 2009 virus, this residue changed into a human-like R. Signatures that separate swine viruses from human viruses were also present. Continuous monitoring of these signatures in nonhuman species will help with influenza surveillance and with evaluation of the likelihood of further adaptation to humans.

A recent outbreak of pandemic (H1N1) 2009, previously known as the swine-origin influenza A, has infected >296,000 persons worldwide; 3,486 deaths have been reported (*1*). An increased number of infected humans can potentially alter virulence in the human population. The genomic sequences of many of the new strains of pandemic (H1N1) 2009 virus have revealed important information for promoting medical diagnosis, drug-resistance monitoring, clinical and basic research, and vaccine development. Nevertheless, analyzing adaptive mutation of the new pandemic (H1N1) 2009 virus is a priority so that researchers can evaluate the likelihood that viruses from other nonhuman species will further adapt to humans.

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Pandemic (H1N1) 2009 virus consists of multiple reassorted virus genes from different origins. Of its 8 segmented genomic RNAs, 2 polymerase genes, PB2 and PA, were from the avian virus of North American lineage and were introduced into swine populations around 1998. The other polymerase gene, PB1, also evolved recently from a human seasonal influenza (H3N2) virus around the same year. This particular H3N2 PB1 gene is known to have originated from an avian virus that entered humans in 1968. However, hemagglutinin (HA), nucleoprotein (NP), and nonstructural (NS) protein genes of pandemic (H1N1) 2009 virus descended directly from the classic swine influenza A virus of North American lineage, which can be traced back to the 1918 virus. Originating from the Eurasian swine virus, the remaining 2 genes, neuraminidase (NA) and matrix (M), were introduced from birds around 1979 (*2*,*3*). Limited information is available as to how this unique combination of gene segments evolved from 1998 until it was identified in April 2009 or on the molecular transitions or evolutionary path of this virus before it was transmitted among humans.

Our previous study developed an entropy-based computational scheme to identify host-specific genomic signatures of human and avian influenza viruses (*4*). This method is based on an entropy threshold computed from the amino acid composition at the well known PB2-627 position of avian influenza viruses (entropy value of 0.4 was based on 95 avian influenza genomes, as of early 2006), which contains mostly glutamic acid in the native avian hosts of the viruses. This threshold was then used to identify the 52 species-associated positions at which each of the 2 viruses settles as a distinct amino acid residue that is characteristic of the host. Although the origin of the gene segments in pandemic (H1N1) 2009 virus has been determined (*2*,*3*), the mechanism of transformation of the host-specific ami-

no acid signatures is unclear, because the new viral genes evolved after they were introduced into the swine population some years ago.

By adopting the entropy profiling approach, this study attempts to update influenza A viral signatures on the basis of all influenza sequences from the National Center for Biotechnology Information (NCBI). In addition to providing an updated list of human-avian signatures, this study also computes the human-swine signatures and analyzes the amino acid sequences of pandemic (H1N1) 2009 virus at the host species–specific positions to elucidate the adaptive mutation of influenza A viruses in these host species. As more new influenza virus isolates are collected and their sequences analyzed, the signatures at the host species–specific positions serve as predictors of adaptive mutation, subsequently providing valuable information to help in preparing for potential pandemics.

Materials and Methods

Influenza Virus Sequences

All influenza A virus protein sequences from the NCBI, as of May 28, 2009, were downloaded and analyzed. These full-length or partial sequences were grouped according to the hosts from which the viruses were isolated: humans, avian, and swine. In particular, to observe how these viruses vary in terms of residues, the newly deposited pandemic (H1N1) 2009 virus sequences were considered separately from the human isolates. For each host-specific group, sequences belonging to each viral protein were aligned using the program ClustalW (*5*). Based on the proposed signature identification procedure, 2 surface proteins, HA and NA, were not analyzed because their extensive genetic diversity prevents satisfactory multiple alignment within either human or avian viruses. As an alternatively translated protein product from the PB1 gene, PB1-F2 is also not included in the analysis because it terminates prematurely at position 12. For each of the 4 groups of data, i.e., human, avian, swine, and pandemic (H1N1) 2009, eight alignments were analyzed: PB2, PB1, PA, NP, M1, M2, NS1, and NS2. The total number of sequences varied from gene to gene and from host to host, subject to their availability at the NCBI. For human-isolated viruses (excluding strains of pandemic (H1N1) 2009 virus), >3,000 sequences of the 8 proteins were analyzed. For avian-isolated, swine-isolated, and pandemic (H1N1) 2009 viruses, the numbers of sequences were ≈3,500, 350, and 70, respectively.

Recent Ancestors of Pandemic (H1N1) 2009 Viruses

Smith et al. (*6*) performed evolutionary analysis of the early development of the pandemic, indicating that sporadic infection of humans with triple reassortant and other subsequent reassortant swine viruses occurred before the 2009 human outbreak. To elucidate the transition of amino acid residues along this evolutionary course, we collected and analyzed the protein sequences of 18 recent ancestral swine viruses of the new H1N1 viruses (hereinafter termed "recent ancestral swine viruses") for 1999–2009 from the ancestral lineages of the new pandemic (H1N1) 2009 strains. The sampling was based on the phylogenetic trees published in a study by Smith et al. (*6*). Although a number of swine virus origins have been reported, resulting in various genetic lineages and subtypes, we are most interested in identifying a swine virus population from which the current pandemic (H1N1) 2009 virus might have evolved directly. Not only are those 18 strains chronologically closer (after the years 1997–1998) to the pandemic (H1N1) 2009 viruses but their PB2 and PA genes are also descendants of avian viruses, which complies with the conclusion drawn from recent publications. The online Appendix Table, available from http://www.cdc.gov/EID/content/15/12/1897-appT.htm, summarizes the strain names and accession numbers of recent ancestral swine viruses included in this study.

Entropy-based Signature Identification

For each amino acid position of the aligned sequences of the same virus type, i.e., avian, human, swine, or pandemic (H1N1) 2009, an entropy value was computed by using the formula $-\Sigma P_i \times \ln(P_i)$, as described by Chen et al. (*4*). This formula follows the definition of Shannon entropy (*7*) that has been used to evaluate the diversity of a system. In this study, an entropy was used to measure the variability of aligned amino acid residues at a given genomic position, where $i = 1$ to 20 represents 20 different amino acid residues, and P_i represents the probability density of the respective residue. An entropy value ranges from 0 (only 1 residue present at that position) to 2.996 (all 20 residues are equally represented). As is assumed, a position at which the entropy is less than or equal to a prespecified threshold has a consensus residue for that virus type. When viruses isolated from 2 host species are compared, a species-specific signature position is considered to have different consensus amino acid residue from each of the 2 viruses at the same position. In this study, an entropy threshold of 0.33 was used, based on the PB2-627 position of 3,391 avian influenza sequences.

Results

In 2006, we reported 52 avian-human signatures based on a small set of influenza sequence data of 15,785 protein sequences. The selection was based on an entropy threshold value of 0.4 set at position 627 in the PB2 gene (82 Es and 13 Ks from 95 avian PB2 sequences) because that position has been considered associated with host-restriction (*8–11*). Of the 52 positions, 45 are in the genes PB2, PB1, PA, NP, M1, M2, NS1, and NS2 examined in this work.

Today, >100,000 influenza protein sequences are available at NCBI, and a new entropy threshold of 0.33 was set based on the currently available avian sequences of PB2-627, which contain 3,113 Es, 228 Ks, 46 Vs, 2 As, and 2 Gs. This threshold was adopted to update the list of 47 avian-human signatures in Table 1 for the 8 proteins of interest. Consistent with our earlier findings, most signatures are located on the NP gene (15 positions), followed in number by PA (10 positions), PB2 (9 positions), M2 (5 positions), M1 (3 positions), PB1 (2 positions), NS2 (2 positions), and NS1 (1 position). The 20 signatures associated with PB1, NP, and M1 do not differ between the 2 datasets of 2006 and 2009. In PB2, two new signatures are identified at positions 567 and 702. These 2 positions were only just omitted

Table 1. Amino acid residues of pandemic (H1N1) 2009 virus strains at 47 positions where avian–human signatures are located*

Gene	Position	Avian virus residue	Human virus residue	Pandemic (H1N1) 2009 virus residue
PB2	44	**A**(2,838), S(39), T(1)	**S**(2,734), A(30), L(2)	**A**(61)
	199	**A**(2,816), S(22), D(4), T(2), V(1)	**S**(2,781), A(8)	**A**(61)
	271	**T**(2,758), I(47), A(21), M(5), Q(1)	**A**(2,770), T(15), S(1)	**A**(61)
	475	**L**(3,355), M(25), I(1)	**M**(2,747), L(8), I(1)	**L**(61)
	567	**D**(3,116), E(257), N(18), V(3), G(3), A(2), K(1)	**N**(2,736), D(18), S(1)	**D**(61)
	588	**A**(3,175), T(91), I(72), V(43), S(3), P(1), D(1)	**I**(2,734), A(8), V(6),T(4), L(1), S(1)	**T**(61)
	613	**V**(3,343), A(29), I(19)	**T**(2,651), I(71), A(23), V(9), S(1)	**V**(61)
	627	**E**(3,113), K(228), V(46), A(2), G(2)	**K**(2,746), E(6), R(3)	**E**(61)
	702	**K**(3,232), R(131), Q(1)	**R**(2,731), K(22), G(1), I(1)	**K**(61)
PB1	327	**R**(3,340), K(54), G(1)	**K**(2,489), R(275)	**R**(80)
	336	**V**(3,350), I(26), A(16)	**I**(2,595), V(168), T(1)	**I**(80)
PA	28	**P**(2,915), S(7), L(5), T(1)	**L**(2,736), P(19), R(2), S(2), Q(1)	**P**(61)
	55	**D**(2,906), N(29)	**N**(2,752), D(13)	**D**(61)
	57	**R**(2,849), Q(77), K(4), W(3), L(2)	**Q**(2,736), R(20), L(6), K(3)	**R**(61)
	100	**V**(2,759), A(109), I(68), F(1)	**A**(2,727), V(27), T(7), I(2), S(1)	**V**(61)
	225	**S** (2,854), N(7), C(6), G(1)	**C**(2,736), S(29), G(1)	**S**(61)
	268	**L**(3,317), F(14), I(2), V(1)	**I**(2,724), L(35), V(2)	**L**(61)
	356	**K**(3,309), R(34), N(7), E(1), I (1)	**R**(2,705), K(30)	**R**(61)
	404	**A**(3,098), S(220), T(10), P(4), R(1), V(1)	**S**(2,706), A(28), P(1)	**A**(61)
	409	**S**(3,100), N(191), G(4), I(1), R(1), K(1)	**N**(2,723), S(11), I(1)	**N**(61)
	552	**T**(3,304), A(1), N(1)	**S**(2,721), T(10), N(2), R(1), I(1)	**T**(61)
NP	16	**G**(3,379), S(58), D(8), C(1)	**D**(2,884), G(16)	**G**(120), D(1)
	33	**V**(3,173), I(284), A(1), D(1)	**I**(2,876), V(25)	**I**(121)
	61	**I**(3,419), M(30), V(19), L(6)	**L**(2,881), I(19)	**I**(121)
	100	**R**(3,422), K(34), V(23), S(1)	**V**(2,842), I(52), R(4), A(3), L(1), M(1)	**V**(68), I(46)
	109	**I**(3,407), V(48), T(22), M(2), S(1)	**V**(2,820), I(77), A(3), T(3)	**I**(114)
	214	**R**(3,282), K(52), T(3), L(1)	**K**(2,897), R(25)	**R**(114)
	283	**L**(3,309), F(4), P(3), I(3)	**P**(3,062), L(19), S(3)	**L**(114)
	293	**R**(3,275), K(40)	**K**(3,020), R(65)	**R**(114)
	305	**R**(3,238), K(32), S(2)	**K**(3,052), R(33)	**K**(114)
	313	**F**(3,191), L(43), S(10), Y(1), C(1), I(1)	**Y**(3,064), F(21)	**V**(114)
	357	**Q**(2,766), K(33), T(3), R(2)	**K**(3,052), R(46), Q(5)	**K**(114)
	372	**E**(2,742), D(69), G(3), K(2)	**D**(3,051),E(51), N(1)	**E**(114)
	422	**R**(2,818), K(2)	**K**(2,891), R(51)	**R**(114)
	442	**T**(2,793), S(12), A(5)	**A**(2,890), T(51), R(1)	**T**(114)
	455	**D**(2,792), N(3), E(1)	**E**(2,890), D(51), T(1)	**D**(114)
M1	115	**V**(3,794), I(15), G(2), L(2), M(1)	**I**(3,586), V(19)	**V**(151)
	121	**T**(3,684), A(126), P(4)	**A**(3,599), T(7)	**T**(151)
	137	**T**(3,806), D(12), A(8), P(1), S(1)	**A**(3,577), T(25)	**T**(146)
M2	11	**T**(2,890), I(190), S(8), E(1)	**I**(3,805),T(102)	**T**(55)
	20	**S**(3,032),N(76), K(12), R(3), I(2)	**N**(3,859), S(49)	**S**(55)
	57	**Y**(3,040), H(5), N(1), C(1)	**H**(3,804),Y(65), D(25), Q(5), R(5), N(4)	**Y**(55)
	86	**V**(2,894), A(6), I(4), D(1), L(1), F(1), S(1)	**A**(3,781), V(26), T(10), D(1)	**V**(55)
	93	**N**(2,710), T(13), D(3), H(3), S(3), Y(2), I(1)	**S**(3,699), N(69), Q(2), R(1), H(1), I(1)	**N**(55)
NS1	81	**I**(2,652), V(43), T(8), M(2), S(1), Y(1), G(1)	**M**(2,860), I(59), V(4)	**I**(93)
	227	**E**(3,080), G(60), K(31), S(1)	**R**(2,863), G(8), K(2), W(1), E(1)	Delete
NS2	107	**L**(3,147), P(2), S(2), F(1), Q(1)	**F**(2,850), L(45), S(1), V(1)	**L**(93)

*__Boldface__ indicates dominant amino acid residue type. PB, polymerase B; PA, polymerase A; NP, nucleoprotein; M, matrix; NS nonstructural.

from the 2006 list because their entropy values (0.490 and 0.404, respectively) exceeded the 0.4 threshold, based on the 95 avian sequences examined at that time. New entries in Table 1 also include PA-100, M2-93, and NS1-81. In 2006, although PB2-674 was reported as a signature, it is disqualified here because the entropy of 0.3376 (3,146 As, 88 Es, 87 Ts, 38 Ss, 13 Gs, 4 Vs, and 1 K for avian virus) exceeds the new 0.33 threshold at this position. Similarly, PA-382 (2,421 Ds, 311 Es, 2 Vs, and 1 N in human viruses, with an entropy of 0.3633) and NS2-70 (2,898 Ss, 352 Gs, 28 Rs, and 1 D in avian viruses, with an entropy of 0.3483) were both removed from the 2006 list.

Taubenberger et al. (*12*) identified 10 polymerase gene positions that separate avian viruses from human influenza A viruses. Table 1 shows 8 of them (PB2 199, 475, 567, 627, and 702; PA 55, 100, and 552), suggesting that the method is robust in finding these signatures. Two other polymerase gene positions that Taubenberger et al. also reported are PB1-375 and PA-382; the latter has already been mentioned above. The other missing position in Table 1, PB1-375, has an entropy value of 0.8865 for human and 0.6338 for avian viruses. This position was also excluded from the 2006 list because of an entropy of 0.698 from avian viruses, which substantially exceeded the 0.4 threshold.

To elucidate the potential adaptive mutations of the pandemic (H1N1) 2009 viruses, we studied the amino acid sequences of pandemic (H1N1) 2009 viruses at the positions that represent the so-called species-specific signatures of avian and human viruses. As shown in to the last column of Table 1, 36 of the 47 positions display avian-like signatures in the pandemic (H1N1) 2009 virus. Two positions, PB2-588 and NP-313, exhibit neither avian- nor human-like signatures. Eight human-like signatures were found in pandemic (H1N1) 2009 strains, except for NS1-227, in which all new viruses have an early-terminating NS1 protein and, therefore, contain no residue.

Table 1 presents the updated avian-human signatures for influenza A viruses; Table 2 summarizes the swine-human signatures. Medical literature documents that the swine virus population has distinct evolutionary lineages that originated from the classic 1918 virus referred to as classic or North American swine virus, and the others of post-1979 Eurasian swine virus and subsequent triple reassortants. Because the residue diversity at many positions markedly increased for these swine viruses because of their distinct origins, only 8 swine-human signatures met the 0.33 threshold. Unlike some positions in which human-like signatures of pandemic (H1N1) 2009 were found (Table 1), in this study, all 8 locations of the swine-human signature of this new virus are characteristic of swine. Notably, Table 1 lists all 8 positions in Table 2, with each having the same signature as in the avian virus. Restated, avian and swine viruses contain the same amino acid residue at the 8 human-swine signature positions.

We attempted to further elucidate the transition of the amino acid residue on the pandemic (H1N1) 2009 virus that have human signatures by sampling 18 recent ancestral swine viruses (online Appendix Table). Doing so enables us to examine more closely the prevalence of amino acid residues specifically with pandemic (H1N1) 2009 viruses. Table 3 summarizes the amino acid statistics of these recent ancestral swine viruses together with avian, human, and pandemic (H1N1) 2009 sequences at the 8 positions containing human residues for pandemic (H1N1) 2009 virus in Table 1. Consider PB2-271, for example, avian viruses have signature T, whereas human viruses have signature A. Although pandemic (H1N1) 2009 viruses also have the human signature A, their predecessors, i.e., the recent ancestral swine viruses, have already acquired the human signature A at this position. PB1-336, along with PA-409, NP-33, -100, -305, and -357, follows the same residue transition, all showing human-characteristic residues in both recent ancestral swine and pandemic (H1N1) 2009 viruses. PA-356 is the only exception, where the residue in recent ancestral swine viruses still maintains an avian-characteristic K before changing to a human residue R in pandemic (H1N1) 2009 viruses. Of particular interest is whether the transition from K to R at position PA-356 is responsible for the ability of pandemic (H1N1) 2009 viruses to replicate and transmit efficiently in humans.

After all 8 human residue-containing positions of pandemic (H1N1) 2009 viruses were found to be within PB2,

Table 2. Amino acid residues of pandemic (H1N1) 2009 virus strains at 8 positions where swine–human signatures are located*

Gene	Position	Swine virus residue (all subtypes)	Human virus residue	Pandemic (H1N1) 2009 virus residue
PB2	44	**A**(301), S(27), C(1)	**S**(2,734), A(30), L(2)	**A**(61)
PA	268	**L**(325), I(31), T(1)	**I**(2,724), L(35), V(2)	**L**(61)
	552	**T**(280), S(25)	**S**(2,721), T(10), N(2), I(1), R(1)	**T**(61)
M1	137	**T**(429), A(39)	**A**(3,577), T(25)	**T**(146)
M2	57	**Y**(343), H(23), R(2)	**H**(3,804),Y(65), D(25), Q(5), R(5), N(4)	**Y**(55)
	86	**V**(324), A(24), S(1)	**A**(3,781), V(26), T(10), D(1)	**V**(55)
	93	**N**(320), S(23)	**S**(3,699), N(69), Q(2), R(1), H(1), I(1)	**N**(55)
NS2	107	**L**(299), F(25)	**F**(2,850), L(45), S(1), V(1)	**L**(93)

*__Boldface__ indicates dominant amino acid residue type. PB, polymerase B; PA, polymerase A; M, matrix; NS, nonstructural.

Table 3. Position-specific residue transitioning for influenza A virus among avian, recent ancestral swine, pandemic (H1N1) 2009, and human strains, for those 8 positions of pandemic (H1N1) 2009 virus showing human-characteristic signatures*

Gene	Position	Avian virus residue	Recent swine viruses residue†	Pandemic (H1N1) 2009 virus residue	Human virus residue
PB2	271	**T**(2,758), I(47), A(21), M(5), Q(1)	**A**(17), S(1)	**A**(61)	**A**(2,770), T(15), S(1)
PB1	336	**V**(3,350), I(26), A(16)	**I**(16)	**I**(80)	**I**(2,595), V(168), T(1)
PA	**356**	**K**(3,309), R(34), N(7), E(1), I(1)	**K**(16), R(1)	**R**(61)	**R**(2,705), K(30)
	409	**S**(3,100), N(191), G(4), I(1), R(1), K(1)	**N**(17)	**N**(61)	**N**(2,723), S(11),I (1)
NP	33	**V**(3,173), I(284), A(1), D(1)	**I**(18)	**I**(121)	**I**(2,876), V(25)
	100	**R**(3,422), K(34), V(23), S(1)	**V**(17), I(1)	**V**(68), I(46)	**V**(2,842), I(52), R(4), A(3), L(1), M(1)
	305	**R**(3,238), K(32), S(2)	**K**(18)	**K**(114)	**K**(3,052), R(33)
	357	**Q**(2,766), K(33), T(3), R(2)	**K**(17), R(1)	**K**(114)	**K**(3,052), R(46), Q(5)

*__Boldface__ indicates dominant amino acid residue type. PB, polymerase B; PA, polymerase A; NP, nucleoprotein.
†Eighteen recent ancestral swine viruses as listed in the online Appendix Table (available from www.cdc.gov/EID/content/15/12/1897-appT.htm). We consider recent ancestral strains phylogenetically neighboring to the pandemic 2009 strains, in particular for PB2 and PA genes they are clustered together with recent avian strains because the pandemic (H1N1) viruses were reported to originate from avian viruses around 1998. Note that for 1 strain, A/swine/Missouri/4296424/06(H2N3), the PA sequence was not found anywhere near the other 17 recent swine strains of interest. Two PB1 sequences, A/swine/Hong Kong/78/2003(H1N2) and A/swine/Korea/C13/2008(H5N2), were also found distantly located from the other 16 recent swine PB1 sequences. We excluded these 3 sequences from the amino acid statistics in this table because of their genetic deviation from the remaining ancestral swine viruses we have collected here.

PB1, PA, and NP protein genes, all amino acid positions of these 4 proteins were scanned for their residue transitions among the 4 virus populations shown in Table 3. The change in the amino acid that may be associated with the transformation of pandemic (H1N1) 2009 virus is summarized in Table 4. As well as PA-356, already shown in Table 2, two additional positions, PB2-684 and PA-204, showed the same dominant amino acid residue in avian and recent ancestral swine viruses, but a different dominant residue in pandemic (H1N1) 2009 viruses and human viruses. Dominance is defined here as 1 residue containing the largest sequence count compared with other residues at a particular aligned position. The previously used entropy measurement in Tables 1, 2, and 3 does not apply to the positions listed in Table 4, in which we emphasize the amino acid transition of dominant residues instead of highly conserved ones subject to the prescribed entropy threshold 0.33. Other than those 3 positions, PB1-216 was found to contain a human residue G in 8 of 9 recent ancestral swine viruses that are closer to pandemic (H1N1) 2009 viruses in the phylogenetic tree published in a study by Smith et al. (*6*). However, for the other 7 recent ancestors that are more distant from pandemic (H1N1) 2009 viruses, PB1-216 maintains an avian-residue S in 6 of 7 viruses. Our results show that the position-specific transition may serve as a molecular marker for monitoring such adaptive mutations in the future.

Discussion

Although most studies confer that the death rate associated with pandemic (H1N1) 2009 infection is more moderate than that of subtype H5N1 infection, its virulence may vary with adaptive mutations in viral genes, subsequently increasing the likelihood that the new virus alters its virulence in the new host species. Many of the previously identified virulence factors are apparently not involved. For instance, no E to K mutation at position 627 of PB2 is observed, which has been considered an important factor for avian virus to efficiently replicate in mammalian systems (*8*–*11*). Previous studies have indicated that PB1-F2 contributes viral pathogenesis in the mammalian system (*13*,*14*). No PB1-F2, however, is predicted in pandemic (H1N1) 2009 viruses because it terminates prematurely at position 12. Its NS1 protein is truncated at position 220 and,

Table 4. Amino acid positions containing the same residue in avian and recent ancestral swine viruses, yet changed to a different one in pandemic (H1N1) 2009 and human viruses*

Gene	Position	Avian virus residue	Recent swine viruses residue†	Pandemic (H1N1) 2009 virus residue	Human virus residue
PB2	684	**A**(3,278), T(70), S(11), V(9), G(6), D(1), E(1)	**A**(18)	**S**(61)	**S**(1,944), A(806), G(2), P(2), Y(1)
PB1	216	**S**(3,299), G(62), N(37), C(11), I(7)	G(9), **S**(7)‡	**G**(80)	**G**(1,708), S(1,039), N(5), I(4), D(1)
PA	204	**R**(2,202), K(674), E(1), G(1), S(1)	**R**(17)	**K**(61)	**K**(1,776), R(991)
	356	**K**(3,309), R(34), N(7), E(1), I(1)	**K**(16), R(1)	**R**(61)	**R**(2,705), K(30)

*__Boldface__ indicates dominant amino acid residue type. PB, polymerase B; PA, polymerase A.
†Same 18 recent ancestral swine viruses used in Table 3.
‡PB1-216 is dominated by residue G (G[8), S[1]) when considering only a subset of 9 PB1 sequences that are phylogenetically closer to pandemic (H1N1) 2009 virus. This statistic clearly shows the amino acid residue transition from avian to human signature within the population of recent swine viruses.

therefore, lacks a PDZ ligand interacting domain. As suggested recently, the presence of this PDZ ligand domain increases the pathogenicity of avian influenza A viruses (*15*). Regardless of whether these known factors are missed, a previous study has demonstrated that the virulence of pandemic (H1N1) 2009 virus is higher than that of seasonal influenza A viruses (*16*). Although a virulence marker and a host range factor may not be necessarily linked tightly, recent investigations have also demonstrated that altering PB2-627 from E to K in the avian viruses increases its virulence in the mammalian experimental system (*9–11*). For example, avian influenza virus subtype H7N7 reportedly infects humans (*17*). A human isolate from a fatal case had its PB2-627 changed from avian-characteristic E to K. Correspondingly, the species-associated signatures identified in this study may serve as potential molecular targets for further evaluating how they impact the virulence of pandemic (H1N1) 2009 viruses in humans.

As shown in Tables 1 and 2, the number of signature positions decreases significantly from 47 (human vs. avian) to 8 (human vs. swine), and the positions of the latter are a subset of those of the former. These observations may have the following implications. First, the 3 host species of interest differ, with each providing a unique environment for infection by the influenza virus. When the avian virus enters humans or swine, its genetic feature is shaped by a particular evolutionary path. The viruses, therefore, have different signatures. Second, some avian-like signatures are preserved in swine viruses, suggesting that both avian species and swine may provide similar conditions for harboring influenza A viruses. The body temperature may be a determinant. As is generally known, many avian species have a body temperature exceeding 40°C; for most pigs it is variable but still higher than the human body temperature, which is 37°C. Consequently, the signatures are retained when an avian virus enters the swine population, with similar signature-related viral replication mechanisms in both species. Third, the 39 signature positions shown in Table 1, but absent from Table 2, may be correlated with certain functional domains that interact with host factors unique in humans while differing significantly from those of avian and swine. Finally, the number of signature positions of swine versus humans is substantially lower than those of avian versus humans, suggesting that the species barrier to humans is easier for a swine virus to cross than for an avian virus.

The entropy-based computation depends strongly on a good multiple sequence alignment. The 2 surface proteins HA and NA are excluded from this analysis because both contain sequences that diverged sufficiently from so many subtypes of a given species. Locating conserved residues at particular positions on the basis of these alignments is extremely difficult. The entropy threshold is the other parameter requiring attention to locate a signature position. In this study, the entropy determined from PB2-627 of the aligned residues of all avian viruses is used because PB2-627 is the most laboratory-proved host-restriction marker (*8–11*). A complete new set of signatures can be reproduced rapidly by using a different entropy threshold based on other factors. The diverse genetic origins of influenza viruses would also have great impact on the reported signatures. The proposed entropy-based method to reach the 8 positions listed in Table 2 was based on all swine viruses of different origins, including North American-(classic 1918) origin strains, Eurasian (post-1979 avian)-origin strains, and recent triple reassortants. A comparison of, for example, all human viruses versus classic 1918-origin swine viruses before 1978 (≈75 strains, or 20% of our swine sequence population) would report 60 signature positions (data not shown). In this work, we included all swine viruses of multiple origins in producing Table 2 to consider only host-specific genomic signatures that have been shaped by the same swine species regardless of origin. For the same reason, we did not subdivide avian or human populations into lineages when reporting avian-human signatures in Table 1.

This study analyzed a complete collection of species-specific influenza A viral sequences, including the long-evolving avian, recent ancestral swine and human viruses, as well as pandemic (H1N1) 2009 viruses, which is still in its infancy. The amino acid sequence transition of pandemic (H1N1) 2009 virus at the signature positions was also elucidated by applying the entropy-based signature analysis to these sequences. They were found mostly to be characteristic of avian species, as presented in Table 1. Notably, 8 of them changed from avian-like signatures to human-like signatures. Close examination of the residue transition at these 8 positions in Table 3 showed that PA-356, unlike the other 7 positions, retained an avian-like signature in the recent ancestral swine population and changed to a human-like signature only in pandemic (H1N1) 2009. This finding suggests that PA-356 may be related to host-restriction factors from swine to human species. Similarly, all ribonucleoprotein positions were scanned for the same transitioning pattern as in PA-356, i.e., a retained avian-like residue in the recent ancestral swine population and a change to the human residue in pandemic (H1N1) 2009 viruses. Table 4 lists them all. Although 1 of the positions, PB1- 216, was not dominated by the residue S as we would have expected, it exhibited a mixture of 2 residues involving a transition from avian to human viruses. In summary, Table 4 provides a list of candidate host-restriction factors that we believe are important to adaptive mutation of influenza A viruses among the 3 host species. Continuous monitoring of these signatures in nonhuman species will help in influenza surveillance and in evaluating the likelihood of further adaptation to humans.

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Tick-borne Agents in Rodents, China, 2004–2006

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A total of 705 rodents from 6 provinces and autonomous regions of mainland People's Republic of China were tested by PCRs for tick-borne agents (*Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, spotted fever group rickettsiae, and *Francisella tularensis*). Infection rates were 5.5%, 6.7%, 9.1% and 5.0%, respectively. Eighteen (2.6%) rodents of 10 species were positive for 2 or 3 agents. Sequence analysis of PCR products confirmed the presence and genotypes of detected agents. These findings demonstrate that these tick-borne agents cocirculate and that a variety of rodent species may be involved in their enzootic maintenance.

Anaplasma phagocytophilum, *Borrelia burgdorferi* sensu lato, spotted fever group (SFG) rickettsiae, and *Francisella tularensis* are the causative agents of human granulocytic anaplasmosis, Lyme disease, spotted fever, and tularemia, respectively. These agents are naturally maintained in animal reservoirs and considered emerging or reemerging pathogens with serious public health implications. Although these agents could infect humans through various routes, ticks play a major role in transmission from animal hosts to humans.

Co-infection with these agents has been found in many tick species including *Ixodes scapularis* in northeastern United States, *I. pacificus* and *I. spinipalpis* in the western United States *I. ricinus* in Europe, and *I. persulcatus* in Asia (*1*). Patients co-infected with 2 tick-borne pathogens usually show more severe clinical signs of longer duration (*1*). Experimental concurrent infections with *A. phagocytophilum* and *B. burgdorferi* may suppress interleukin-2 (IL-2) and interferon-γ production, promote IL-4 response, increase pathogen load, and intensify Lyme arthritis (*2*–*4*). Natural infection and co-infection with these 4 agents have been reported in the People's Republic of China in various tick species (*5*–*7*) such as *I. persulcatus*, *Dermacentor silvarum*, *Haemaphysalis concinna*, *H. longicornis*, and *H. warburconi*, which are known to feed on small mammals as well as humans.

We hypothesize that multiple agents might be present in rodents from tick-infested areas. The purpose of this study was to identify *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* in rodents from mainland China and to better understand the public health role of these emerging and reemerging pathogens.

Materials and Methods

Sample Collection

During 2004–2006, rodents were collected at 6 study sites in Heilongjiang Province, Inner Mongolia Autonomous Region, Jilin Province, Zhejiang Province, Guizhou Province, and Xinjiang Autonomous Region (Figure) at various times according to peak seasons of tick species. The first 3 sites were forested highlands in the Small Xing'an Mountains and the Great Xing'an Mountains of northeastern China, where local residents worked and were exposed to rodents and ticks. The study sites in Zhejiang and Guizhou provinces were forested rolling hills with typical temperate zone vegetation; these regions attract hundreds of thousands of tourists per year. The study site

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in Xinjiang Autonomous Region was a forest with a rural resident population. Rodents were trapped by using peanuts as bait. After captured rodent species were identified, spleen specimens were collected and stored at –20°C until DNA was extracted.

Extraction of DNA

Total DNA was extracted from spleen samples by using Trizol agent (Invitrogen, Carlsbad, CA, USA) following the instructions of the manufacturer. Briefly, ≈300 mg of spleen tissue from each rodent was crushed in Trizol reagent, and DNA was separated from RNA by centrifugation. DNA was precipitated after washing twice in a solution containing 0.1 M sodium citrate in 10% ethanol. The DNA pellet was then washed in 75% ethanol and kept at room temperature for 10–20 min. After centrifugation at 2,000 × *g* at 2–8°C for 5 min, DNA was dissolved in 8 mmol/L NaOH and centrifuged to remove insoluble material. The supernatant containing DNA was removed and adjusted with HEPES buffer to a pH of 7–8.

PCR

Nested PCR was conducted with primers designed to amplify part of the 16S rRNA gene of *A. phagocytophilum*, as described (*8*). For amplification of *B. burgdorferi* DNA, a nested PCR was performed with primers derived from *B. burgdorferi* 5S–23S rRNA intergenic spacer (*9*). PCR was performed by using primers Rr 190.70p and Rr 190–701n to amplify a fragment of the gene encoding a 190-kDa outer membrane protein A (*ompA*) gene specific for SFG rickettsiae (*10*). Samples were tested for *F. tularensis* by a nested PCR specific for the outer membrane protein (*fopA*) gene, as described (*11*). All PCRs were performed by using a model 2700 thermal cycler (Perkin-Elmer, Waltham, MA, USA). PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and examined under UV light. To avoid contamination, we performed DNA extraction, reagent setup, amplification, and agarose gel electrophoresis in separate rooms and included negative controls (distilled water) were in each amplification.

DNA Sequencing and Analysis

PCR products of positive samples were sequenced directly by using a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer). To limit errors in sequencing, we performed 2 sequencing reactions of each PCR product. When different sequences were obtained, additional sequencing reactions were conducted to generate a consensus sequence. Sequences obtained in the present study were compared with the corresponding sequences deposited in GenBank by using the BLAST program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure. Study sites (triangles) in the People's Republic of China where rodents were collected, 2004–2006. Numbers in parentheses are co-infection rates of rodents with 2 or 3 tick-borne agents

Statistical Analysis

Chi-square or Fisher exact tests were used to compare proportions. p values <0.05 were considered statistically significant.

Results

A total of 705 rodents were captured. The number of rodents tested and infectivity rates at different survey sites are shown in the Table. *A. phagocytophilum* was detected only in rodents captured in eastern regions of China (Heilongjiang, Jilin, and Zhejiang provinces) (Figure). *B. burgdorferi* was detected in rodents captured at all 6 survey sites. SFG rickettsiae were detected in rodents captured at all sites except Jilin Province. *F. tularensis* was detected in rodents captured only in northern China (Heilongjiang and Jilin provinces and Inner Mongolia and Xinjiang autonomous regions; Figure).

In Heilongjiang Province, all 4 agents were detected in rodents at similar frequencies (χ^2 2.80, df 3, p = 0.424). No SFG rickettsiae were detected in rodents from Jilin Province. The infectivity rates for the 3 agents in Jilin Province did not significantly differ (χ^2 2.23, df 2, p = 0.328). Infectivity rates for SFG rickettsiae were significantly higher than those for *B. burgdorferi* and *F. tularensis* in rodents from Inner Mongolia Autonomous Region (χ^2 39.76, df 2, p<0.001). Infectivity rates for the 3 agents in Xinjiang Autonomous Region did not differ significantly (χ^2 5.01, df 2, p = 0.082). Except for *F. tularensis*, the other 3 agents showed similar infectivity rates for Zhejiang Province (χ^2 1.30, df 2, p = 0.523). Only *B. burgdorferi* and SFG rickettsiae were found in Guizhou Province, and the difference

Table. Infection rates for 4 tick-borne agents in rodents, People's Republic of China, 2004–2006*

Study site	No. rodents tested	No. (%) rodents positive				p value
		Anaplasma phagocytophilum	*Borrelia burgdorferi*	SFG rickettsiae	*Francisella tularenesis*	
Heilongjiang Province	64	3 (4.7)	3 (4.7)	1 (1.6)	5 (7.8)	0.424
Jilin Province	205	20 (9.8)	17 (8.3)	0	26 (12.7)	0.329
IMAR	148	0	8 (5.4)	32 (21.6)	2 (1.4)	0.0001
XJAR	44	0	1 (2.3)	4 (9.1)	2 (4.5)	0.348
Zhejiang Province	216	16 (7.4)	16 (7.4)	21 (9.7)	0	0.598
Guizhou Province	28	0	2 (7.1)	6 (21.4)	0	0.252
Total	705	39 (5.5)	47 (6.7)	64 (9.1)	35 (5.0)	

*SFG, spotted fever group; IMAR, Inner Mongolia Autonomous Region; XJAR, Xinjiang Autonomous Region.

in their infectivity rates was not significant (p = 0.525, by Fisher exact test).

A total of 18 (2.6%, 95% confidence interval 1.4%–3.8%) rodents from all survey sites except Xinjiang Autonomous Region were positive for 2 or 3 agents, among which 15 were positive for 2 agents. A *Clethrionomys rufocanus* rodent from Heilongjiang Province was positive for *A. phagocytophilum*, *B. burgdorferi*, and SFG rickettsiae, and 2 rodents (*Apodemus agrarius* and *Tamias sibiricu*) from Jilin Province were positive for *A. phagocytophilum*, *B. burgdorferi*, and *F. tularensis* (online Appendix Table 1, available from www.cdc.gov/EID/content/15/12/1904-appT1.htm).

Overall, except for 6 unclassified rodents, 23 species of rodents captured at the 6 survey sites were identified. Rodent species composition varied greatly at different sites (online Appendix Table 2, available from www.cdc.gov/EID/content/15/12/1904-appT2.htm). *Rattus norvegicus* rodents were found at all survey sites except Xinjiang Autonomous Region. *A. agrarius*, *A. peninsulae*, *Clethrionomys rufocanus*, *Mus musculus*, and *T. sibiricu* rodents were found in northeastern China; *A. sylvaticus*, *Niviventer confucianus*, and *R. losea* were found mainly in southern China; and *Meriones unguieulataus* and *M. musculus* were found mainly in western China.

The dominant rodent species differed at various study sites. *C. rufocanus* (57.8%) was dominant in Heilongjiang Province, *A. agrarius* (36.2%) and *A. peninsulae* (27.1%) in Jilin Province, *A. agrarius* (29.7%) and *Microtus maximowiczii* (23.7%) in Inner Mongolia Autonomous Region, *M. musculus* (50.0%) and *M. unguieulataus* (34.1%) in Xinjiang Autonomous Region, *N. confucianus* (53,0%) in Zhejiang Province, and *R. norvegicus* (32.14%) and *M. musculus* (28.6%) in Guizhou Province (online Appendix Table 2).

To confirm the presence and determine genotypes of detected organisms, PCR products were sequenced and analyzed. A 919-bp partial 16S rRNA gene fragment for *A. phagocytophilum* was obtained from each positive specimen (*8*). *A. phagocytophilum* sequences detected in rodents from Heilongjiang and Jilin provinces (GenBank accession no. DQ342324) were identical and differed from those from Zhejiang Province (GenBank accession no. DQ458808) by 2 bp, from those from ticks in United Kingdom and Sweden (GenBank accession nos. AY082656 and AJ242784.1, respectively) by 2 bp, and from other known *A. phagocytophilum* sequences by >3 bp.

Sequence analysis of the *B. burgdorferi* 5S–23S rRNA intergenic spacer showed that agents isolated from rodents in Heilongjiang Province, Inner Mongolia Autonomous Region, Jilin Province, and Xinjiang Autonomous Region belonged to the *B. garinii* genospecies, similar to agents detected in ticks (GenBank accession no. DQ150540) in northern China. Of 16 *B. burgdorferi* detected in Zhejiang Province, 12 belonged to the *B. garinii* genospecies and the other 4 belonged to the *B. valaisiana*–related group (GenBank accession nos. EU160458 and EU160459). The 2 strains found in Guizhou Province also belonged to the *B. valaisiana*–related group (GenBank accession no. EU247840).

For identification of SFG rickettsiae, partial nucleotide sequences of the *ompA* gene were obtained from positive specimens in Heilongjiang Province and Inner Mongolia Autonomous Region. All sequences were identical to those of the *R. sibirica* genotype (GenBank accession no. U43807). Nucleotide sequences of 35 specimens positive for *F. tularensis* were identical to each other and to published sequences for the *F. tularensis* subsp. *holarctica* strain (GenBank accession no. AF247642.2).

Discussion

We detected *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* in diverse species of rodents from different areas of China. Our findings and previous evidence (*6*,*9*,*12*–*15*) suggest that several tick-borne agents cocirculate in mainland China, and a variety of rodent species may be involved in enzootic maintenance of these agents.

This study was not intended to be a comprehensive survey on active infections with *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis*. Rather, it was designed to investigate the presence and extent of these

agents in China. If one considers that human infections with *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* have been reported in various regions of China (*16–19*), the presence of these agents in rodents in the study areas suggests a potential threat to humans, and the public health role of these findings should be further investigated.

Although infectivity rates varied at different survey sites (Table, online Appendix Table 1), we could not determine the geographic diversity of these agents in rodents. The number of rodents examined was limited; therefore, infectivity rates in the current study could be biased. In addition, because intensity of circulation of any vector-borne agent fluctuates dramatically throughout the year and from year to year, even at the same location (*20,21*), we could not justify comparing infectivity rates between different sites on the basis of unsynchronized single collections over a 3-year period. A randomized sampling scheme and further collection of rodents are required to clarify this issue. Unfortunately, we did not collect the ticks from captured rodents for additional testing of the tick-transmitted agents. This limitation prevented us from understanding vector potential.

In this study, *A. phagocytophilum* was detected only in eastern China (Table, Figure), where it coexists with the other 3 agents (online Appendix Table 1). *A. phagocytophilum* detected in Heilongjiang, Jilin, and Zhejiang provinces were closely related to each other by 16S rRNA gene sequence analysis, but less related to other known strains in other countries. *B. burgdorferi* was detected in rodents from all 6 survey sites. As observed in a previous study (*9*), *B. garinii* was the dominant genospecies in mainland China, and the *B. valaisiana*–related group was present in southern China.

SFG rickettsiae, including ≈20 species of rickettsiae, can be transmitted to animals and humans not only by ticks but also by other arthropods such as infected lice, fleas, and mites (*10*). In this study, we amplified the *ompA* gene, which is present in most SFG rickettsiae (*10,22*). The overall infectivity rate for SFG rickettsiae was highest (9.1%) among the 4 agents tested (online Appendix Table 1). Sequence analysis identified the *Rickettsia* sp. detected in Heilongjiang Province and Inner Mongolia Autonomous Region as a genotype of *R. sibirica*, which is known to cause Siberian tick typhus (*18*). However, we did not sequence PCR products amplified from rodents at other study sites because of a limited amount of samples. Although sequence analysis of the *ompA* gene fragment is not sufficient to identify the agent (*22*), it is commonly used to recognize tick-borne *Rickettsia* spp. in field surveys (*23*).

F. tularensis was found only in northern China, which verifies our belief that *F. tularensis* is present only north of 30°N latitude. In many disease-endemic areas, ticks are known to play a role in transmitting *F. tularensis* from animal hosts to humans, although other arthropods such as deer flies, fleas, mites, and mosquitoes are known to carry the bacterium. Sequence analysis showed that all *F. tularensis* detected in this study belong to the subspecies *holarctica*.

Interference of infections among *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* in rodent hosts is not clear. Our findings indicate that infection with *A. phagocytophilum* does not intensify risk for transmission of the other 3 agents and vice versa. *B. burgdorferi* in rodents appears to increase risk for infection with *F. tularensis* but does not increase the possibility of infection with SFG rickettsiae or *A. phagocytophilum*. Further investigations are needed to demonstrate positive or negative interactions of the pathogens and to establish whether this interference is associated with the animal species.

Of 705 rodents tested in this study, 15 were infected with 2 agents and 3 were infected with 3 agents. These findings indicate that mixed natural foci of tick-borne agents are present at the study sites. Because *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* were found in ticks collected in the study areas (*6–9,12–14*), it is not surprising that multiple agents were detected in rodents. Coexistence of multiple agents might be caused by a single bite of a tick infected with several agents or multiple bites of ticks infected with at least 1 agent. The presence of 4 pathogens in the study areas demonstrates the risk for multiple infections in humans, which may lead to variations and exacerbation of clinical signs (*1*). Therefore, differential diagnoses should be made for febrile patients with a history of tick bites in these areas, particularly when clinical signs are atypical for 1 disease or a related disease.

Among 23 rodent species trapped in this study, 21 were infected with ≥1 agent (online Appendix Table 2). Only 2 species (*Cricetulus migratourius* and *N. fulvescens*) were negative for all 4 agents. Which species is the main host of each agent remains unknown, because none of the agents are predominantly associated with 1 or a few related rodent species, regardless of their geographic origin. However, *A. phagocytophilum*, *B. burgdorferi*, SFG rickettsiae, and *F. tularensis* in various rodent species illustrate the potential roles of various rodents in maintaining these tick-borne agents. Systematic epidemiologic studies that investigate characteristics of natural foci and the role of small wild animals in transmission of these agents to humans are needed.

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Cost-effectiveness Analysis of Hospital Infection Control Response to an Epidemic Respiratory Virus Threat

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The outbreak of influenza A pandemic (H1N1) 2009 prompted many countries in Asia, previously strongly affected by severe acute respiratory syndrome (SARS), to respond with stringent measures, particularly in preventing outbreaks in hospitals. We studied actual direct costs and cost-effectiveness of different response measures from a hospital perspective in tertiary hospitals in Singapore by simulating outbreaks of SARS, pandemic (H1N1) 2009, and 1918 Spanish influenza. Protection measures targeting only infected patients yielded lowest incremental cost/death averted of $23,000 (US$) for pandemic (H1N1) 2009. Enforced protection in high-risk areas (Yellow Alert) and full protection throughout the hospital (Orange Alert) averted deaths but came at an incremental cost of up to $2.5 million/death averted. SARS and Spanish influenza favored more stringent measures. High case-fatality rates, virulence, and high proportion of atypical manifestations impacted cost-effectiveness the most. A calibrated approach in accordance with viral characteristics and community risks may help refine responses to future epidemics.

Pandemic influenza A (H1N1) 2009 virus is a new influenza virus of swine origin that was first detected in April 2009. Within 4 months of its appearance in Mexico, it had spread to >100 countries, with >200,000 confirmed cases globally, including >2,000 deaths (*1*). When the World Health Organization (WHO) raised its global influenza pandemic alert to phase 5 (imminent pandemic) on April 27, 2009, many countries followed suit and activated their pandemic preparedness plans, although this varied between countries. Many countries with direct experience of the 2003 severe acute respiratory syndrome (SARS) outbreak tended toward more stringent measures.

Singapore was one of the countries most affected by SARS and experienced a disproportionate impact of the spread of the disease in hospitals (*2*,*3*). A total of 98 healthcare workers in Singapore were infected with SARS, 6 of whom died (*4*). After the SARS experience, Singapore's Ministry of Health (MOH) developed a pandemic influenza plan with several levels of response that correlated roughly with the WHO Pandemic Alert Response system (*5*). The Disease Outbreak Response System (DORSCON)-FLU system that MOH devised requires progressively higher levels of infection control in hospitals in addition to border screening, restrictions on visitors to hospitals, and community-based syndromic surveillance for acute febrile illnesses (Table 1).

In accordance with the progressive elevation of WHO pandemic alert levels, Singapore raised its own pandemic alert level to Yellow on April 27, 2009, and further elevated it to Orange 2 days later. At this level, all hospital staff were required to wear N95 masks when dealing with all patients. Patients were restricted to 1 registered and screened visitor, all medical and nursing student rotations and local medical conferences were cancelled, leave restrictions for healthcare workers (HCWs) were put in place, inter-hospital movement of patients and HCWs was banned, and

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Table 1. Characteristics of Singapore MOH influenza outbreak response system*

Singapore MOH DORSCON alert level	WHO pandemic alert level	Global/local situation	Hospital measures	Community measures
Green 0	1	No novel influenza virus circulating	Triage and isolation of febrile patients, use of PPE as appropriate	Surveillance, maintenance of antiviral drug stockpile
Green 1	2–3	Novel virus but predominantly animal disease with limited transmission to humans	Full PPE for suspect cases, contact tracing for confirmed cases, antiviral treatment for all confirmed cases	Enhanced surveillance, communication, readiness measures
Yellow	4	Inefficient human-to-human transmission of novel virus	Full PPE for HCWs in high risk contact, visitor restriction, restrict movement of patients and HCWs	Enhanced surveillance, public health education, border body temperature screening, surveillance of returned travelers from affected areas
Orange	5	Global or local clusters but transmission still localized	PPE stepped up to cover "medium-risk" patients, no visitors, no interhospital movement of patients or HCWs, post-exposure prophylaxis for contacts	Body temperature screening at community areas, consider school closure, body temperature screening at borders, enhanced public health education
Red	6	Pandemic under way, import into Singapore is inevitable	As above with establishment of 18 influenza clinics	As above with possible use of masks in the community

*MOH, Ministry of Health; DORSCON, Disease Outbreak Response System; WHO, World Health Organization; PPE, personal protective equipment; HCWs, healthcare workers. Adapted from (*5*).

further limitations were placed on elective surgery. These measures were aimed primarily at avoiding a repeat of the SARS epidemic where nosocomial transmission originated with patients whose SARS infections were undiagnosed in hospital, and because influenza may be contagious before symptoms develop in infected patients. In fact, nosocomial influenza has been well documented since the 1957 Asian influenza pandemic (*6*). Based on studies conducted primarily in the United States, it has been estimated that 1 nosocomial case of influenza in a pediatric unit can cost up to $7,500 (US) (*7*). A recent review (*8*) of 28 nosocomial outbreaks of seasonal influenza summarized the evidence for nosocomial transmission of influenza in hospitals with accompanying illness and death (*8*,*9*).

When it subsequently became apparent that the case-fatality rate for pandemic (H1N1) 2009 was much lower than previously thought, especially in settings of industrialized countries, the alert level in Singapore was lowered to Yellow on May 11, 2009, even as WHO moved to alert level 6 after the pandemic was declared.

The risks and impacts of an outbreak will no doubt depend on the transmissibility, virulence, and clinical severity of illness. Thus, the benefits of a high alert status response at the onset of an outbreak as a "safe rather than sorry" strategy is not unreasonable when faced with an unknown novel potentially lethal virus. Yet, on the other hand, preventive measures from a hospital perspective come with a price. Direct costs include activation as well as ongoing administrative, manpower, and logistic resources, such as use of enhanced personal protective equipment, as part of the alert response measures.

We made use of this unique opportunity to evaluate the real costs of our primary prevention interventions and their potential cost-effectiveness against different models of influenza virulence and transmissibility in a simulated outbreak in our 1,000-bed tertiary teaching hospital to understand the relative incremental cost per additional death averted at different alert status levels. The key variables that affected the cost-effectiveness ratio the most were identified and studied. The same analysis was subsequently repeated for a parallel 1,500-bed tertiary teaching hospital. Using the outcome variables of disease cases, deaths, and incremental cost per death averted, we sought to determine if a calibrated and measured response plan based on characteristics of the virus in the outbreak could be better defined.

Methods

To determine the cost incurred per day over the period where hospitals were at DORSCON Yellow and Orange, we obtained actual direct and indirect costs from the Operations and Finance Departments of the hospitals. Excess costs were measured by comparing these with operating costs and results over the same period in 2008.

To simulate a hospital outbreak, we used a decision analysis model to perform cost-effectiveness analysis to determine the impact of an outbreak from a single index case that was not detected by hospital surveillance and was found in the general ward. The Markov decision model was built using Treeage software (www.treeage.com), and simulation was performed based on hospital staff and inpatients (n = 7,500) over a time horizon of 30 days. Each person

would transit between exclusive Markov states of Susceptible, Exposed, Incubation, Infectious, Isolated, Atypical, Recovered, or Dead. (Figure 1). The scenario assumes that all clinical cases will be identified and isolated. Infection is thus transmitted in the preclinical infectious phase or by atypical or subclinical cases not recognized and hence not isolated, as well as through the failure of personal protective equipment. Variables studied in the model included the number of persons exposed per infected patient, secondary attack rate, percentage of atypical and subclinical cases, duration of preclinical infectious period, and infectious period, as well as case-fatality rate.

Based on preliminary data available at the time of writing, comparisons were made between 3 respiratory viruses: a SARS-like virus, a 1918 Spanish influenza–like virus, and a pandemic (H1N1) 2009–type virus. Validation of the model was performed by comparing generated reproduction numbers to reported estimates from actual SARS data in Singapore (*10*) and Spanish influenza (for confined areas) (*11*) and showed consistent case and death numbers. We compared 4 different strategies: 1) no additional measures; 2) Green Alert response, which mandated personal protective equipment (PPE) for HCWs in direct contact with patients suspected of having avian influenza or other emerging infectious diseases; 3) Yellow Alert response, which mandated enhanced PPE at all high-risk areas; and 4) Orange Alert response, which mandated N95 masks for all patient contact and the restrictions described above (Table 1). Outcome measures were number of patients infected, number of deaths, cost (in US$) per case prevented, and cost per death prevented compared to baseline where no preventive measures were implemented, as well as incremental cost per death averted compared with the corresponding lower level of alert status. Multivariate sensitivity analysis was performed to understand the impact of viral characteristics as well as different hospital response policies on cost-effectiveness outcomes. The number of persons exposed in hospital and protective gear failure rates were from an actual outbreak simulation exercise performed at our hospital (*12*). Because there were no cases of pandemic (H1N1) 2009 virus infections during this period, estimates were all based on those in the literature (*13*). Details of the input variables are included in Table 2.

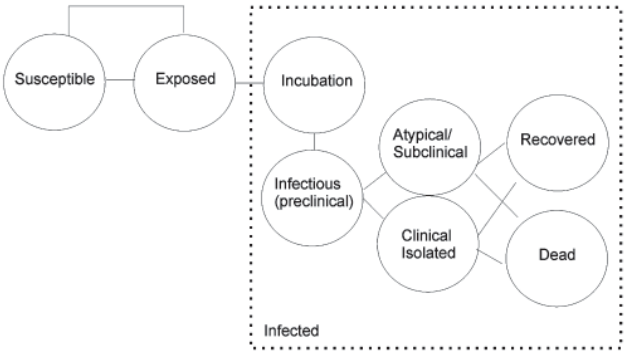

Figure 1. Markov model simulating a stochastic simulation of epidemics approach for an outbreak in a hospital institution.

Results

An outbreak of pandemic (H1N1) 2009 from introduction by an HCW, a patient with undiagnosed infection, or a visitor in our hospital at base case with no protection measures will result in 2,580 infected patients at 30 days. This finding would be similar to that of seasonal influenza and correspond to a 30% attack rate. With a 0.4% mortality rate, there would be 10 deaths from infection with pandemic (H1N1) 2009 virus. In contrast, Spanish influenza would result in 3,210 infected patients and 161 deaths (case-fatality rate 5%). The increased number of infections in the Spanish influenza model is driven by the short incubation time of the epidemic and results in more rounds of infection rather than an increase in basic reproductive number (average number of secondary cases per index case) (*14*). On the other hand, because SARS has a longer incubation period and lower transmissibility rate, the number of infected patients is lower at 825 but, owing to the high case-fatality rates, 82 deaths may ensue (Table 3).

Green Alert status mandates PPE for HCWs in direct contact with patients suspected of having the infection. Transmission will thus be only through preclinical cases before they are identified and patients can be isolated or through atypical or subclinical cases that are missed. We assumed pandemic (H1N1) 2009 has a lowered 50% transmissibility for atypical or subclinical cases (*15*); this rate effectively reduced the infected patients to 316 with only 1 death (Figure 2, panel B). This resulted in additional costs of $95 to prevent 1 additional infected patient and $23,600 to prevent 1 death. Moving to Yellow Alert would reduce infected patients to 59 and avert all deaths. The costs to prevent additional infection and death are $3,221 and $828,000, respectively. Activating Orange alert with full PPE gear, restricting visitors, and cancelling elective procedures would halve the infections to only 24 cases with no deaths. However, the additional cost over Yellow Alert would escalate to $7,153 per infection prevented and a staggering $2.5 million to infinity for 1 death averted (Figure 3).

Simulation for Spanish influenza showed a decreased number of deaths from 31 at Green Alert to 6 at Yellow Alert and 3 at Orange Alert (Figure 2, panel B). This finding translated to $50,000 per death averted moving from Green to Yellow and $153,000 per death averted moving from Yellow Alert to Orange. For SARS, on the other hand, the incremental cost of moving from Green Alert to Yellow

Table 2. Variables used in Markov mode (base case and sensitivity analysis) to compare outbreak estimates, Singapore*

Variable	Description	Base case	Sensitivity analysis
Exposure	No. persons exposed in 1 day in hospital per index case (nonlinear)	15 (average for 2 days) 6 (average for 5 days)	2–30
Secondary attack rate	No. persons exposed/infected	30% Spanish influenza 10% SARS 30% pandemic (H1N1) 2009	10–100%
Incubation period	Time to symptoms	Spanish influenza: 2 days SARS: 4 day Pandemic (H1N1) 2009: 3 days	1–7
Infectious period preclinical	Incubation–latent	Spanish influenza: 1 day SARS: 0 day Pandemic (H1N1) 2009: 1 day	1–3
% Clinical versus asymptomatic		Spanish influenza: 95% SARS: 100% Pandemic (H1N1) 2009: 95%	70–100%
% Atypical (missed)		Spanish influenza: 5% SARS: 20% Pandemic (H1N1) 2009: 5%	0–50%
% Complication		10× mortality rate	
Infective atypical	Infective	4 days	1–7
Case-fatality rate	% death	Spanish influenza: 5% SARS: 10% Pandemic (H1N1) 2009: 4%	
Isolation failure	Transmission despite PPE/isolation	5%	0–10%
Exposure reduction	% reduction in exposure rate	Alert Green 50% Alert Yellow 80% Alert Orange 90%	0–100%
Cost based on alert policy, direct and indirect	Once Daily recurring	Activation: US$110,000 Green: US$4,000 Yellow: US$76,000 Orange: US$100,000	
Cost by type of treatment, based on actual financial charges	Isolation Treatment antiviral/day Uncomplicated influenza Complicated influenza Respiratory failure with mechanical ventilation	US$230 US$25 Mean: US$600, Median: US$420 Mean: US$1800, Median: US$220 Mean: US$5,500, Median:US$4,660	

*SARS, severe acute respiratory syndrome; PPE, personal protective equipment.

is $120,000 per death averted; this drops to $75,000 when moving from Yellow to Orange. This finding is mainly due to the high (10%) case-fatality rate and the relatively higher percentage of atypical patients who are missed and not isolated, a lesson learned from the actual SARS experience (Figure 3).

Sensitivity analysis showed that the factors that impacted the cost-effectiveness ratio most are case-fatality rate, patient exposure rate, and secondary attack rate (Figure 4). In the pandemic (H1N1) 2009 scenario, the case fatality-rate ranging from 0.1% (seasonal influenza) to 10% (SARS) results in the cost per death averted moving from infinity (no death) to $35,000 per death averted (Orange Alert). Similarly, changing the exposure rate from 1.5 persons/day (10% PPE failure rate, Orange Alert) to 30 persons/day (0% reduction) per infected patient changed the incremental cost-effectiveness ratio from $2.5 million per death averted to $23,000. If pandemic (H1N1) 2009 had a higher 50% transmission rate, Orange Alert would become the most cost-effective strategy. The other variables had an impact on cost per case prevented but did not impact the incremental cost per death averted ratio.

To determine the impact of hospital size on our model, we modeled our simulation on the nation's other tertiary hospital with 1,500 beds using their actual cost records. The model estimates that 10 expected deaths in the outbreak would be reduced to 1 death under Green Alert and none in Yellow and Orange Alerts. The incremental cost/death averted is $32,000, $1.9 million, and $5.4 million when moving from Green to Yellow to Orange, respectively. Although the cost ranking is consistent with that predicted by base-case simulation, the actual incremental cost index is much higher, reflecting the higher cost for activating alert status in a bigger hospital.

Discussion

Singapore and many of the other countries badly affected by the SARS epidemic of 2003 launched comprehensive

Table 3. Results of cost-effectiveness analysis of potential outbreaks and responses, Singapore*

Alert level and disease	No. infected	No. deaths	Additional cost	Cost/case prevented†	Cost/death prevented†	Incremental cost/case‡	Incremental cost/death‡
None							
Pandemic (H1N1) 2009	2,580	10	25,200				
Spanish influenza	3,210	161	80,000				
SARS	825	83	99,200				
Green							
Pandemic (H1N1) 2009	316	1	326,430	95	23,644		
Spanish influenza	624	31	468,000	107	2,140		
SARS	105	11	220,500	120	1,195		
Yellow							
Pandemic (H1N1) 2009	59	0.2	1,485,500	414	103,274	3,221	827,907
Spanish influenza	120	6	2,212,000	493	9,857	2,472	49,829
SARS	43	4	1,188,000	995	9,945	11,146	121,241
Orange							
Pandemic (H1N1) 2009	24	0.1	1,836,000	506	126,807	7,153	2,503,600
Spanish influenza	59	2.95	2,856,000	629	12,590	7,541	153,333
SARS	12	1.2	1,537,000	1,263	12,601	8,041	7,541

*SARS, severe acute respiratory syndrome. All costs given in US$.
†Compared with no policy.
‡Compared with 1 alert level down.

pandemic response plans based on a SARS model (*5*). The lessons of the SARS epidemic, in particular the effect of protecting HCWs from patients with undiagnosed, unisolated respiratory viral infections (*16*,*17*), have been applied rigorously to the pandemic plans of the Singapore Ministry of Health. Although it is difficult to quantify the impact of these interventions when they are taken as a whole, data from our modeling show that a nuanced approach that concentrates on administrative measures to isolate patients and selectively use PPE when working with patients suspected of having novel strains of pandemic (H1N1) 2009 virus would have a relatively favorable cost-effectiveness ratio.

On the other hand, the psychological and economic impact of SARS has been described as one of Singapore's most traumatic experiences and one that left deep scars on the healthcare system of this country (*18*). It could be and has been argued that a draconian approach that seeks to protect all HCWs fully ensures that every case of influenza is identified early and contacts traced. Any healthcare facility ensuring no second- or third-generation transmission would provide intangible gains that exceed the economic costs of such a strategy. Nevertheless, this desire must still be balanced against the community impact of a disease such as influenza, which has a different epidemiology than SARS (*7*). We currently believe that pandemic (H1N1) 2009 virus causes predominantly community-based disease (*13*). Data from the United States, where infection control recommendations (*19*) are similar to our DORSCON Green, have not shown any evidence to date of large nosocomial outbreaks.

In our model, we have shown that cost-effectiveness ratio is dependent on the interplay between exposure rate, transmissibility (secondary attack rate), case-fatality rate, and risk of transmission from atypical cases. Infectious diseases with high fatality rates and transmission from atypical cases (such as SARS) will need the full benefit of PPE to reduce mortality rates. This finding is reflected in Orange Alert having a better cost-effectiveness ratio than Yellow Alert. Mild diseases with low fatality rates, such as pandemic (H1N1) 2009, and low incidence of atypical or subclinical infectious cases have the best cost-effectiveness ratio at Green Alert provided surveillance measures are able to identify infected patients and isolate them early. The cost-effectiveness ratio increases exponentially after that due to the much higher costs incurred. However, although Yellow Alert comes at a heavier price tag, it effectively averts any deaths. Activating Orange Alert increases cost with minimal benefit in mortality rate reduction. In reality, our model suggests that parallel efforts in contact tracing and voluntary quarantine may further reduce the exposure rate and break the chain of transmission.

Our base model took into account only direct costs associated with each alert status. In real situations, indirect costs such as lost revenues from cancellation of elective surgeries to free up hospital resources, decreased elective admissions and outpatient attendances, administrative costs associated with senior staff meetings, and lost clinical teaching time, add up to more than the direct costs and would magnify the incremental cost-effectiveness ratio further. In fact, if direct and indirect costs were included in the modeling, the incremental cost/death averted ratio of moving from Yellow Alert to Orange in pandemic (H1N1) 2009 increased to a staggering $8–$81 million for both hospitals. Although these indirect costs are not part of the infection control process per se, surge capacity response plans to ensure that the healthcare system has the reserve

capacity to react to a full-blown community outbreak are critical to all pandemic plans (*20*) and contribute serious costs to the hospital.

The major limitations of our study are that we have simulated a situation in which community infection is still relatively low and the outbreak in hospital arises from 1 index case. When a community epidemic is established, the incidence of new index cases entering the institution increases, especially if there are prevalent atypical or subclinically infected persons. In such a scenario the cost-effectiveness ratio of higher alert status will decrease, and it may become more beneficial to escalate protective measures.

Cost-effectiveness analyses merely provide a mathematical projection to better understand the key factors that affect outcomes. The actual magnitude of the cost-effectiveness will vary depending on institutional cost, which varies between different sized hospitals and whether direct or indirect costs are included. Nonetheless, knowledge of the exponential relationship of the different viruses on the cost-effectiveness ranking is critical in charting response policy. Indirect costs of an uncontrolled pandemic are also economic and social, especially in Singapore where the economy is dependent on trade and tourism. A higher cost-effectiveness ratio does not imply that additional lives are not worth saving. In the case of pandemic (H1N1) 2009, if it costs $2.5 million to prevent 1 death, using a median age of 37 years for persons who died (*21*) and expected life expectancy of 80 years (*22*), the incremental cost-effectiveness ratio works out to $40,000 per life-year saved. In addition, preventive measures go beyond saving lives and include resultant savings from reducing hospitalization of infected patients and prolonged intensive care with mechanical ventilation for severe cases, as well as the logistic costs of further contact tracing and quarantine.

We have not factored the cost of influenza antiviral prophylaxis or the costs and effectiveness of novel vaccinations that may be required, nor did we include the costs of work-days lost from staff taking medical leave due to their

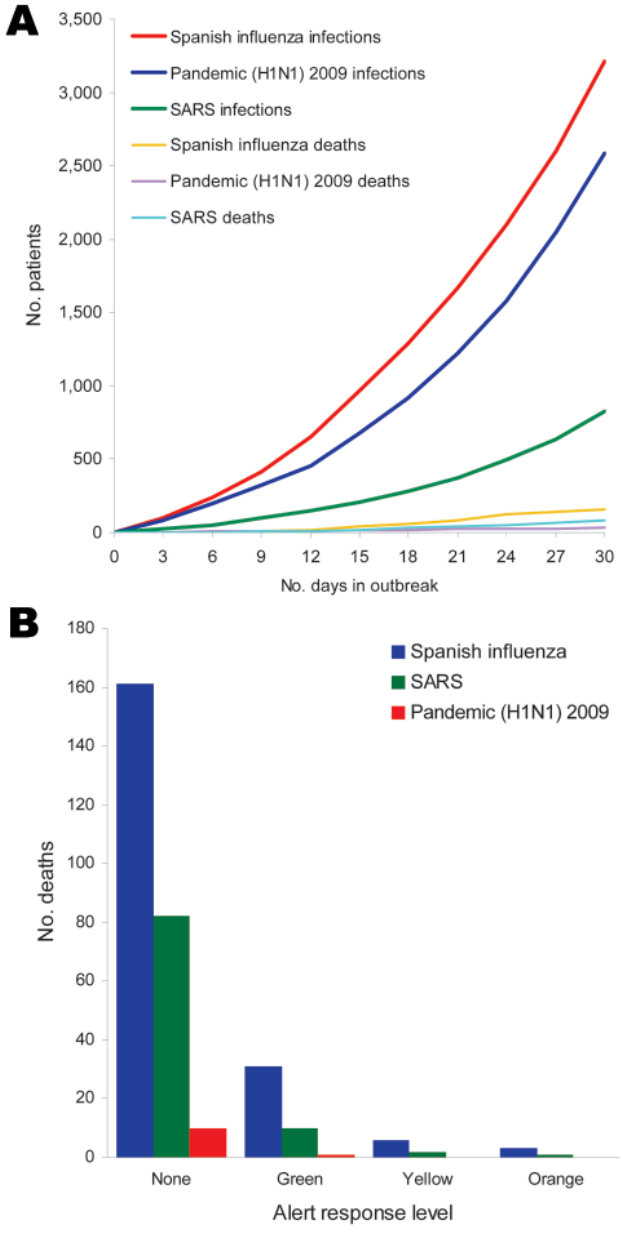

Figure 2. Epidemic simulation. A) Base case simulation assuming no protection over 30 days (n = 7,500). B) Number of deaths for pandemic (H1N1) 2009, Spanish influenza, and severe acute respiratory syndrome (SARS) with different levels of alert status.

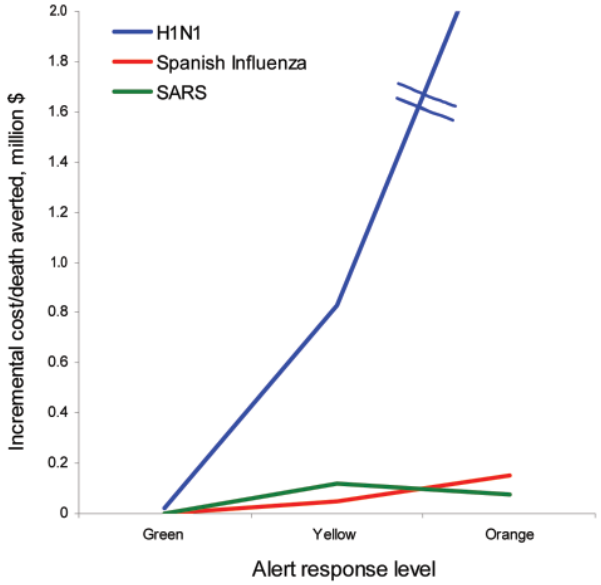

Figure 3. Incremental cost/death for 3 viruses with different alert status. Incremental cost to avert 1 additional death moving through ascending levels of alert status. Cost-effectiveness increases exponentially for pandemic (H1N1) 2009 while maintaining an almost linear fashion for both Spanish influenza and severe acute respiratory syndrome (SARS). The incremental cost/death averted ratio is lower for Alert Orange compared to Alert Yellow for SARS.

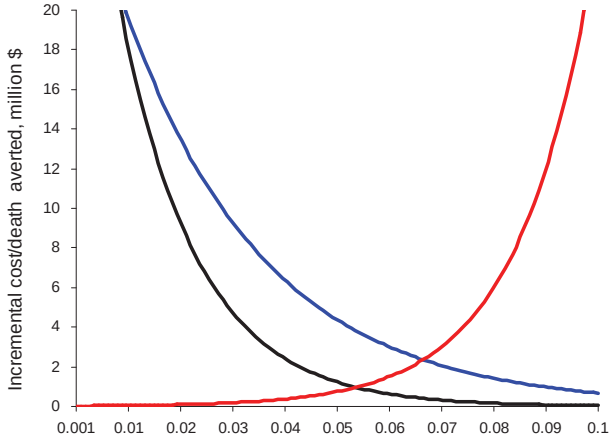

Figure 4. Sensitivity analysis for case-fatality rate (black line), % exposure reduction (red line), and secondary attack rate (blue line). Exponential graphs show poor cost-effectiveness at extremes of low case-fatality rate and low transmissibility (high % exposure reduction and low secondary attack rate).

being infected or being placed in quarantine. The impact of lax border controls, subclinical patients carrying the virus into the community, and closure of community institutions or even hospitals due to an outbreak were also not computed. We assumed that the hospital is a closed community with a fixed number of staff and patients. This obviously is not true in real life but is mitigated in our analysis because the same assumption is applied to every response measure and the outcomes are incremental indices over another level of protection.

From the perspective of a healthcare institution, how do we predict the virulence of new virus early in the outbreak and adopt the most cost-effective response policy? If a mild epidemic spreads rapidly through the community, there might be multiple points of entry into the hospital; however, such a mild community outbreak might present more commonly to primary healthcare clinics and presentations to hospital may be few. Thus a step-up approach from Green to Yellow in accordance with predicted risks as we have shown may be the most cost-effective approach.

It is not known for certain how pandemic (H1N1) 2009 will behave in subsequent waves. Although the new virus seems to have relatively low virulence, the virus might re-emerge with a case-fatality rate more like that of the 1918 influenza pandemic or the SARS pandemic. Our model shows that DORSCON Green, which focuses on infection control for suspected cases, will achieve a relatively high degree of protection for our staff, patients, and visitors even in the setting of a higher case-fatality rate. The main advantage of DORSCON Yellow and Orange is that undetected infected persons that are not isolated are less likely to become a source of transmission if there is universal use of N95 masks. This has to be balanced with the degree of compliance that can be achieved by the use of full-scale PPE for patients with no risk of the disease (e.g., patients with trauma or other medical or surgical conditions) and the well known adverse effects of prolonged use of N95 masks (*23*).

However, it is useful to also note that although a step-up approach may be the most cost-effective for the healthcare institution, the appropriate policy stance at the national level may not necessarily be the same. Our model did not take into account the psychological and economic impact to the country and the larger healthcare system, which are serious factors to consider when making a policy decision on the appropriate response across the healthcare system. Singapore, Hong Kong, and China were among the settings most severely affected by the SARS outbreak in 2003. In the initial face of an unknown virus with a perceived high mortality rate in Mexico, Singapore's response to first err on the side of safety and make adjustments dynamically as the situation became clearer therefore would be reasonable when viewed from the larger perspective.

Such actions, however, were not without their own adverse effects in terms of cost and in overall patient care at the healthcare institution. We had the opportunity to perform a cost-effectiveness analysis using the actual costs incurred from this heightened infection control response. We have quantified how the virulence or case-fatality rate of a respiratory viral infection has a serious impact on the hospital infection control response. This impact occurs at 2 levels, first, the actual number of deaths and ill persons, and second, the direct and indirect costs on the hospital in terms of activation, logistics, and lost revenue. This impact is reflected in the subsequent responses of Singapore and other countries when the virulence of the novel influenza virus appeared to be much less than previously feared. Understanding the key factors that affect the cost-effectiveness ratio will enable us to make better informed decisions as we prepare to respond to future epidemics.

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Possible Interruption of Malaria Transmission, Highland Kenya, 2007–2008

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Highland areas where malaria transmission is unstable are targets for malaria elimination because transmission decreases to low levels during the dry season. In highland areas of Kipsamoite and Kapsisiywa, Kenya (population ≈7,400 persons), annual household indoor residual spraying with a synthetic pyrethroid was performed starting in 2005, and artemether/lumefantrine was implemented as first-line malaria treatment in October 2006. During April 2007–March 2008, no microscopy-confirmed cases of malaria occurred at the sites. In 4 assessments of asymptomatic persons during May 2007–April 2008, a total of <0.3% of persons were positive for asexual *Plasmodium falciparum* by microscopy or PCR at any time, and none were positive by PCR at the last 2 sample collections. Our findings show that in such areas, interruption and eventual elimination of malaria transmission may be achievable with widespread annual indoor residual spraying of households and artemisinin combination therapy.

Widespread implementation of malaria control interventions such as insecticide-treated bed nets (ITNs) and artemisinin combination therapy (ACT) have resulted in dramatic reductions of transmission in areas where this disease is endemic (*1*–*3*). For the first time since the 1950s, the World Health Organization and other organizations are promoting malaria eradication (*4*). In highland areas (>1,500 m above sea level) in Africa, malaria transmission is unstable, with a low incidence of malaria during dry seasons (*5*). These areas may be ideal initial targets for attempting the interruption of malaria transmission. Also in these areas, the combination of ACT, which may decrease transmission by reducing gametocyte load in infected persons, and annual indoor residual spraying (IRS) with long-lasting insecticides, which can reduce indoor vector density for a prolonged period, could act synergistically to interrupt malaria transmission.

We have conducted malaria epidemiology studies in the adjoining highland areas of Kipsamoite and Kapsisiywa, Nandi Hills District, Kenya, since 2003. Starting in 2005, the Ministry of Health of Kenya introduced new interventions to reduce malaria transmission and improve malaria treatment in these areas. Interventions introduced included IRS, distribution of ITNs to pregnant women and their children ≤5 years of age, and provision of artemether/lumefantrine (co-artemether) as first-line therapy for uncomplicated malaria. The present study documents the changes in malaria transmission that occurred during the period of these interventions and provides evidence that malaria transmission was interrupted during April 2007–March 2008.

Methods

Study Site

The study was conducted in the adjacent highland areas of Kipsamoite (7 villages) and Kapsisiywa (9 villages) in the Nandi Hills District of Kenya. Study site characteristics have been described (*6*). Elevation ranges from 1,829 m to 2,132 m. In both areas, malaria transmission is unstable. A malaria epidemic occurred in Kipsamoite in 2002 (*7*). Historically, Kapsisiywa has had more malaria cases than Kipsamoite, likely because its elevation is lower, a swamp surrounds it, and it has more areas where water is likely to pool (*6*).

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Study Population and Recruitment

Persons living in these areas are predominantly Nandi, a Kalenjin subtribe. Demographic analysis, malaria surveillance, and collection of weather and vector density information were performed in these areas as part of 2 related studies, a study of malaria early warning systems and a study of malaria transmission and immunity. Written informed consent for study participation was obtained from consenting heads of households in the area for demographic studies and from persons (or parents/guardians of persons <15 years of age) for other studies. Ethical approval for the study was obtained from the Kenya Medical Research Institute National Ethical Review Committee and the Institutional Review Boards for Human Studies at Case Western Reserve University, the Centers for Disease Control and Prevention, and the University of Minnesota.

Demography and Surveillance for Clinical Malaria and Asymptomatic Persons

Demographic surveys of all households were started in April 2003 and conducted every 4 to 6 months. Starting in 2005, these surveys included assessments of travel, ITN use, and IRS treatment of houses. Clinical malaria surveillance was conducted during 2003–2008 Kenyan Ministry of Health dispensaries, the only healthcare facilities within the study area. Free malaria diagnosis and treatment were available to all persons with symptoms of malaria. Persons with symptoms of malaria (fever, chills, severe malaise, headache) who did not have a clear alternative diagnosis by history and physical examination were assessed for malaria by microscopic examination of blood smears.

Clinical malaria was defined as symptoms of malaria and a positive blood smear for *Plasmodium falciparum* or *P. malariae*. Primary treatment for uncomplicated malaria was given according to Kenya Ministry of Health provisions for the clinics: sulfadoxine-pyrimethamine (2003–2004), amodiaquine (2004–2006), and co-artemether (2006–2008). Because of changes in studies, forms, and clinic personnel, clinical surveillance was not conducted in January and July–November 2006, in Kipsamoite; and July–December 2005, and January, March, and June–November 2006, in Kapsisiywa.

Four surveys for parasitemia in asymptomatic persons were conducted during 2007–2008. In May 2007, samples were collected from all consenting persons who were living on site during the time of collection (5,788 persons). In August 2007, samples were collected from 605 persons randomly selected from the overall population for active surveillance of malaria. Testing was repeated in samples from 577 and 538 of these persons in the cohort in November 2007 and April 2008, respectively. PCR was performed on 400 randomly selected samples from each period and on any sample that was parasite positive by microscopy.

Microscopy and PCR

Microscopy testing for *Plasmodium* species was performed as described (*7*) by using 2 independent readings and a third reading for slides with discordant results. For PCR testing, genomic DNA was isolated with a QIAamp 96 DNA blood kit (QIAGEN Inc., Valencia, CA, USA) from dried blood spots collected on Whatman 903 filter paper (Whatman Corporation, Florham Park, NJ, USA). *P. falciparum* infection was detected by nested PCR specific for the small subunit RNA gene as described (*8*). In previous surveys in these areas, we documented low frequencies of *P. malariae* infection (<1%) and no *P. vivax* or *P. ovale* infections (*7*,*9*) by microscopy and PCR. Thus, we did not further test for these infections.

Assessment of Rainfall, Temperature, and Indoor Resting Vector Density

Daily rainfall was measured with standard metal rain gauges, and daily minimum and maximum temperatures were recorded with maximum/minimum mercury thermometers placed in 7 villages in Kipsamoite. Values from the 7 villages were averaged.

Household indoor resting density of *Anopheles* spp. was measured in 4 cluster areas at each site; each cluster area comprised approximately equal numbers of persons. Three index households were randomly selected for each cluster, and the index house plus its 4 nearest neighbors were sampled. Sixty households in Kipsamoite and 60 households in Kapsisiywa were sampled every 2 weeks. Pyrethrum knockdown captures were conducted by using standard methods (*10*). *Anopheles* spp. mosquitoes were identified taxonomically, first in the field by trained field assistants and then by an entomologist from the Kenya Medical Research Institute.

Data Analysis

Annual malaria incidence, rainfall, temperature, and vector density during April 2003–March 2004, were used as baseline data and compared with data from subsequent years beginning in April 2004. Annual malaria incidence was compared by using negative binomial regression analysis, except for April 2007–March 2008, when it was compared by Fisher exact test because no cases occurred during that year. For years with missing months of incidence data, malaria incidence in the recorded months was compared with incidence in the same months in the reference year (April 2003–March 2004). For periods when no cases were detected, a 1-sided 95% confidence interval was constructed. Mean daily temperature and mean monthly rainfall were compared by using the 2-sample *t* test. Me-

dian monthly vector density was compared by using the Wilcoxon rank-sum test. All data analysis was conducted by using Stata version 10.0 software (Stata Corp., College Station, TX, USA).

Results

IRS, ITNs, and Malaria Treatment with Co-artemether at Health Centers

The population ranged from 3,250 to 4,253 in Kipsamoite and from 3,412 to 3,841 in Kapsisiywa. The Ministry of Health first conducted limited indoor residual spraying in 2005. The number of households sprayed increased markedly in 2007 (Table 1). The primary insecticide used in both sites was lambda cyhalothrin at a dose of 10–20 mg/m^2.

ITNs were distributed per Ministry of Health policy to pregnant women and their children ≤5 years of age when the women came to the health centers for antenatal care. A copayment was required in most instances. Short-lasting nets treated with α-cypermethrin were provided during 2005–2006, and long-lasting nets treated with deltamethrin or permethrin have been provided since 2006. Persons were asked, "Have you slept under a treated bednet regularly since the last demography survey?" During 2006–2008, the percentage of persons who reported sleeping under an ITN decreased from 17.3% to 11.1% in Kipsamoite and from 27.9% to 15.1% in Kapsisiywa.

Co-artemether became official first-line treatment for uncomplicated malaria by Kenyan Ministry of Health policy in May 2004, but it was not implemented in these sites until October 23, 2006 (Kapsisiwya) and February 6, 2007 (Kipsamoite). Co-artemether was also not widely available in local shops or neighboring clinics before these dates.

Table 1. Indoor residual spraying of households in Kipsamoite and Kapsisiywa, Kenya, 2005–2007

Year, area	Months of spraying	No. households sprayed/total (%)
2005		
Kipsamoite	Apr–May	37/770 (4.8)
Kapsisiywa	Apr–Jun	374/713 (52.5)
2006		
Kipsamoite	Feb–May	119/786 (15.1)
Kapsisiywa	Feb–May	327/716 (45.70
2007		
Kipsamoite	Apr–Jul	545/773 (70.5)
Kapsisiywa	Apr–Jun	656/690 (95.1)

Malaria and Symptoms Consistent with Malaria, 2003–2008

When compared with the April 2003–March 2004 reference year, malaria incidence in Kipsamoite increased in 2004, returned to 2003 levels in 2005, and decreased in 2006 (Table 2, Figure 1, panel A). In contrast, malaria incidence decreased slightly in Kapsisiywa in 2004 and then decreased in 2005 and 2006 (Table 2, Figure 1, panel B). Malaria incidence in Kapsisiywa was higher than in Kipsamoite in 2003 ($p<0.001$) and 2004 ($p = 0.06$), but not in any other year. At both sites during March 20, 2007–March 30, 2008, microscopically confirmed cases of malaria were found among symptomatic persons (Table 2, Figure 1, panels A and B).

Overall clinic attendance, although seasonal and variable, did not decrease during 2007–2008 at either site (Figure 1, panels C and D). During March 20, 2007–March 30, 2008, when 0 of 416 symptomatic persons had evidence of *P. falciparum* parasitemia by microscopy (0%, 1-sided 95% confidence interval 0%–0.9%), 17 (7.3%) of 231 symptomatic persons who provided blood samples for PCR testing were positive for *P. falciparum*. All PCR-positive samples were obtained during March–June, 2007, except

Table 2. Annual cumulative incidence of *Plasmodium falciparum* malaria in Kipsamoite and Kapsisiwya, Kenya, April 2003–March 2008*†

	Kipsamoite			Kapsisiywa		
Year	Cumulative incidence	Incidence ratio (95% CI)	p value	Cumulative incidence	Incidence ratio (95% CI)†	p value
2003 Apr–2004 Mar	23.20	Ref	Ref	106.03	Ref	Ref
2004 Apr–2005 Mar	42.53	1.83 (1.36–2.47)	<0.001	82.58	0.78 (0.66–0.91)	0.002
2005 Apr–2006 Mar	18.79‡	0.81 (0.57–1.16)§	0.229	8.03¶	0.10 (0.07–0.15)#	<0.001
2006 Apr–2007 Mar	9.30**	0.47 (0.30–0.71)††	<0.001	8.99‡‡	0.19 (0.12–0.27)§§	<0.001
2007 Apr–2008 Mar	0.00	0.00	<0.001	0.00	0.00	<0.001

*CI, confidence interval; Ref, reference.
†Annual cumulative incidence/1,000 persons was compared by using negative binomial regression, except for 2007–2008, which was compared by using Fisher exact test.
‡Available data were for all months except January 2006.
§Compared with same months in 2003–2004 (incidence 23.20/1,000).
¶Available data were for April–June 2005 and February 2006.
#Compared with same months in 2003–2004 (incidence 79.41/1,000).
**Available data were for April–June 2006 and December 2006–March 2007.
††Compared with same months in 2003–2004 (incidence 19.99/1,000).
‡‡Available data were for April–May 2006 and December 2006–March 2007.
§§Compared with same months in 2003–2004 (incidence 48.19/1,000).

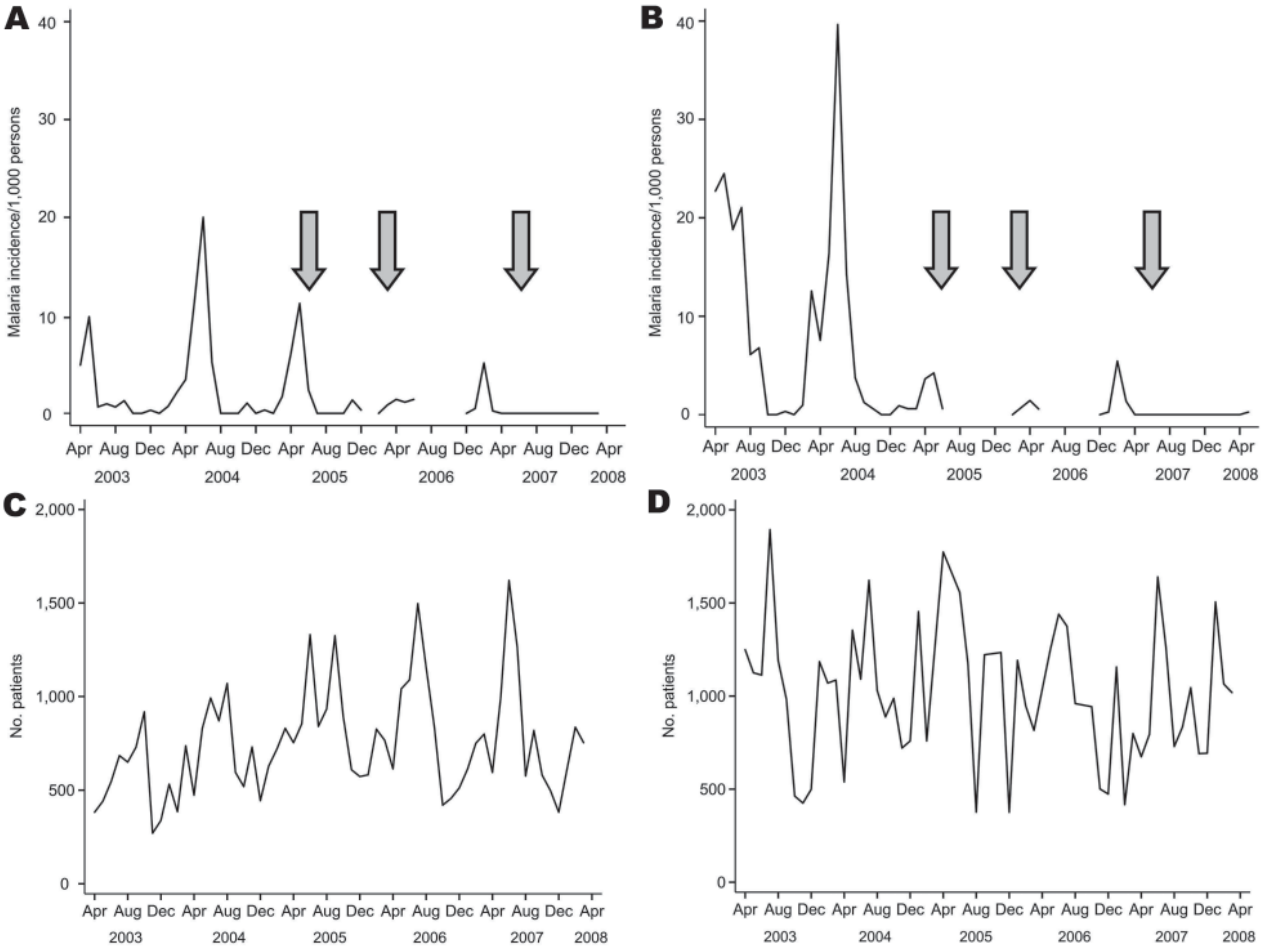

Figure 1. Malaria incidence and number of patients seen at health dispensaries in 2 highland areas of western Kenya, April 2003–March 2008. A) Monthly incidence of malaria/1,000 persons in Kipsamoite. B) Monthly incidence of malaria/1,000 persons in Kapsisiywa. C) No. patients who came to the Kipsamoite health dispensary. D) No. patients who came to the Kapsisiywa health dispensary. Gaps in panels A and B indicate that no data were collected during these periods. Arrows indicate when indoor residual spraying was conducted in the 2 areas.

for 2 samples, which were obtained in December 2007. In symptomatic persons, gametocyte prevalence, assessed by microscopy, was low in all years (2.8%, 2.9%, 0.8%, 0.8%, and 0% in Kipsamoite and 0.4%, 1%, 0%, 0%, and 0% in Kapsisiywa during 2003, 2004, 2005, 2006, and 2007, respectively).

Asexual Parasitemia and Gametocytemia in Asymptomatic Persons, 2007–2008

In 4 surveys of asymptomatic persons during 2007–2008, a total of <0.3% were positive for *P. falciparum* trophozoites or gametocytes by microscopy during any period. In the last 2 periods, no person was positive for asexual *P. falciparum* by PCR (Table 3). The person positive for *P. falciparum* trophozoites by microscopy in April 2008 was also positive for gametocytes. PCR showed that this person did not have an asexual *P. falciparum* infection.

Changes in Rainfall, Temperature, and Vector Density, 2003–2008

During 2003–2008, no consistent pattern of increased or decreased temperature was shown, and the average daily temperature during 2007–2008, the year in which no malaria cases were documented by microscopy, was most similar to that in 2003, the year of highest malaria incidence (Figure 2, panel A, Table 4). Average monthly rainfall did not differ between years (Figure 2, panel B, Table 4). A decrease in anopheline mosquito density was seen in 2004, before any widespread IRS treatment program, but sustained low levels of mosquito density were first seen after IRS treatments (Figure 2, panel C; Table 5). Of 447 anopheline mosquitoes caught over this 5-year period, 439 (98.21%) were identified as *An. gambiae*, 6 (1.34%) were identified as *An. funestus*, and 2 (0.45%) were not identified.

Table 3. Prevalence of *Plasmodium falciparum* parasitemia by microscopy and PCR in asymptomatic persons in Kipsamoite and Kapsisiywa, Kenya, May 2007–April 2008

Characteristic	2007 May	2007 Aug	2007 Nov	2008 Apr
Microscopy				
No. *P. falciparum* positive	14*	0	0	1
No. tested	5,788	605	577	538
% *P. falciparum* positive	0.24	0	0	0.19
Gametocytes				
No. positive	5†	0	0	1
No. tested	5,788	605	577	538
% positive	0.09	0	0	0.19
PCR				
No. *P. falciparum* positive	1	1	0	0
No. tested	414	400	400	401
% *P. falciparum* positive	0.24	0.25	0	0

*Twelve persons who tested positive by microscopy were from Kipsamoite and 2 were from Kapsisiywa.
†Four persons who tested positive by gametocyte were from Kipsamoite and 1 was from Kapsisiywa.

Discussion

Implementation of highly effective methods of decreasing malaria transmission, including ITNs, IRS, and ACT, has led to renewed discussion about global eradication of malaria (*4*,*11*). A major goal in moving toward eradicating malaria is interruption or elimination of local malaria transmission. The World Health Organization has stated that "elimination has been achieved when the 'prevention of reintroduction', without local transmission by mosquitoes, has been successful for three or more consecutive years" (*12*). Interruption of local transmission is the step before elimination, in which it is documented that local transmission of malaria is absent in a previously malaria-endemic area for a specific period. The present study provides microscopy evidence of interruption of local malaria transmission in 2 adjacent highland areas of unstable transmission. Malaria could recur in these areas, and it is unclear precisely how much specific factors (e.g., IRS, ACT, and changes in rainfall and temperature) affected malaria transmission and incidence. Overall, however, data support the idea that in unstable transmission settings, combining regular, widespread IRS campaigns and use of ACT as first-line antimalarial treatment has the potential to interrupt local malaria transmission.

IRS probably played a major role in reducing malaria transmission in these areas for several reasons. First, sustained decreases in indoor resting *Anopheles* spp. mosquito density were seen after the IRS campaigns. Second, in both sites, malaria incidence decreased only after IRS was widely applied. In Kipsamoite, no large reduction in malaria incidence was seen until 2007, when spraying covered >70% of households. In Kapsisiywa, ≈50% of households were sprayed in 2005 and 2006, and a large decrease in malaria incidence was observed in both years. In 2007, after >90% of households were sprayed, malaria transmission was interrupted for the subsequent year. Third, in contrast to IRS, 2 factors that could affect vector density and therefore malaria incidence, rainfall and temperature, showed no clear relationship with either vector density or malaria incidence.

A reduction in vector density was seen in both sites in 2003 before the IRS campaigns. Potential reasons for this reduction include an unusual decrease in temperature during July–September 2003 (Figure 2). This decrease in temperature coincided with the first decrease in vector density; another possible reason for the decrease was pyrethrum spray catch testing of anopheline vectors conducted by our team, which started in April 2003. Although spraying with short-term insecticide does not usually affect vector density in areas of high transmission, spraying of approximately one sixth of all households every 2 weeks may have had an effect on the adult vector population, which led to a smaller breeding pool and lower overall vector density in this area of low transmission. The decrease in malaria incidence in Kipsamoite in 2005, after only 15% of households were sprayed, may in part reflect the effects of greater spraying in neighboring Kapsisiywa. The decrease may also reflect the combined effect of partial coverage with ITNs and additional coverage by IRS. Reduction of incidence in Kipsamoite in 2005 was not caused by concentrated spraying in areas of malaria clustering (*5*) because spraying was nearly absent in these areas. Reductions in malaria incidence were seen in Kapsisiywa in 2005–2006 after spraying of 40%–50% of households, but the small peak in incidence seen subsequently in these 2 years, but not 2007 (Figure 1), suggests that for interruption, a higher percentage of households (>70%) must be sprayed.

Treatment of malaria patients with co-artemether reduces gametocyte carriage and density in children and makes them less infectious to mosquitoes than treatment with sulfadoxine-pyrimethamine plus chloroquine (*13*). The effect of co-artemether on gametocytes may have been synergistic with the effect of widespread IRS on the *Anopheles* spp. vector in reducing malaria transmission. In

symptomatic persons, gametocyte prevalence was always low, and it decreased before introduction of co-artemether, but it did not decrease to undetectable levels in Kipsamoite until after introduction of co-artemether. Among asymptomatic persons, studies during 1999–2002 generally demonstrated higher gametocyte prevalence (0%–5.7%) (*7*). Lower prevalence among asymptomatic persons in the current study (0%–0.2% during May 2007–April 2008) could reflect effects of co-artemether on gametocyte prevalence after introduction of co-artemether in late 2006–early 2007, but without interim data from 2002–2006, an association cannot be clearly demonstrated. As with IRS, however, absence of microscopy-positive malaria cases occurred only after introduction of co-artemether. Because co-artemether was first used during a time of low transmission of malaria, the contribution of ACT to the absence of malaria incidence could not be quantified in the present study. However, a much larger study in South Africa in which IRS treatment and ACT treatment of persons with clinical malaria were introduced sequentially demonstrated an additional reduction of malaria incidence after introduction of ACT (*14*) and this supports the idea of synergy between these 2 interventions.

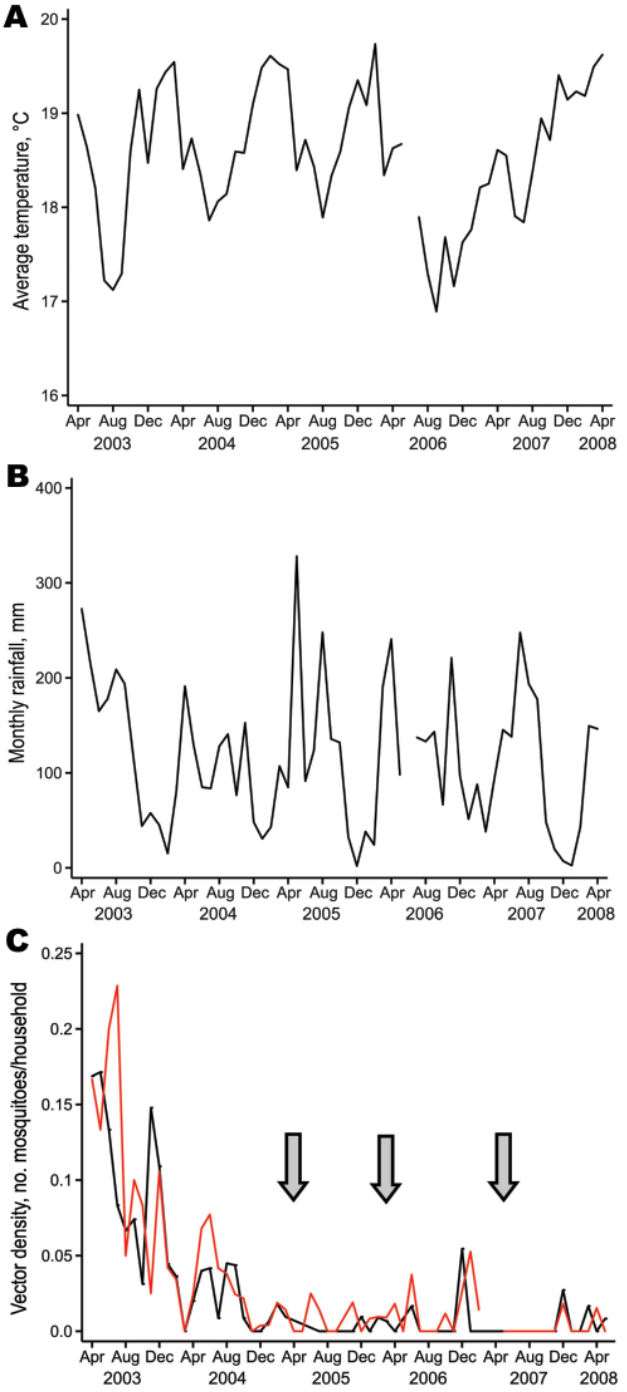

Figure 2. Temperature, rainfall, and vector density in 2 highland areas of western Kenya, April 2003–May 2008. A) Average daily temperature (°C) in Kipsamoite. B) Monthly rainfall (mm) in Kipsamoite. C) Median biweekly vector density (no. *Anopheles* spp. mosquitoes/household) in Kapsisiywa (red line) and Kipsamoite (black line). Gaps in panels indicate that no data were collected during these periods. Arrows indicate times when indoor residual spraying was conducted.

Although ITNs are the preferred intervention for preventing malaria-related illness and death in areas of high transmission (*1*,*15*,*16*), ITNs probably did not play a major role in interrupting malaria transmission in the highland areas we studied. ITN coverage never exceeded 30% in either area, and use actually decreased over the study period. In areas of unstable transmission, IRS treatment once a year is likely to be easier, more effective, and more accepted than ITNs. The Roll Back Malaria program currently recommends IRS as the preferred method of reducing malaria in areas of low transmission (*17*); our study supports this recommendation.

Insecticide treatment using IRS is not without problems; chief among them is potential development of resistance to the insecticide. Lambda-cyhalothrin, the insecticide used for IRS in these areas by the Kenyan Ministry of Health, was used in Mozambique for IRS starting in 1993, but resistance developed to such an extent that lambda cyhalothrin was replaced by bendiocarb in 2000. By 2006, however, lambda cyhalothrin resistance had decreased in many areas (*18*). In a recent study in nearby areas of western Kenya, no phenotypic resistance to pyrethroid insecticides was seen, but 27% of anophelines carried the knockdown resistance (*kdr*) mutation associated with increased resistance to pyrethroids (*19*). Assessment for insecticide resistance in the highland areas of the present study will enable better policy decisions to be made about continued use of lambda cyhalothrin, use of alternatives such as bendiocarb or DDT, or cycling of insecticides when certain resistance thresholds are reached for a particular insecticide. *P. falciparum* resistance to sulfadoxine-pyrimethamine was present in 27% of infections in western Kenya as early

Table 4. Average monthly rainfall and average daily temperature for Kipsamoite, Kenya, April 2003–March 2008*

Year	Mean (SD) monthly total rainfall, mm†	p value	Mean (SD) daily temperature, °C†	p value
2003 Apr–2004 Mar	132.8 (83.6)	Ref	18.5 (0.9)	Ref
2004 Apr–2005 Mar	101.4 (48.9)	0.274	18.7 (0.6)	0.510
2005 Apr–2006 Mar	119.2 (97.6)	0.717	18.8 (0.6)	0.346
2006 Apr–2007 Mar	130.5 (76.7)	0.957	17.8 (0.6)	0.039
2007 Apr–2008 Mar	105.2 (81.6)	0.422	18.8 (0.6)	0.352

*Ref, reference.
†Means were compared by using the 2-sample *t* test.

as 1999 (*20*). Resistance to co-artemether has not yet been documented in Kenya, but development of resistance to components of artemether/lumefantrine in nearby populations with higher levels of transmission would also pose a threat to this highland population. Monitoring of drug resistance to ACT in all areas in which malaria is endemic will be critical for sustaining reduction of malaria incidence in sub-Saharan Africa.

Limitations of our study include missing monthly malaria incidence data among symptomatic persons during 2005–2006, the observational nature of the study, and the possibility that lack of parasitemia by microscopy and PCR in symptomatic and asymptomatic persons was caused by seasonal variation common in highland areas (*21*) and not by interruption of local transmission. However, 3 pieces of evidence from studies of asymptomatic and symptomatic persons support interruption of local transmission rather than seasonal variation. First, no parasitemia was seen in 2 successive microscopy and PCR surveys of asymptomatic persons, whereas in 5 earlier surveys of asymptomatic persons in Kipsamoite, the area of lower transmission, the frequency of infected persons was never zero (range 5.9%–14.5% by PCR) (*7*). Second, over 7 years of clinic surveillance of symptomatic persons in Kipsamoite and 5 years in Kapsisiywa, there was never a >4-month period in which there were no microscopy-positive cases of *P. falciparum* before March 2007. Thus, even with seasonal variation, a year with no microscopy-positive cases in these areas is unprecedented. Third, absence of gametocytemia by microscopy was documented among asymptomatic persons in 2 of 4 assessments and among symptomatic persons for a year (April 2007–March 2008), which suggested that the potential for local transmission was low or absent.

Because reverse transcription–PCR methods for detection of gametocytes have documented higher rates of gametocyte infection than microscopy (*22*,*23*), we are developing this testing method in our laboratory to confirm the absence of gametocytemia in the most recent samples from study participants. The presence of asexual *P. falciparum* infection by PCR in 15 symptomatic persons during April–June 2007 could reflect prolonged detection by this more sensitive method. The presence of only 2 PCR-positive cases in a 9-month period (July 2007–March 2008) suggests that malaria transmission was either interrupted, if these cases were caused by patients' travel, or reduced to almost undetectable levels.

Sustained elimination of local malaria transmission in these areas will require ongoing surveillance of malaria incidence, anopheline vector density, and anopheline insecticide resistance, and correctly timed IRS campaigns with broad coverage of the area (*24*). The longer populations at these sites are unexposed to malaria, the more susceptible they are to malaria epidemics, which could occur if an increase in vector density occurs in conjunction with the arrival of infected persons or mosquitoes from an area of higher transmission of malaria. Because travel is increasingly frequent, true elimination of malaria in this and other highland areas will require reduction and eventual elimination of malaria in surrounding areas. Co-artemether must be consistently available to treat any infected and symptomatic travelers or immigrants to the area. Finally, as malaria cases decrease, microscopists will need to receive training to remain proficient in detection of malaria in blood smears.

In summary, this study demonstrates pronounced reduction and possible interruption of malaria transmission in 2 highland areas of Kenya for a 1-year period and pro-

Table 5. Median monthly *Anopheles* spp. mosquitoes per household in Kipsamoite and Kapsisiwya, Kenya, April 2003–March 2008*

	Kipsamoite		Kapsisiywa	
Year	Median monthly *Anopheles* spp. mosquito density (IQR)†	p value	Median monthly *Anopheles* spp. mosquito density (IQR)†	p value
2003 Apr–2004 Mar	0.079 (0.100)	Ref	0.091 (0.112)	Ref
2004 Apr–2005 Mar	0.014 (0.033)	0.003	0.023 (0.031)	0.005
2005 Apr–2006 Mar	0.000 (0.007)	<0.001	0.009 (0.012)	<0.001
2006 Apr–2007 Mar	0.000 (0.004)	<0.001	0.006 (0.018)	0.002
2007 Apr–2008 Mar	0.000 (0.000)	<0.001	0.000 (0.000)	0.002

*IQR, interquartile range (difference between 75th and 25th percentile values); ref, reference.
†Median vector density was compared by using the Wilcoxon rank-sum test.

vides evidence that interruption of transmission was related to widespread annual IRS insecticide treatment and use of ACT as first-line treatment for uncomplicated malaria. Although both areas remain at risk for recurrence of malaria epidemics, our study provides evidence that interruption and eventual elimination of malaria in areas of unstable transmission may be achievable.

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Community-associated Methicillin-Resistant *Staphylococcus aureus* in Outpatients, United States, 1999–2006

Eili Klein, David L. Smith, and Ramanan Laxminarayan

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Learning Objectives

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

- Specify characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) compared with hospital-acquired MRSA
- Recognize recent trends in MRSA among outpatients
- Identify anatomic sites most commonly associated with infection with MRSA resistant only to oxacillin
- Recognize recent trends in MRSA among inpatients

Editor

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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has become a major problem in US hospitals already dealing with high levels of hospital-associated MRSA (HA-MRSA). Using antimicrobial drug susceptibility data for 1999–2006 from The Surveillance Network, we characterized the relationship between outpatient and inpatient levels of CA-MRSA nationally. In outpatients, the frequency of CA-MRSA isolates has increased >7× during 1999–2006, which suggests that outpatients have become a major reservoir for CA-MRSA. However, contrary to results in other reports, although CA-MRSA increases are associated with decreases in the frequency of HA-MRSA in hospitals, the decreases are only modest. This finding suggests that instead of replacing HA-MRSA in the hospital, CA-MRSA is adding to the overall presence of MRSA already found within the hospital population.

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The past decade has seen a large increase in infections with hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) (*1*). MRSA is one of the most common causes of nosocomial infections, especially invasive bacterial infections (*2*), and is now endemic and even epidemic to many US hospitals, long-term care facilities (*3*), and communities (*4*–*6*). Although community-associated

MRSA (CA-MRSA) strains have been recognized as a leading cause of skin and soft tissue infections (*1*,*6*), especially in patients with no established healthcare risk factors (*7*,*8*), they also cause severe invasive infections (*9*,*10*). Recent reports based on genotypic evidence have suggested that CA-MRSA is likely spreading within hospitals as well, blurring the line between CA-MRSA and HA-MRSA infections (*11*).

Molecular typing studies have identified 2 MRSA clones, USA300 and USA400, as the primary types that cause CA-MRSA infections (*12*). Evidence suggests that emergence of these strains was independent of hospital strains (*13*). Thus, understanding the role of outpatients, who are among the likely carriers of CA-MRSA into a hospital, is useful for understanding the changing epidemiology of MRSA in hospitals. Outpatients, who outnumber inpatients by ≈3:1, may play a major role in the spread of CA-MRSA strains from the community to the hospital through their interaction with hospital staff or use of similar hospital resources, such as surgical rooms. However, limited information hinders understanding of long-term trends in CA-MRSA in outpatients in the context of changing epidemiology of inpatients. This lack of information hinders the ability to evaluate infection control methods in the face of a possible emerging epidemic of nosocomial infections caused by CA-MRSA.

Knowledge of trends in antimicrobial drug resistance rates for emerging pathogens are useful to clinicians to ensure high-quality care, which is essential for antimicrobial drug therapy, in which different drugs can have different costs and effectiveness. These trends can also help hospital administrators and policy makers make infection control investments to address the role that large influxes of outpatients with CA-MRSA infections may play with regard to overall MRSA infection rates in the hospital.

Methods

To analyze trends in frequency of CA-MRSA and HA-MRSA, we studied changes in the proportion of isolates of each type that were found in inpatient and outpatient settings from a nationally representative sample of US hospitals during 1999–2006. Although genotypic analysis is the most reliable way of identifying MRSA strains, historical genotypic data on isolates are not available at the national level. An alternative approach is to ascertain strain type by using phenotypic susceptibility profiles. *S. aureus* susceptibility profiles are determined by the staphylococcal cassette chromosome (SCC) types on which the methicillin resistance gene, *mec*A, is carried. Because CA-MRSA and HA-MRSA strains typically have different SCC*mec* types, rules have been developed for determining the likely genetic makeup of an isolate on the basis of susceptibility results (*11*,*14*–*16*).

Phenotypic susceptibility results were obtained from The Surveillance Network (TSN) Database-USA (Focus Diagnostics, Herndon, VA, USA). TSN is an electronic repository of antimicrobial drug susceptibility data from a national network of >300 microbiology laboratories in the United States. Participating laboratories are geographically dispersed and make up a nationally representative sample based on patient population and number of beds. Patient isolates are tested on site as part of routine diagnostic testing for susceptibility to different antimicrobial agents by using standards established by the Clinical and Laboratory Standards Institute (*17*) and approved by the US Food and Drug Administration. Results are then filtered to remove repeat isolates and identify microbiologically atypical results for confirmation or verification before being included in the TSN database. Data from the database have been used extensively to evaluate antimicrobial drug resistance patterns and trends (*1*,*18*–*22*).

Genotypic analysis of phenotypically defined strains has found that in general, isolates of the USA300 strain, the one most commonly associated with CA-MRSA infections, are resistant to fewer antimicrobial drugs (*14*–*16*). Naimi et al. (*15*) tested genetically determined CA-MRSA isolates against several antimicrobial drugs and found that they were typically susceptible to ciprofloxacin (79%) and clindamycin (83%). Similarly, King et al. (*14*) found that 88% of CA-MRSA strains were resistant only to a β-lactam and erythromycin or a β-lactam only. Popovich et al. (*16*) also found that susceptibility to a fluoroquinolone had a 90% positive predictive value for predicting a community-associated strain. Additionally, the number of antimicrobial drugs to which an isolate was susceptible was a reliable predictor of the genotype (*16*).

We analyzed *S. aureus* isolates that were tested for susceptibility to oxacillin (a proxy for all β-lactam antimicrobial drugs). Isolates classified as resistant according to Clinical and Laboratory Standards Institute breakpoint criteria were considered MRSA (<0.01% had intermediate resistance and were classified as susceptible). MRSA isolates, regardless of source (outpatient or inpatient), that were tested against ciprofloxacin or clindamycin, and ≥3 other drugs and found to be resistant only to oxacillin were classified as CA-MRSA strains. Isolates resistant to oxacillin and ≥1 other drug were assumed to be HA-MRSA strains. Other drugs tested were gentamicin, tetracycline, sulfamethoxazole/trimethoprim, and vancomycin.

Using this framework, we determined that the mean number of outpatient isolates analyzed annually was >50,000. Isolates were stratified on the basis of source (blood, lungs, skin, and other organs). Confidence intervals (CIs) for TSN data were calculated by using the Wilson score method incorporating continuity correction as detailed by Newcombe (*23*). Statistical analysis was performed by using Stata version 10 software (StataCorp LP, College Station, TX, USA).

Results

Susceptibility to clindamycin, ciprofloxacin, gentamicin, tetracycline, sulfamethoxazole/trimethoprim, and vancomycin was used to infer genotypes of MRSA isolates during 1999–2006. During this period, there was a statistically significant reduction ($p<0.001$) in the proportion of MRSA isolates in outpatient areas resistant to ciprofloxacin (84% to 56%), clindamycin (67% to 30%), gentamicin (30% to 3%), and sulfamethoxazole/trimethoprim (16% to 2%). Our phenotypic rule, which was based on susceptibility to all drugs, found qualitatively similar results, with the proportion of MRSA isolates resistant to ≥1 other drug decreasing from 87% to 46% during the period (Figure).

For outpatient data, the proportion of all *S. aureus* infections that were MRSA infections nearly doubled, from 26.8% (95% CI 26.3%–27.3%) to 52.4% (95% CI 52.0%–52.9%), over the study period. This increase was caused almost entirely by increases in isolates resistant only to oxacillin, which increased >7× from 3.6% (95% CI 3.5%–3.7%) to 28.2% (95% CI 28.0%–28.5%). The proportion of isolates resistant only to oxacillin increased for skin and soft tissue infections. However, increases were also observed in invasive blood and lung infections and other infections. Isolates resistant to ≥1 other drug increased ≈5% during 1999–2001 from 23.2% (95% CI 23.0%–23.5%) to 28.2% (95% CI 28.0%–28.5%) before reaching a plateau. In 2005, the proportion of isolates resistant to oxacillin and 1 other drug then decreased back to almost the same percentage it started at. This pattern was driven by overall increases at all infection sites during 1999–2001 and later decreases at all collection sites except skin infections (Table).

Among inpatients, the proportion of *S. aureus* isolates that were MRSA increased 25% from 46.7% (95% CI 46.2%–47.2%) to 58.5% (95% CI 58.0%–58.9%). Again, the increase was driven primarily by increases in the rate of isolates resistant only to oxacillin, which increased >7× from 3.3% (95% CI 3.1%–3.4%) to 19.8% (95% CI 19.4%–20.1%). Similar to outpatient data, the frequency of skin and soft tissue infections increased for isolates resistant only to oxacillin, although increases in blood, lung, and other infections were also observed. For isolates resistant to ≥1 other drug, a slightly different pattern was observed than for the pattern of outpatient isolate resistance. Instead of a large increase, the proportion of MRSA isolates resistant to ≥1 drug remained the same (≈43%–44%) until 2003 before decreasing >5% from 44.1% (95% CI 43.7%–44.5%) to 38.5% (95% CI 38.2%–38.9%) during 2003–2005. This decrease was largely caused by reductions in lung infections, although decreases were also seen in blood and other infections. Also different was the increase in MRSA skin isolates resistant to multiple drugs. There was an increase from 1999, but the increase was less (only 3%–4%) and appeared to plateau at ≈12%–13%.

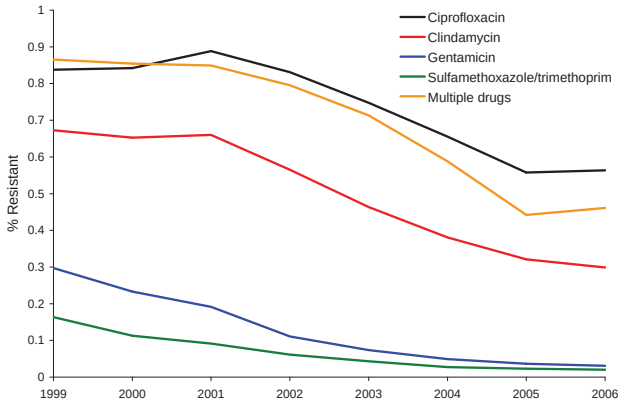

Figure. Resistance of methicillin-resistant *Staphylococcus aureus* isolates to clindamycin, ciprofloxacin, gentamicin, and sulfamethoxazole/trimethoprim in outpatient areas of hospitals, United States, 1999–2006. Multiple drugs indicates isolates that were tested against ciprofloxacin or clindamycin and ≥3 other drugs and found to resistant only to oxacillin. The p values were calculated by using the χ^2 test. Differences in all comparisons were significant ($p<0.001$).

Discussion

We found during 1999–2006 that the percentage of *S. aureus* infections resistant to methicillin increased >90%, or ≈10% a year, in outpatients admitted to US hospitals. This increase was caused almost entirely by CA-MRSA strains, which increased >33% annually. Increases in the proportion of HA-MRSA isolates among outpatients were more variable, increasing ≈10% per year during 1999–2001 before the increase slowed; the proportion then decreased over the second half of the study period. This reduction in the growth of HA-MRSA isolates corresponds to a steep increase in the frequency of CA-MRSA skin and soft tissue infections among outpatients over an extremely short period, mostly during 2003–2005.

The frequency of CA-MRSA among inpatients increased nearly in conjunction with outpatient rates, overall and at each infection site. However, increases in blood and lung infections increased more among inpatients than in outpatients, which likely reflected the more severe status and increased likelihood of open wounds in inpatients. During this same period, rates of HA-MRSA decreased only ≈10%. Most of this decrease occurred during 2003–2005 and was mainly the result of a decrease in the frequency of HA-MRSA lung infections. This decrease was more likely the result of changes in empirical antimicrobial drug therapy for ventilator-associated pneumonia (*24*) than a consequence of any changes in the epidemiology of MRSA.

Despite increases in the proportion of CA-MRSA strains among inpatients, the continuing high level of HA-

Table. Frequency of MRSA in hospitals, by unit, United States, 1999–2006*

Unit	% Patients (95% confidence interval)							
	1999	2000	2001	2002	2003	2004	2005	2006†
Outpatient								
All MRSA	26.8 (26.3–27.3)	29.4 (29.0–29.9)	33.4 (33.0–33.9)	35.7 (35.3–36.2)	40.7 (40.2–41.2)	47.7 (47.3–48.1)	52.7 (52.3–53.1)	52.4 (52.0–52.9)
HA-MRSA	23.2 (23.0–23.5)	25.1 (24.9–25.4)	28.2 (28.0–28.5)	28.4 (28.2–28.7)	29.3 (29.0–29.5)	28.4 (28.2–28.7)	24.1 (23.8–24.3)	24.2 (24.0–24.5)
Blood	2.7	2.8	4.0	3.7	3.1	2.8	2.1	1.9
Lungs	4.7	5.3	6.5	5.5	5.4	4.2	3.5	2.9
Skin	9.3	10.6	10.1	11.6	13.8	15.5	14.2	15.5
Other source	6.4	6.5	7.6	7.7	7.0	6.0	4.2	4.0
CA-MRSA	3.6 (3.5–3.7)	4.3 (4.2–4.4)	5.2 (5.1–5.4)	7.3 (7.1–7.5)	11.4 (11.3–11.6)	19.3 (19.1–19.5)	28.7 (28.4–28.9)	28.2 (28.0–28.5)
Blood	0.3	0.3	0.6	0.7	0.8	0.8	0.9	0.8
Lungs	0.4	0.5	0.9	0.9	0.7	0.6	0.8	0.7
Skin	2.3	3.0	2.9	4.8	9.0	16.6	25.3	25.4
Other source	0.5	0.5	0.8	0.9	1.0	1.3	1.6	1.4
Inpatient								
All MRSA	46.7 (46.2–47.2)	47.6 (47.2–48.1)	50.0 (49.6–50.4)	52.2 (51.8–52.6)	54.9 (54.6–55.3)	58.3 (57.9–58.6)	59.5 (59.2–59.9)	58.5 (58.0–58.9)
HA-MRSA	43.4 (43.0–43.9)	43.2 (42.8–43.6)	44.1 (43.7–44.5)	43.9 (43.5–44.3)	44.1 (43.7–44.5)	41.9 (41.6–42.3)	38.5 (38.2–38.9)	38.7 (38.3–39.1)
Blood	7.1	7.2	7.5	7.5	7.5	7.0	6.2	6.3
Lungs	21.4	19.9	19.2	18.5	17.6	16.5	14.7	14.5
Skin	9.3	10.5	11.4	12.0	12.9	13.1	13.0	13.1
Other source	5.6	5.6	6.0	5.9	6.1	5.3	4.5	4.7
CA-MRSA	3.3 (3.1–3.4)	4.5 (4.3–4.6)	5.8 (5.6–6.0)	8.4 (8.2–8.6)	10.9 (10.6–11.1)	16.3 (16.1–16.6)	21.0 (20.7–21.3)	19.8 (19.4–20.1)
Blood	0.6	0.7	1.0	1.2	1.2	1.7	2.1	2.0
Lungs	1.2	1.6	2.1	2.3	2.1	2.6	3.3	3.8
Skin	1.1	1.7	2.1	4.0	6.6	10.9	14.4	12.8
Other source	0.4	0.5	0.7	0.9	1.0	1.2	1.3	1.2

*MRSA, methicillin-resistant *Staphylococcus aureus*; HA-MRSA, hospital-associated MRSA; CA-MRSA, community-associated MRSA.
†Data through October only. Data for blood, lungs, skin, and other source refer to the percentage of each source tested that was estimated to be CA-MRSA or HA-MRSA.

MRSA suggests that in contrast to reports from local institutions (*11*), CA-MRSA strains are adding to the problem of MRSA rather than replacing HA-MRSA strains. The fact that the frequency of HA-MRSA has decreased implies that some crowding out of HA-MRSA strains within the hospital may be occurring. However, lack of a decrease suggests that within the hospital, HA-MRSA strains may be more fit, and thus CA-MRSA strains are unable to replace them fully. The result is a coexistence of both strains in the hospital and maintenance of CA-MRSA because of the large influx of colonized and infected patients.

This finding is consistent with the biology of the 2 strains, which suggests differential fitness on the basis of the size of SCC*mec*. In CA-MRSA strains, the predominant SCC*mec* elements are types IV and V, which are smaller than the SCC*mec* types typically found in HA-MRSA strains. These smaller genetic elements may increase the fitness of CA-MRSA strains outside hospital-related antimicrobial drug pressures, presumably by increasing mobility and growth potential (*25*). However, their increased susceptibility to antibacterial agents in the hospital leaves them at a fitness disadvantage. The result is that, although the community has effectively become a reservoir for the CA-MRSA strains that are continually introduced into the hospital population, without genetic changes, they are unlikely to replace HA-MRSA strains in the hospital.

The large proportion of infections caused by CA-MRSA strains in hospitals with high frequencies of HA-MRSA has implications for drug-prescribing patterns within hospitals. Because CA-MRSA strains are generally susceptible to more antimicrobial drugs, persons with these infections may be able to be treated with less expensive antimicrobial drugs with fewer adverse outcomes. Moreover, appropriate therapy can reduce the likelihood of emergence of other resistant pathogens, such as vancomycin-resistant enterococci. Initial empiric therapy of infections with the suspected etiology of CA-MRSA must be tailored to antimicrobial drug susceptibility patterns within the local community and be based on efficacy studies that suggest specific effectiveness targets.

Kaplan suggested that empiric therapy should be modified if >10%–15% of CA-MRSA isolates become resistant

to a specific empiric therapy (*26*). Conversely, it may be appropriate to reintroduce a specific agent when susceptibility levels increase above a threshold. However, cycling strategies may not always be optimal (*27*), and no efficacy studies have been conducted to establish this target. In addition, we urge caution in applying national results to the CA-MRSA antibiogram of a specific area. Although results showed an overall trend at the national level, specific results at individual testing centers tended to be more variable. Moreover, local health officials and hospitals should coordinate their efforts to identify susceptibility patterns at the community level, rather than at the hospital level, to optimize the gains from investments in infection control (*28*).

The results of our study should be interpreted with caution because TSN provides information concerning only the site of isolate collection and not the infection. In addition, TSN only provides information on the collection location (i.e., outpatient or inpatient) and not case histories. Thus, some isolates may be difficult to classify in situations such as when an isolate was collected in the emergency department and then the patient was admitted or the patient was discharged and then returned as an outpatient. However, the effect of these situations is likely to be small because most isolates are from patients who can be classified as inpatients or outpatients.

A further limitation of the study is that although CA-MRSA isolate drug susceptibility patterns are technically genetically determined, the data enabled only phenotypic classification of isolates. In addition, as with any large time-series database, changes in surveillance or bias in the types of infections cultured over time, such as more severe or unusual infections, could alter the results. These findings suggest that more complicated bacteriology could alter the results. However, no general trend in the number of isolates collected was seen at individual testing centers, and resistance results from the TSN database were comparable to results of other national studies (*1*). Furthermore, the striking increases over the study period suggest that the trends are likely robust to any bias.

In summary, we examined the frequency of CA-MRSA and HA-MRSA in inpatient and outpatient settings. Our results indicate that outpatients may be a major reservoir of CA-MRSA, which will continue to enter hospitals, exacerbating the problem of MRSA. However, although CA-MRSA isolates have undoubtedly spread within hospitals and are likely to continue to do so, without changes in the fitness of different strains, CA-MRSA strains are unlikely to displace HA-MRSA strains within the hospital.

Our findings have implications for local and national policies aimed at containing and preventing MRSA. More rapid diagnostic methods are urgently needed to better aid physicians in determining appropriate empiric therapy. Strategies for prevention of infection and treatment of patients with CA-MRSA within healthcare settings should be coordinated primarily at the local level in accordance with local susceptibility profiles. Lastly, infection control policies should take into account the role that outpatients likely play in the spread of MRSA and promote interventions that could prevent spread of MRSA from outpatient areas to inpatient areas.

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Risk for Infection with Highly Pathogenic Avian Influenza Virus (H5N1) in Backyard Chickens, Bangladesh

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To evaluate risk factors for infection with highly pathogenic avian influenza A virus (H5N1) in backyard chickens in Bangladesh, we conducted a matched case–control study. We enrolled 25 case farms (cases March–November 2007) and 75 control farms (June–November 2007). We used a questionnaire to collect farm data, which were analyzed by matched-pair analysis and multivariate conditional logistic regression. Factors independently associated were offering slaughter remnants of purchased chickens to backyard chickens (odds ratio [OR] 13.29, 95% confidence interval [CI] 1.34–131.98, p = 0.027), having a nearby water body (OR 5.27, 95% CI 1.24–22.34, p = 0.024), and having contact with pigeons (OR 4.47, 95% CI 1.14–17.50, p = 0.032). Separating chickens and ducks at night was protective (OR 0.06, 95% CI 0.01–0.45, p = 0.006). Reducing these risks and taking protective measures might reduce the risk for influenza (H5N1) infection in backyard chickens.

Highly pathogenic avian influenza (HPAI) A virus (H5N1) is a deadly zoonotic pathogen; from 1997 through 2008, a total of 413 human cases were reported in 15 countries, and 256 persons died (*1*). By March 2008, the virus had been identified in birds in 61 countries (*2*). The persistence of the virus in poultry over a wide geographic area strengthens the hypothesis that a mutant virus might evolve and initiate a human pandemic. To reduce this threat, every country should have a surveillance system for detecting the virus in poultry, including backyard flocks.

Worldwide, poultry production has recently undergone rapid change, including the introduction of intensive production, new breeds, improved biosecurity, and preventive health measures. In developing countries, however, adoption of this type of production has been limited because of the costs of infrastructures to maintain biosecurity for birds, quality hybrid chicks, balanced feed, biologics, and quality veterinary care (*3*). Up to 80% of the poultry in Africa and Asia are kept in backyard-type systems (*3*,*4*), and these birds represent a substantial economic resource for impoverished rural populations.

In Bangladesh, ≈89% of rural households have backyard poultry (*5*), and many households keep chickens and ducks on the same property (*6*). In the absence of fences or other barriers, backyard chickens roam freely from one property to another. Because backyard chickens are reared in such free-range systems, they are more vulnerable to the HPAI (H5N1) virus infection; and, if they become infected, they can transmit the virus to domestic ducks, in which the virus can perpetuate (*7*–*9*) and infect more backyard chickens. This cycle of virus transmission between backyard chickens and ducks would continue until intercepted. HPAI (H5N1) virus in backyard chickens also poses a serious threat to public health because of the frequent and close contacts between poultry and humans. Little has been published about the risk factors associated with HPAI (H5N1)

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virus infection in backyard chickens in any parts of the world, and to our knowledge, nothing has been published about the risk factors in Bangladesh. Because understanding the risk factors for the virus in backyard chickens and preventive measures might slow or prevent the spread of the virus, we conducted a case-control study to determine the risk factors for HPAI (H5N1) virus infection in backyard chickens in Bangladesh.

Materials and Methods

Study Population and Case Definition

Bangladesh is composed of 4,500 unions (local government units that comprise several villages) and 90,500 villages (*10*). Of the total poultry population in the country (≈222 million birds), 50% are backyard poultry, predominantly indigenous (nondescriptive) chickens and mostly reared in free-range systems on the homesteads in these villages (*10*). In Bangladesh, villagers sometime rear Fayoumi and Sonali (a cross-bred F1 generation of Fayoumi [female] and Rhode Island Red [male]) chickens in a semi-scavenging system (*11–13*) and occasionally in intensive systems. All 25 HPAI outbreaks recorded in indigenous (n = 20 farms), Fayoumi (n = 2), and Sonali (n = 5) chickens in village areas in Bangladesh by November 17, 2007, were considered outbreaks in backyard chickens, and the farms were enrolled in our study as case backyard farms. By date of onset of clinical signs, the first outbreak of HPAI in backyard chickens was recorded on March 22, 2007, the date on which Bangladesh was officially declared HPAI (H5N1) virus infected. In 2007, the numbers of backyard farms infected were 1 farm in March, 3 in April, 7 in May, 7 in June, 2 in July, 1 in September, 3 in October, and 1 in November.

A case backyard farm was defined as one that had a high chicken mortality rate and on which influenza virus A subtype H5 was detected from tracheal samples of 2 chickens by reverse transcription–PCR (RT-PCR) using a primer set hemagglutinin (HA) oligo 5′ (5′-ACACATGCYCARGACATACT-3′) and HA oligo 3′ (5′-CTYTGRTTYAGTGTTGATGT-3′), as described by Lee et al. (*14*). Testing was done at the National Reference Laboratory for Avian Influenza in Bangladesh. Case reporting of HPAI (H5N1) in chickens in Bangladesh and detailed laboratory diagnosis, including diagnostic reconfirmation from the Veterinary Laboratory Agency in the United Kingdom, has been described (*15*).

For each case farm, we selected and enrolled 3 control backyard farms, each of which was within 1–10 km of a case farm. Each unaffected village in this zone of selection was assigned a unique number, and 2 were randomly selected by lottery. One villager from each selected village was asked to give 10 names of the backyard farm owners in the village who had reared village chickens for >1 year. These 10 names with distinct numbers were used as the sample frame for the village. From the sample frame of the first selected village, we randomly chose 2 backyard farm owners who had adult chickens (>6 months of age) and chicks (<1 month of age) and whose chickens had not died during the clinical phase of HPAI on the case farm. Likewise, 1 backyard farm owner was selected from the sample of the second village. To find control farms with Sonali or Fayoumi chickens, the same (1–10 km) zone of selection was used, but the names of the farm owners were drawn from the local upazila (a lower administrative unit in Bangladesh) veterinary office and used as the sample from which to randomly select 3 farms. Because biologics were scarce, serologic testing to confirm the noninfected status of the control farms was not attempted.

Global positioning system coordinates from the case and control farms were collected during farm visits and entered into a digitized map of Bangladesh. A geographic information system program (Arc View 9.1; Environmental System Research Institute, Redlands, CA, USA) was used (Figure).

Data Collection and Survey Method

A questionnaire designed for this study was pretested at 5 case farms. The questionnaire was then modified according to new variables encountered during pretesting. In the final questionnaire, 59 variables were surveyed (spreadsheet available from P.K.B.). The questionnaire was then administered on the case and control farms by 2 veterinarians trained to administer questionnaires; they interviewed farm owners or, if owners were absent, any adult family members. Variables collected addressed geographic location, stock information, flock health history, and overall farm management. All interviews were conducted in Bengali, the only spoken language in the study area, during June–November 2007.

Statistical Analysis

The collected data were entered into a spread sheet program (Excel; Microsoft, Redmond, WA, USA) and transferred into Epi Info 2000 (Centers for Disease Control and Prevention, Atlanta, GA, USA) for analysis. To estimate the strength and statistical significance of associations between risk factors and HPAI (H5N1) virus infection, we used the Mantel-Haenszel matched-pair analysis (McNemar) test. An association was considered significant if 2-sided tests of significance had a p value ≤ 0.05. To examine independence of effects, we conducted multivariate conditional logistic regression using the conditional logistic regression (CLogit) function in Stata 9.0 for Windows (Stata Corp., College Station, TX, USA). Any variables with $p \leq 0.2$ after matched-pair analysis were included in

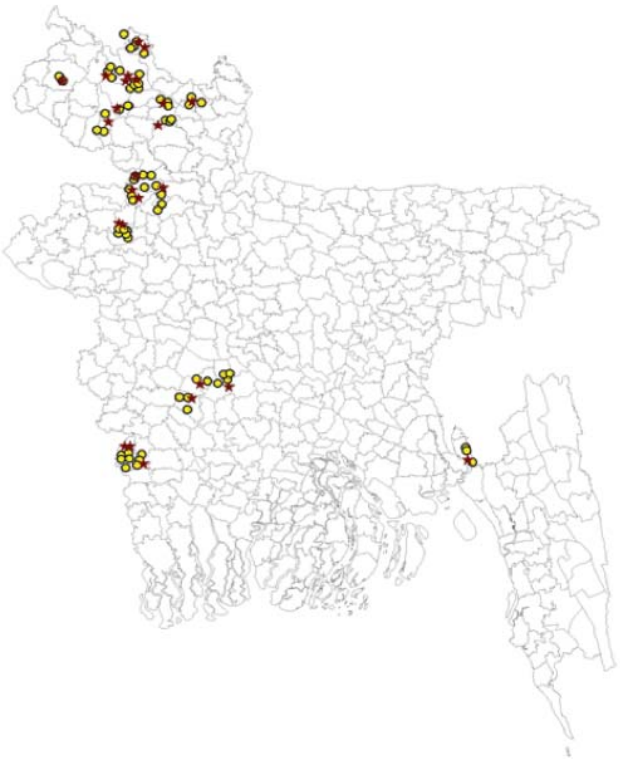

Figure. Locations of 25 backyard farms where outbreaks of highly pathogenic avian influenza A virus (H5N1) infection occurred during March–November 2007 (red stars) and 75 control backyard farms (yellow circles), Bangladesh.

the initial model. A backward stepwise variable–selection strategy was used to construct a final model with a significance level of $p \leq 0.05$.

Results

Population Statistics

Indigenous, Fayoumi, and Sonali chickens were raised on 20, 2, and 3 backyard farms, respectively. Median number (and range) of indigenous chickens was 24 (3–88) on case farms and 14 (5–50) on control farms and of Fayoumi/Sonali chickens was 950 (125–1,970) on case farms and 2,200 (1,500–3,500) on control farms. Chickens of various ages (mean 35.7 weeks [range 3.5–130]) were raised on 10 of the case farms, but precise ages of chickens on 15 case farms were not provided by the owners; 6 said they had only adult chickens, and 9 said they had adult chickens and young chicks. Adult and young indigenous chickens were raised on the 60 control farms.

Matched-Pair Analysis

The results of matched-pair analysis (Table 1) showed that offering slaughter remnants of purchased chickens to backyard chickens (within 21 days of the onset clinical signs in case farms) had the strongest point estimate of effect (matched odds ratio [OR] 22.1) and high statistical significance ($p<0.001$) despite wide 95% confidence intervals (CIs) of 2.7–177.7. Other factors positively associated with case farms were migratory birds around a farm (OR 7.5, 95% CI 1.5–38.7, p = 0.010), rodents on the farm (OR 5.8, 95% CI 2.0–16.8, p = 0.001), contact with pigeons (OR 5.5, 95% CI 1.9–16.0, p = 0.001), and a nearby body of water (OR 3.7, 95% CI 1.5–9.5, p = 0.004). Protective factors (OR <1) for case backyard farms were placing chickens and ducks in different shelters at night (OR 0.1, 95% CI 0.1–0.5, p = 0.001) and having a commercial farm within 0.5 km (OR 0.3, 95% CI 0.1–0.9, p = 0.028).

Multivariate Analysis

Eight variables with $p \leq 0.2$ were considered for inclusion in the conditional logistic regression model to estimate independence of effects (Table 2). The final conditional logistic regression model identified 3 variables as independent risk factors for HPAI (H5N1) infection of backyard chickens in Bangladesh (Table 3). They were 1) offering slaughter remnants of purchased chickens to backyard chickens (within 21 days of the clinical onset of the disease) (OR 13.29, 95% CI 1.34–131.98), 2) having nearby body of water (OR 5.27, 95% CI 1.24–22.34), and 3) having contact with pigeons (OR 4.47, 95% CI 1.14–17.50). The final model also identified a protective factor: placing chickens and ducks in different shelters at night (OR 0.06 95% CI 0.01–0.45).

Discussion

We used analytic epidemiologic techniques to unveil the possible risk factors associated with influenza (H5N1) infection for backyard chickens in Bangladesh so that effective risk management can be advocated. A few published reports quantify the risk factors for influenza (H5N1) infections in commercial chickens (*16*,*17*), but to our knowledge, analytic epidemiologic reports quantifying risk factors for backyard chickens are few, if any. The results of this study should contribute to the understanding of risk factors associated with influenza (H5N1) infections in backyard chickens in other developing countries, particularly in southern Asia.

Although only 1 case of influenza (H5N1) in a human has been reported in Bangladesh, the country's poultry sector has been severely affected; by July 2009, a total of 325 outbreaks had been reported in chickens, 51 of which were in backyard chickens (www.mofl.gov.bd/daily_birdflu_report.pdf). Because of limited manpower, the country relies predominantly on passive surveillance to detect HPAI outbreaks in chickens. Thus, the possibility of unreported cases occurring in backyard chickens in some parts of the

Table 1. Matched-pair analysis of potential risk factors for highly pathogenic avian influenza virus (H5N1) in backyard chickens, Bangladesh, 2007

Risk factor	Case farms (n = 25), no. (%)	Control farms (n = 75), no. (%)	Matched OR (95% CI)*	p value
Farm ≤5 km from nearest veterinary hospital	15 (60.0)	41 (54.7)	1.3 (0.5–3.2)	0.636
Nearby (<0.1 km) body of water	16 (64.0)	23 (30.7)	3.7 (1.5– 9.5)	0.004
Farm ≤0.5 km from larger body of water	15 (60.0)	50 (66.7)	0.7 (0.3–1.9)	0.543
Commercial farm within 0.5 km	9 (36.0)	44 (58.7)	0.3 (0.1–0.9)	0.028
Migratory birds seen around farm	6 (24.0)	5 (6.7)	7.5 (1.5–38.7)	0.010
Local live bird market within <5-km radius	24 (96.0)	73 (97.3)	0.7 (0.1–7.4)	0.747
Farm ≤1 km from live bird market	17 (68.0)	50 (66.7)	1.1 (0.4–3.0)	0.895
Contact with ducks	22 (88.0)	55 (73.3)	4.0 (0.8–20.1)	0.062
Contact with pigeons	16 (64.0)	21 (28.0)	5.5 (1.9–16.0)	0.001
Presence of rodents	12 (48.0)	9 (12.0)	5.8 (2.0–16.8)	0.001
Chickens and ducks on the same farm	11 (44.0)	40 (53.3)	0.7 (0.3–1.7)	0.415
Chickens and ducks in different night shelters	2 (8.0)	31 (41.3)	0.1 (0.1–0.5)	0.001
Frequent (≈1×/wk) cleaning of shelter	18 (72.0)	44 (58.7)	1.8 (0.7–4.9)	0.221
No disinfection in shelter	4 (16.0)	10 (13.3)	3.0 (0.2–48.0)	0.448
Disposal of bird in open space	19 (76.0)	58 (77.3)	0.9 (0 .3– 3.2)	0.869
Recently purchased chickens brought in†	5 (20.0)	10 (13.3)	1.7 (0.5–5.7)	0.421
Offering slaughter remnants of purchased chickens†	8 (32.0)	2 (2.7)	22.1 (2.7–177.7)	0.000
Death of neighbor's chickens	7 (28.0)	20 (26.7)	1.1 (0.4–2.8)	0.900
Source of chicks = own hatched	5 (20.0)	20 (26.7)	0.4 (0.1–2.0)	0.226

*Matched-pair analysis using McNemar (Mantel-Haenszel) test statistics. OR, odds ratio; CI, confidence interval.
†Within 21 days of the onset clinical signs on case backyard farms.

country cannot be ruled out. These hidden and unreported infections in backyard chickens can help perpetuate the virus, posing a serious challenge to eradication efforts. Strengthening active instead of passive surveillance and generating awareness at the rural level of risk factors for the HPAI (H5N1) virus infection in backyard chickens, and their management, might help reduce the virus load in poultry in the country.

Stalls with live poultry can be found at virtually every kitchen or village market in Bangladesh. At the local markets, villagers can sell their poultry to local persons or to poultry vendors, who buy poultry in bulk to sell at larger city markets. When villagers fear a disease outbreak, they start selling apparently healthy and even clinically diseased chickens. Diseased chickens are cheaper, encouraging other villagers to buy them for meat. They purchase live chickens and slaughter them at home. They then offer the slaughter remnants, inedible for humans, to their own backyard chickens, which scavenge and forage around the slaughter places. Such practice occurred at 8 (32%) case farms ≤21 days of the onset of the clinical signs (Table 1); this practice appears to be strongest risk factor for HPAI (H5N1) infection in backyard chickens in Bangladesh.

Another risk factor was domestic ducks, which are considered a "Trojan horse" for the HPAI (H5N1) virus (*7*–*9*). Their main feed sources are vegetation, small fish, amphibians, snails, oysters, and other crustaceans, found in and around water. The water bodies and their banks might become contaminated with the HPAI (H5N1) virus by virus-shedding ducks that congregate at these places. Backyard chickens might be exposed to the virus while sharing the same banks near the body of water, which could explain why a nearby body water appeared to be an independent risk factor.

The influence of 2 kinds of bodies of water on the HPAI outbreaks in backyard chickens was assessed by in-

Table 2. Initial results from multivariate analysis of potential risk factors for highly pathogenic avian influenza virus (H5N1) in backyard chickens, Bangladesh, 2007*

Risk factor	Odds ratio	95% Confidence interval	p value
Nearby (≤0.1 km) body of water	3.64	0.82–16.18	0.089
Commercial farm within 0.5 km	3.57	0.34– 37.82	0.291
Migratory bird seen around farm	3.37	0.05–234.59	0.575
Contact with ducks	1.47	0.15–14.29	0.740
Contact with pigeons	7.64	1.00–58.48	0.050
Presence of rodents	7.94	0.89–72.61	0.067
Chickens and ducks in different night shelters	0.08	0.01–0.71	0.023
Offering slaughter remnants of purchased chickens†	9.02	0.77–105.79	0.080

*Conditional logistic regression; initial set with 8 variables entered; χ^2 for likelihood ratio test = 43.85; p>0.001; no. observations = 100.
†Within 21 days of the onset clinical signs in case backyard farms.

Table 3. Results of final model with potential risk factors for highly pathogenic avian influenza virus (H5N1) in backyard chickens, Bangladesh, 2007*

Risk factor	Odds ratio	95% Confidence interval	p value
Nearby (<0.1 km) body of water	5.27	(1.24–22.34)	0.024
Contact with pigeons	4.47	(1.14–17.50)	0.032
Chickens and ducks in different night shelters	0.06	(0.01–0.45)	0.006
Offering slaughter remnants of purchased chickens†	13.29	(1.34–131.99)	0.027

*Conditional logistic regression; final model with 4 variables entered; χ^2 for likelihood ratio test = 38.82; p>0.001; no. observations = 100.
†Within 21 days of the onset clinical signs in case backyard farms.

corporating 2 variables: 1) presence of a nearby (<0.1 km) body of water and 2) distance ≤0.5 km from a larger body of water. The latter variable was meant for any larger water-logged paddy or open field, lagoon, marsh, river, lake, or canal where water and migratory birds live or take refuge. These bodies of water are sometimes shared by domestic ducks; but generally, ducks on backyard farms feed on nearby bodies of water, predominantly ponds made by the birds' owners for household purposes or aquaculture. Secondarily, ducks roam in these ponds to collect feed. Presence of a larger body of water within 0.5 km of a backyard farm seems to have no causal association with the occurrence of HPAI in backyard chickens in Bangladesh, but the presence of a nearby pond might.

In 1997, domestic pigeons (*Columbia* spp.) were largely resistant to infection with an HPAI (H5N1) virus isolated from Hong Kong Special Administrative Region, People's Republic of China (*18*); other studies showed that they appeared to be more resistant to infection than many other avian species (*19*,*20*). In Bangladesh, many backyard farmers rear chickens, ducks, pigeons, and sometimes other poultry in groups of mixed ages. Domestic pigeons are a major source of meat in Bangladesh, and not 1 pigeon in the country has been reported dead of influenza (H5N1) infection. The risk factor of contact with pigeons included 2 categories: the owners' own domestic pigeons and neighbors' visiting pigeons. In a complex of backyard farms in Bangladesh, pigeons are allowed to feed with other farm poultry; in addition, pigeons of 1 backyard farm frequently visit others for additional feed. Oronasal secretions and feces from sick, or dead, backyard chickens with HPAI, have a high virus titer, thereby polluting the farm. Pigeons' feeding and behavior probably allows them to come in close contact with the secretions of the infected or dead chickens or with fomites, enabling them to transmit the virus mechanically. Nettles et al. (*21*) reported that pigeons and some wild birds—crows, mourning doves, vultures, and others—are not responsible for dissemination of influenza virus (H5N2) among poultry farms. However, in contrast to the findings of Nettles et al. (*21*), dead crows in different areas of Bangladesh were found to be positive for influenza virus (H5) (neuraminidase was not determined) (*22*). Because of the lack of evidence of mechanical transmission of influenza virus (H5N1) through pigeons in backyard chickens, the hypothesis that they are mechanical transmitters of influenza virus (H5N1) under the prevailing conditions of backyard chicken farms in Bangladesh cannot be confirmed without a thorough virologic study.

Some owners also offer supplementary feeds, predominantly cereals or their byproducts, to their chickens and ducks, usually in the evening when they are placed in the night shelters to protect them from predators. No domestic duck in Bangladesh has been reported dead of influenza virus (H5N1) infection. Placing chickens and ducks in separate night shelters appeared to be a protective factor. On the contrary, an association with influenza (H5N1) was found with rearing them on the same farm.

In developing countries, including Bangladesh, biosecurity enhancement, according to the Food and Agriculture Organization of the United Nations, poultry production system 4 is impossible to adopt. However, practical ways to minimize the risk factors identified in this study are possible in these countries, as follows. Chickens must not be fed remnants of slaughtered chickens that have been purchased from markets or mobile poultry vendors, and inedible portions thus produced must be disposed of hygienically. Villagers should not buy any obviously or apparently sick chickens, although they are cheaper, because these birds pose a serious threat to the villagers' health and to the health of their backyard chickens. Enactment of laws with punitive measures for selling clinically sick chickens anywhere in the country and strict implementation of these laws are vital for limiting the spread of the virus from live bird markets to backyard chickens and vice versa. Individual backyard farm owners should be encouraged to rear only chickens or ducks; but if that is impractical, the owners should be advised to construct separate night shelters for ducks and chickens. Chickens should be limited or prevented from scavenging along the banks of bodies of water. During feeding, a family member can prevent pigeons from joining the flock; any remaining feed must be removed carefully.

Because backyard chickens are a vital economic resource in Bangladesh, backyard farmers cannot be prevented from rearing them. Therefore, avoidance of the risk factors identified in this study, and implementation of protective factors, might reduce the risk for influenza (H5N1) infection in backyard chickens in the country.

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Landscape Epidemiology of Tularemia Outbreaks in Sweden

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Summer outbreaks of tularemia that occurred from 1995 through 2005 in 2 locations in Sweden affected 441 persons. We performed an epidemiologic investigation of these outbreaks using a novel strategy, involving high-resolution genotyping of *Francisella tularensis* isolates obtained from 136 patients (using 18 genetic markers developed from 6 *F. tularensis* genome sequences) and interviews with the patients. Strong spatial associations were found between *F. tularensis* subpopulations and the places of disease transmission; infection by some subpopulations occurred within areas as small as 2 km^2, indicating unidentified environmental point sources of tularemia. In both locations, disease clusters were associated with recreational areas beside water, and genetic subpopulations were present throughout the tularemia season and persisted over years. High-resolution genotyping in combination with patients' statements about geographic places of disease transmission provided valuable indications of likely sources of infection and the causal genotypes during these tularemia outbreaks.

Traditional objectives of investigations of infectious disease outbreaks are to identify ways to control ongoing outbreaks and to prevent future outbreaks. However, a paucity of epidemiologic and ecologic knowledge hampers the investigation of tularemia outbreaks caused by the intracellular bacterium *Francisella tularensis*, although it is one of the most virulent pathogens known. The Centers for Disease Control and Prevention lists this pathogen as one of the most potentially dangerous bioterrorism bacteria (*1*). Little is known about natural reservoirs of tularemia, *F. tularensis* transmission mechanisms to humans, and factors influencing the often irregular pattern of outbreaks. Because tularemia is a zoonosis and little ecologic information exists about the causal organism, its prevention and control may require the development of novel outbreak investigation strategies.

In nature, *F. tularensis* is associated with an extremely wide range of hosts and arthropod vectors; a recent review listed 304 susceptible species (*2*). Transmission to humans may occur through a number of routes; skin inoculation by blood-feeding arthropod vectors is one of the most common routes (*3*). The infectious dose can be as low as 10 bacterial cells (*4*). Human tularemia naturally occurs only in biotopes in the Northern Hemisphere. We describe an investigation of a large number of *F. tularensis* isolates from humans. Patients were infected mainly from mosquito bites and had an influenza-like illness, a primary skin ulcer, and enlargement of lymph nodes, the ulceroglandular form of tularemia (*5*,*6*). Tularemia is endemic in Sweden, with seasonal outbreaks and a patchy geographic distribution. The number of infected humans ranged from 27 to 698 per year from 1998 through 2007 in a population of ≈9.1 million (annual incidence rate 0.30–7.78/100,000 persons) (*7*). For comparison, 20–64 humans were reported with tularemia from 2000 through 2006 in the tularemia-endemic US states of Arkansas and Missouri, from a population of ≈8.3 million (annual incidence rate 0.23–0.76/100,000 persons) (*8*). *F. tularensis* subsp. *holarctica* causes tularemia all over the Northern Hemisphere. This is a severe febrile disease but does not generally result in death. A more virulent variety, *F. tularensis* subsp. *tularensis*, exists in North America. It was associated with a human mortality rate of 5%–15% before the advent of effective antimicrobial drug

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treatments (*4*). *F. tularensis* has a clonal genetic structure, a property that should facilitate tracking the spread of tularemia by genotyping (*9*,*10*).

We demonstrate a strategy to enhance epidemiologic investigations of tularemia by combining geographic data collected from patient interviews and high-resolution genotyping of *F. tularensis* subsp. *holarctica* isolates recovered from tularemia patients. We found that geographic distributions of specific *F. tularensis* subsp. *holarctica* subpopulations were highly localized during outbreaks (infections by some genotypes were restricted to areas as small as 2 km^2), indicating distinct point sources of infection.

Materials and Methods

Study Locations

We studied tularemia, which must be reported under Swedish law, in 2 locations 364 km apart: the Municipality of Ljusdal and the County of Örebro (19,384 and 273,956 inhabitants, respectively, in 2005) (*11*) (Figure 1). The human tularemia incidence rates in Ljusdal and Örebro cited here were based on the annual number of human tularemia cases reported to the County Medical Officer for Communicable Diseases. Tularemia has been endemic for several decades in Ljusdal with repeated outbreaks (*12*), whereas in Örebro, incidence of tularemia has been low since the disease was first reported in Sweden in the 1930s (*5*). From 1990 through 1999, Örebro reported only 8 cases.

Figure 1. Locations of the 2 tularemia outbreak areas in Sweden, showing Ljusdal and Örebro 364 km apart.

Study Period, Isolate Information, and Preparation of DNA

From 1995 through 2005, clinicians in Ljusdal and Örebro sent patient specimens for tularemia serologic analysis, *F. tularensis* culture, and PCR detection to the laboratories at the Swedish Defense Research Agency, Umeå, Sweden; Umeå University Hospital, Umeå; or Örebro University Hospital, Örebro. Culture and PCR diagnostics of ulcer specimens were performed as described elsewhere (*13*). Blood culture was performed by using the instrumented BD Bactec Plus system (Becton Dickinson, Franklin Lakes, NJ, USA). All *Francisella* culture work was performed under BioSafety level 3 laboratory conditions. A tube agglutination test or an ELISA measuring immunoglobulin (Ig) M and IgG was used for serologic analysis, as previously described (*14*). For DNA preparation, *F. tularensis* isolates were recultured, then a loopful of bacteria was suspended in phosphate-buffered saline and heat-killed, and a chaotropic salt method was applied (*6*).

Identification and Selection of Markers

We used 3 types of genetic markers to provide high-typing resolution and robust categorization of *F. tularensis* subsp. *holarctica* into genetic subpopulations. As described elsewhere, we previously identified 280 insertion/deletion (INDEL) and variable number of tandem repeat (VNTR) markers by multiple alignments of the *Francisella* genomes U112, FSC147, SCHU S4, OSU18, and LVS (*15*). For the current study, we selected 20 of these 280 markers (17 INDELs and 3 VNTRs) that were polymorphic among *F. tularensis* subsp. *holarctica* isolates from Europe and North America. We then added 11 VNTR markers showing polymorphism among subsp. *holarctica* isolates of worldwide origin, and 1 INDEL (Ft-M19) shown to be specific to subsp. *holarctica* (*9*). Finally, we added 2 single nucleotide polymorphism (SNP) markers identified in a comparative BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of *F. tularensis* subsp. *holarctica* genomes of a worldwide origin including FSC200 (human, Ljusdal, 1998), LVS, RC530, FSC022, FTA, OR96-0246, and OSU18. The 2 SNP markers were selected to be phylogenetically informative while lacking homoplasy among 67 *Francisella* strains of diverse origin. Collectively, the selection process yielded a set of 34 genetic markers that were applied to 48 of the 136 study isolates (24 each from Örebro and Ljusdal). Markers found to be polymorphic among the 48 selected isolates were finally used to genotype all 136 isolates.

DNA Fragment Analysis and Real-time PCR SNP Assay

DNA fragment analysis of INDELs and VNTRs were performed on a Genetic Analysis System CEQ 8800 (Beckman Coulter Inc., Fullerton, CA, USA) machine, as previously described (*15*). Genomic positions for SNPs and their corresponding PCR amplification primers are presented in Table 1. The SNP states were determined by real-time PCR by using a set of 2 forward primers with different single nucleotide extensions at the 3′ end, and a common reverse primer (*16*). We could read the SNP state by a differential PCR amplification efficiency.

Genetic Groups, Subgroups, and Cluster Analysis

We assigned isolates to genotypes by using the binary character output for each INDEL marker, the repeat copy number at each VNTR marker, and the results of the SNP assay. We analyzed the associations among genotypes by their degree of genetic character sharing by using the eBURST algorithm (*17*). Genetic groups were defined as genotypes sharing 16 of 18 characters and subgroups sharing 17 of 18 characters. In addition, the genetic associations between the isolates were assessed by cluster analysis by using the unweighted pair group m with arithmetic mean (*18*) algorithm implemented in BioNumerics version 5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) using the categorical coefficient.

Patient Interviews and Geographic Mapping

To obtain geographic data on likely places of disease transmission in Ljusdal, all patients in whom *F. tularensis* subsp. *holarctica* was diagnosed from 1995 through 2005 completed a questionnaire distributed during 2007 and marked on an accompanying map the location of the site where they believed they had been infected. In Örebro, patients admitted to hospital with tularemia from 2000 through 2004 were interviewed on admission, interviewed by telephone, or issued questionnaires, as previously described (*5*). All patients were asked for alternative places of disease transmission and to self-estimate the spatial data quality on a 3-category scale. A pair of RT-90 cartographic coordinates (*19*) was then assigned to each patient and the corresponding *F. tularensis* subsp. *holarctica* isolate, specifying the locations of first-choice place of transmission with the highest self-estimated quality. RT 90 is a standardized 2-dimensional Swedish map reference coordinate system. For patients who indicated multiple places of infection with identical data quality estimates, the coordinates of the place closest to the spatial mean center of tularemia in Ljusdal or Örebro were used (online Appendix Table 1, available from www.cdc.gov/EID/content/15/12/1937-appT1.htm, and online Appendix Table 2, available from www.cdc.gov/EID/content/15/12/1937-appT2.htm). If no geographic information was available for a patient, the residential address was used.

We visualized and analyzed geographic and genotype data by using ArcView software in ArcGIS version 9.3 (ESRI, Redlands, CA, USA), and calculated spatial mean centers of disease occurrence (the average x and y values for the input coordinates) for all genetic groups and subgroups of isolates. The directional trend was examined by using the directional distribution tool in ArcGIS Spatial Statistics Tools (ESRI) and an ellipse size of 1 standard deviation.

Results

Descriptive Epidemiology

We isolated *F. tularensis* from 136 of 441 patients with laboratory-verified tularemia in the Municipality of Ljusdal during 1995–2005 (n = 56) and in the County of Örebro during 2000–2004 (n = 80). This finding constituted 34% (56/163) and 29% (80/278) of all patients with laboratory-verified tularemia during the outbreak years in Ljusdal (1995, 1998, 2002, 2005) and Örebro (2000, 2002–2004), respectively (Figure 1).

Analysis per outbreak year showed that the number of patients with culture-verified tularemia were proportional

Table 1. Attributes of 7 novel typing markers for *Francisella tularensis* subsp. *holarctica**

Marker category	Marker name	Genomic position†	Size, bp	Forward primer sequence (5′ →3′)	Reverse primer sequence (5′ → 3′)
INDEL	Ftind39	798173-4	11	ATAATTACTATCAAATGCCCCAAC	CAAGATTTACCTCAAGAAATGGAT
	Ftind40	1502502-821	73	ATATGATTGCTCCAGTATTTATTTC	TTGTAAGGTGATCGGAGTATTT
	Ftind41	1494030-328	20	CCAAGAGCAGAGCATAATTCTAA	GCCTGACRCAATGACATATTTAC
	Ftind42	1849905-1850278	187	AGTAATAACGGTACGATCACAAAG	GGCTTTAGCTTACCAACASAAC
VNTR	Ft-M26	1833026-37	6‡	AATACTCGCTTCTATCTTTCTGGT	AATCTTTTGGAGAGGTTTTATTCA
SNP	Ft-SNP1	927939	1	ATCCCTGTTGGGATATCCTCGACTAA	ACCAAGGTAGATTTGCAGCTAC**A**§ ACCAAGGTAGATTTGCAGCTAC**G**§
	Ft-SNP2	1044580	1	ATCAGACTTAGGTGTTAGATCAGAGTT	TGAATACTCTACGCGATAAGAT**A**§ TGAATACTCTACGCGATAAGAT**G**§

*INDEL, insertion/deletion; VNTR, variable number tandem repeat; SNP, single nucleotide polymorphism.
†GenBank accession no. AM233362.1 (complete genome of *F. tularensis* subsp. *holarctica* LVS).
‡There were two 6-bp repeats in the genome sequence of LVS.
§The 2 SNP states are in **boldface**, an adenosine nucleotide represent a derived state for both markers.

to the total number of tularemia patients. The ages of patients from whom positive cultures were obtained ranged from 1 to 90 years, distributed evenly among age groups: 20%, 18%, 34%, and 28% in persons 0–20, 21–40, 41–60, and >60 years of age, respectively, with a male:female ratio of 1.2:1 (online Appendix Tables 1, 2). No apparent correlations were found between genotypes and either the gender or age of the patients. The annual incidence rates of tularemia were 21–423 per 100,000 persons in the Municipality of Ljusdal and 12–55 per 100,000 persons in the County of Örebro in outbreak years, but disease-free years were interspersed between outbreaks. Generally, tularemia patients reported that they were infected at places used for outdoor activities, e.g., on walking paths, at bathing places, at golf courses, or in an allotment garden (parcels of land for cultivation) assigned to individuals or families (Figures 2–4; online Technical Appendix, available from www.cdc.gov/EID/content/15/12/1937-Techapp.pdf). A statement of a likely arthropod transmission vector was available for patients from Örebro; indicating mosquitoes (n = 101), ticks (*4*), horse flies (*8*), mosquitoes or ticks (*2*), mosquitoes or horse flies (*19*); 144 patients reported not knowing (the vectors reported by the 80 patients with culture-verified tularemia are listed in online Appendix Table 2). A presumed place of tularemia acquisition was pinpointed by 120 (88%) of 136 of patients and self-estimates of the quality of spatial data were available for 44 of 56 patients in Ljusdal and 76 of 80 patients in Örebro (online Appendix Tables 1, 2) with culture-verified tularemia. Overall, patients felt confident about where they had acquired tularemia; 92 of 136 patients indicated that their place of disease acquisition was certain or probable (Table 2).

Genetic Analysis of Outbreak Isolates

We first identified 34 genetic markers that were polymorphic among *F. tularensis* subsp. *holarctica* isolates of worldwide origin. Among these, 18 markers (6/14 VNTRs, 10/18 INDELs, and both SNPs) were polymorphic among the 136 study isolates. Seven of these polymorphic markers have not been previously described (Table 1); 11 (Ftind30–34, Ftind37, Ft-M3, Ft-M6, Ft-M20, Ft-M22, Ft-M24) have been previously published (*9*,*15*). By applying the 18-marker system to the 136 isolates, we identified 19 genotypes. The 136 isolates were assigned to 3 genetic groups (denoted 1, 2, and 3) corresponding to previously described genetic groups (*9*,*15*,*20*) and 5 subgroups (denoted 1a–e), on the basis of their degree of character sharing (Figure 5). Most isolates from Ljusdal (55/56) and Örebro (68/80) belonged to genetic group 1. In pairwise comparisons, genetic groups 1, 2, and 3 differed at 6–13 of the 18 characters. A mutation at marker Ft-SNP2 was distinctive to the large genetic subgroup 1e from Ljusdal (53 isolates). Cluster analysis based on a distance matrix showed an identical grouping of isolates (Figure 5). Only 1 genotype was represented in both study locations; an isolate from Ljusdal obtained in 2005 was identical to an isolate from Örebro in 2003 (see genotype ID 9; Figure 5). In both study locations, genetic subgroups and genotypes were present throughout a tularemia season and persisted over years (Figures 5, 6).

Phylogeographic Analysis in Ljusdal

The *F. tularensis* isolates recovered from 1995 through 2005 from patients in Ljusdal were genetically monomorphic. The 53 of 56 isolates that belonged to genetic sub-

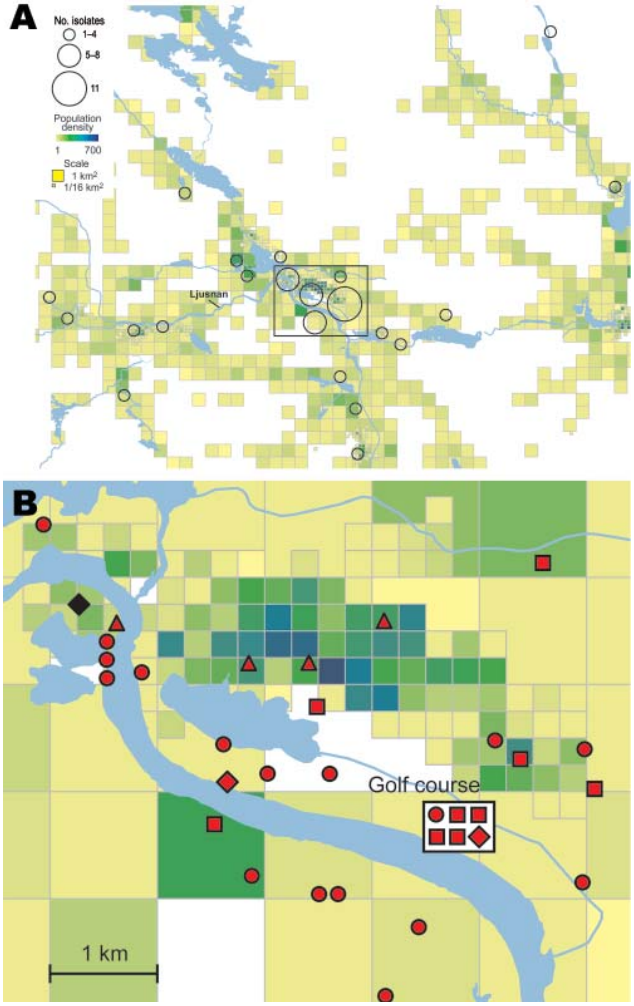

Figure 2. A) Spatial distribution of 56 places of tularemia transmission in Ljusdal, Sweden, 1995–2005, overlaid on a map with color-coded demographic data based on residential addresses. B) Disease cluster in an area of 25 km^2 along the Ljusnan River in Ljusdal. Reported places of disease transmission and corresponding bacterial genotypes are shown. The 33 *Francisella tularensis* isolates belong to genetic group 1e and are of genotype ID 15 (red) or genotype ID 16 (black). Place of disease transmission was reported to be certain (circle), probable (square), or possible (diamond); patient residency was used when transmission data was unavailable (triangle).

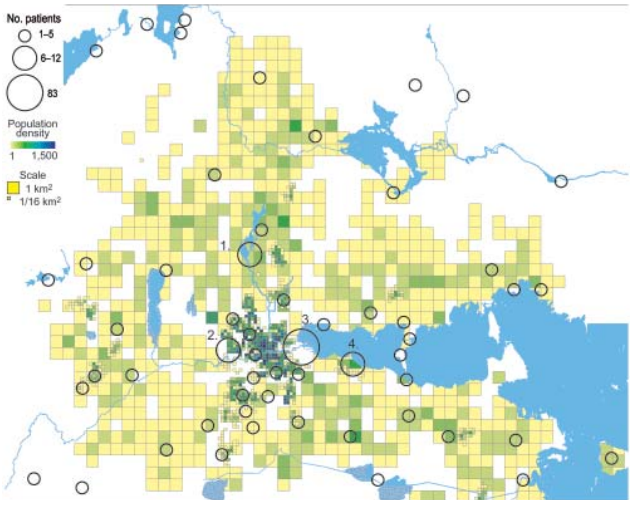

Figure 3. Geographic distribution of 202/240 places of tularemia transmission in Örebro, Sweden, 2000–2004. Four recreational areas were disease cluster sites for tularemia transmission: 1) Lake Lången, 2) Karslundsskogen/Hästhagen, 3) Oset/Rynningevikens nature reserve, and 4) Ekeby-Almby.

group 1e were circumscribed by a 150-km² ellipse (Figure 7, panel A). All 56 isolates were circumscribed by a 230-km² ellipse. Analysis per outbreak year showed that the infection area of 1e isolates was stable, with an east–west distribution along the river Ljusnan (Figure 7). The places of disease transmission for 3 isolates of genetic subgroups 1b, 1c, and 3 were peripheral to the infection area of 1e isolates (Figure 7, panel D). Many patients reported acquiring tularemia from restricted geographic areas, e.g., 33 of 56 isolates were from a 25-km² stretch along the river Ljusnan, with a disease cluster at a golf course (Figure 2).

Phylogeographic Analysis in Örebro

In Örebro, the places of disease transmission for genetic groups 1 and 2 were within 2 partially overlapping geographic areas with distinctly separate mean centers of occurrence (Figure 8, panel A). Closer examination of the areas of genetic group 1 showed that those of subgroups 1a, 1b, and 1d had similar spatial centers but different directional distributions (Figure 8, panels B–D). Infection locations of genetic subgroups 1a and 1b were oriented in an east-west direction along the Svartån River, whereas those of subgroup 1d were oriented in a north-south direction along the Lillån River. The places of disease transmission of genetic group 1 were circumscribed by a concatenated 272-km² ellipse area (Figure 8, panel A); those of genetic group 2 were circumscribed by a 645-km² ellipse (Figure 8, panel E). The proportions of isolates transmitted within the elliptic infection areas of genetic subgroups 1a, 1b, 1d, and 2 were 7 of 12, 9 of 16, 30 of 37, and 7 of 12, respectively (Figure 8, panels B–E). The geographic distribution of genetic subgroup 1a's places of transmission was most restricted, spanning an elliptic area of only 16 km². Seven of 12 transmission locations of genetic subgroup 1a could alternatively be enclosed in a rectangular area of just 2 km² (Figure 8, panel B). The genetic subgroup 1c comprised only 3 isolates with 3 distinct genotypes; places of disease transmission spanned a distance of 58 km (not shown).

In total, 240 of 278 patients in Örebro reported places of tularemia transmission. A spatial distribution plot of the locations reported by 202 of these patients is shown in Figure 3 (those of 38 patients are not shown because they were outside the map area), indicating the existence of 4 tularemia transmission clusters. Two clusters are further detailed in Figure 4. Eighty-three patients reportedly acquired tularemia in the Oset/Rynningeviken nature reserve 2 km from Örebro City center. Twenty-six of 27 cultured *F. tularensis* isolates from this area belonged to genetic group 1. Seventeen patients reported transmission at Lake Lången, 5 km north of Örebro City. Here, multiple patient reports verified that bacteria of genetic groups 1d and 2 coexisted. However, the centers of occurrence were geographically distinct (Figure 8, panel A).

Discussion

In this study of natural outbreaks of human tularemia in 2 locations in Sweden, genetic techniques developed for high-resolution typing of *F. tularensis* were used in con-

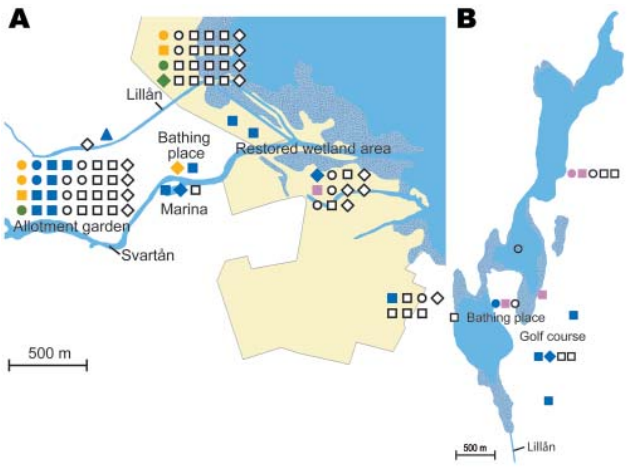

Figure 4. A) Cluster site for tularemia transmission at Oset/Rynningeviken nature reserve in Örebro, Sweden, with 83 patient reports. Twenty-seven *Francisella tularensis* isolates were recovered from these patients. B) Cluster site for tularemia transmission at Lake Lången, Örebro, Sweden, with 17 patient reports. Nine *F. tularensis* isolates were recovered from these patients. Place of disease transmission were reported to be certain (circle), probable (square), or possible (diamond); patient residency (triangle) was used if no such data were available. Genetic groups are indicated by color: yellow (1a), green (1b), blue (1d), or purple (2); white indicates no *F. tularensis* culture.

Table 2. Patient self-estimates of data quality for places of tularemia transmission, Sweden*

Location	Certain	Probable	Possible	Residential address	Total
Örebro	15	38	23	4	80
Ljusdal	23	16	5	12	56
Total	38	54	28	16	136

*Values summarize the information in the column "Patient self-estimate" found in online Appendix Tables 1 and 2 (available from www.cdc.gov/EID/content/15/12/1937-appT1.htm and www.cdc.gov/EID/content/15/12/1937-appT2.htm).

junction with information obtained from patient surveys and interviews to investigate the epidemiology and geographic spread of disease. Places of transmission of specific *F. tularensis* genotypes (Figure 5) were highly localized and restricted to areas as low as 2 km^2, pinpointing likely point sources of infection (Figures 7, 8). The results demonstrate the capability of enhancing epidemiologic investigations of tularemia by combining data from patient interviews with high-resolution genotyping of *F. tularensis* isolates recovered from the same patients.

A recent study in Utah of 5 patients and 11 rabbit carcasses infected with *F. tularensis* indicated that multiple *F. tularensis* subspecies and genetic subgroups may cause tularemia in a localized outbreak (*21*). Other studies have demonstrated that genetic subgroups of *F. tularensis* have distinct frequencies at continental scales throughout the Northern Hemisphere (*20*,*22*–*24*). Our study, in which 136 *F. tularensis* subsp. *holarctica* isolates from 2 localized human outbreaks were examined, shows the phylogeographic structure in *F. tularensis* subsp. *holarctica* populations involved in local outbreaks.

Why is there a phylogeographic structure? We found high genetic diversity and limited spatial distribution of genetic group 1 isolates in Örebro, which suggested recent expansion of local *F. tularensis* subsp. *holarctica* populations. The number of genotypes in genetic group 1 was notably greater than among genetic group 2 isolates from Örebro or among all the isolates recovered in the long-term tularemia-endemic area of Ljusdal (Figure 5). Because previous genotyping data have demonstrated that homoplastic SNP mutations are virtually nonexistent in *F. tularensis* (*20*,*25*), a common mutation at Ft-SNP1 (Figure 5) indicates that the isolates in genetic subgroup 1d in Örebro and 1e in Ljusdal share a more recent common ancestor than they do with isolates of subgroups 1a, 1b, or 1c in Örebro. However, genetic distances among all the group 1 isolates are likely to be small because Ft-SNP1 could be identified only by comparison of complete genome sequences, including a genetic subgroup 1e genome (strain FSC200 from Ljusdal) (*26*,*27*). Altogether, the data imply that genetic groups 1a, 1b, and 1c isolates have a local evolutionary history rather than a recent local disease introduction (as verified by high genetic variation at VNTR markers) and that the 1d isolates appear genetically related with isolates from Ljusdal (as verified by a SNP mutation).

Restoration of a wetland area between 1993 and 2006 (Figure 4) in Örebro may have been a factor in expansion of these genetic subgroups. Because *F. tularensis* subsp. *holarctica* is known to be associated with natural waters, favorable conditions for its replication may have resulted. The large genetic distance between genetic groups 1 and 2 in Örebro (they are consistently distinct at 9 of 18 markers; Figure 5), where tularemia recently has reemerged, compares with distances previously found among *F. tularensis* subsp. *holarctica* isolates of worldwide origin (*9*) and excludes a recent local common origin. The existence of

Clustering tree	Genetic group	Location	No. isolates	Years	INDEL (Ftind) 37	40	41	42	30	31	32	33	34	39	SNP (Ft-SNP) 1	2	VNTR (Ft-M) 22	24	26	20	6	3	Genotype ID
B1/B3	1a	Örebro	7	2003–2004	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	4	4	16	1
			5	2003	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	4	4	19	2
	1b	Örebro	1	2004	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	13	3
			1	2000	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	14	4
			11	2003–2004	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	18	5
			2	2003–2004	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	22	6
			1	2003	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	26	7
		Ljusdal	1	2005	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	4	17	8
	1c	Lj./Ör.	1	2005/2003	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	6	9	9
		Örebro	1	2003	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	6	10	10
			1	2003	1	1	1	1	0	0	0	0	0	0	G	G	4	2	2	3	7	10	11
	1d	Örebro	10	2002, 2003, 2004	1	1	1	1	0	0	0	0	0	0	A	G	4	2	2	3	5	20	12
			26	2002, 2003, 2004	1	1	1	1	0	0	0	0	0	0	A	G	4	2	2	3	5	21	13
			1	2003	1	1	1	1	0	0	0	0	0	0	A	G	4	2	2	3	5	22	14
	1e	Ljusdal	49	1995, 1998, 2002, 2005	1	1	1	1	0	0	0	0	0	0	A	A	4	2	2	3	5	10	15
			4	1998.2005	1	1	1	1	0	0	0	0	0	0	A	A	4	2	2	3	5	11	16
B4	2	Örebro	11	2000, 2002, 2003, 2004	1	1	1	1	1	1	1	1	1	1	G	G	3	1	3	3	4	12	17
			1	2003	1	1	1	1	1	1	1	1	1	1	G	G	3	1	3	3	4	14	18
B2	3	Ljusdal	1	2005	0	0	0	0	1	1	1	1	1	1	G	G	3	2	3	3	4	15	19

Figure 5. Attributes of 19 genotypes of *Francisella tularensis* subsp. *holarctica* identified in this study, and their genetic associations as assessed by a phylogenetic method (the clustering tree) or by an allele-based method (the genetic group designations). The letter and number designations in the clustering tree refer to nomenclatures of *F. tularensis* genetic clades as described by Johansson et al. (*9*). Gray shading indicates the derived genetic marker states. INDEL, insertion/deletion; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; ID, identification.

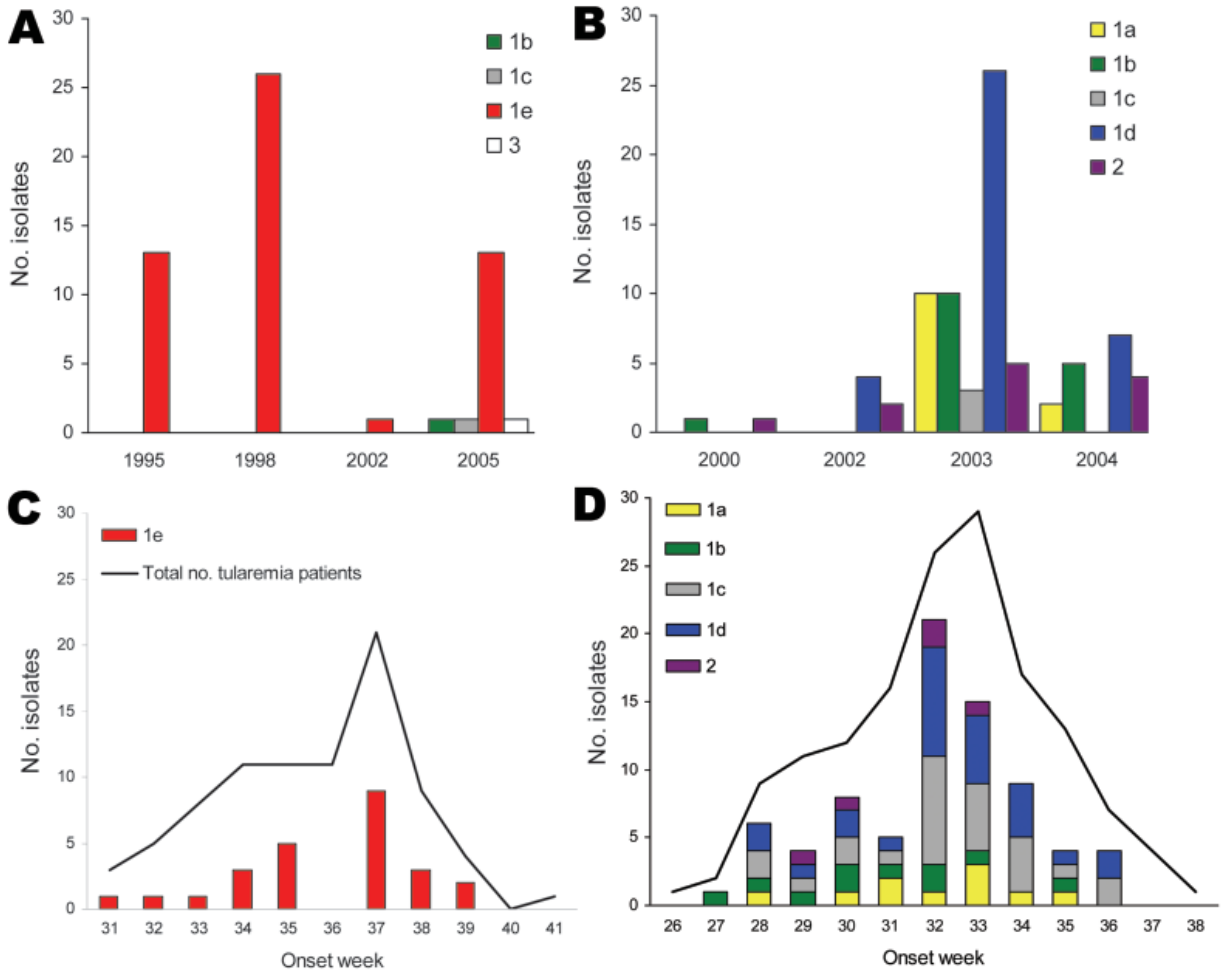

Figure 6. Proportional representation of genetic groups among isolated recovered per year for Ljusdal (A) and Örebro (B) and seasonal distribution of genetic groups of *Francisella tularensis* subsp. *holarctica* in 1998 in Ljusdal (C) and in 2003 in Örebro (D), Sweden. The week of disease onset was available for 84/87 patients in Ljusdal and 148/152 patients in Örebro.

several distinct *F. tularensis* populations active within a single tularemia outbreak is further demonstrated by comparing data of this study with data from previous work by Johansson et al. (*9*) and Kugeler et al. (*24*). All of the group 1 isolates belong to clades named B1/B3 or B.Br.013/014, respectively in these previous publications; group 2 isolates belong to clade B4 or B.Br.007/008 (or a nearby clade), and group 3 isolates to clade B2 or B.Br.OSU18. Kugeler et al. demonstrated the large numbers of SNPs separating group 1 from groups 2 and 3 isolates, thus verifying very distinct genetic populations.

The geographically widely distributed genetic group 2 in Örebro, the subgroup 1d in Örebro, or the subgroup 1e in Ljusdal, may be results of past temporary reductions of population sizes (genetic bottlenecks) or selective events (selective sweeps) that have eliminated genetic variation in originally more diverse populations. The selective sweep hypothesis is particularly attractive; a highly fit genotype over time will increase its frequency relative to other genotypes and may occupy larger geographic areas. This scenario would explain previous findings of low genetic diversity among isolates recovered from areas of Sweden with a long history of tularemia infections (*9*).

The genetic differences of genetic groups and subgroups were mirrored spatially. The genetic groups 1 and 2 in Örebro showed distinct mean centers of occurrence (Figure 8, panel A), and genetic group 1d isolates were the only isolates of group 1 found along the whole stretch of the river Lillån, resulting in a distribution area oriented north-south, opposite to subgroups 1a and 1b, which showed an east-west direction of their distribution areas (Figure 8, panels B–D). Similar pattern of separated disease occurrence center for genetic groups were found in Ljusdal (Figure 7, panel D). Collectively, these observations indicate distinct replication foci and dispersal areas of different *F. tularensis* subsp. *holarctica* populations.

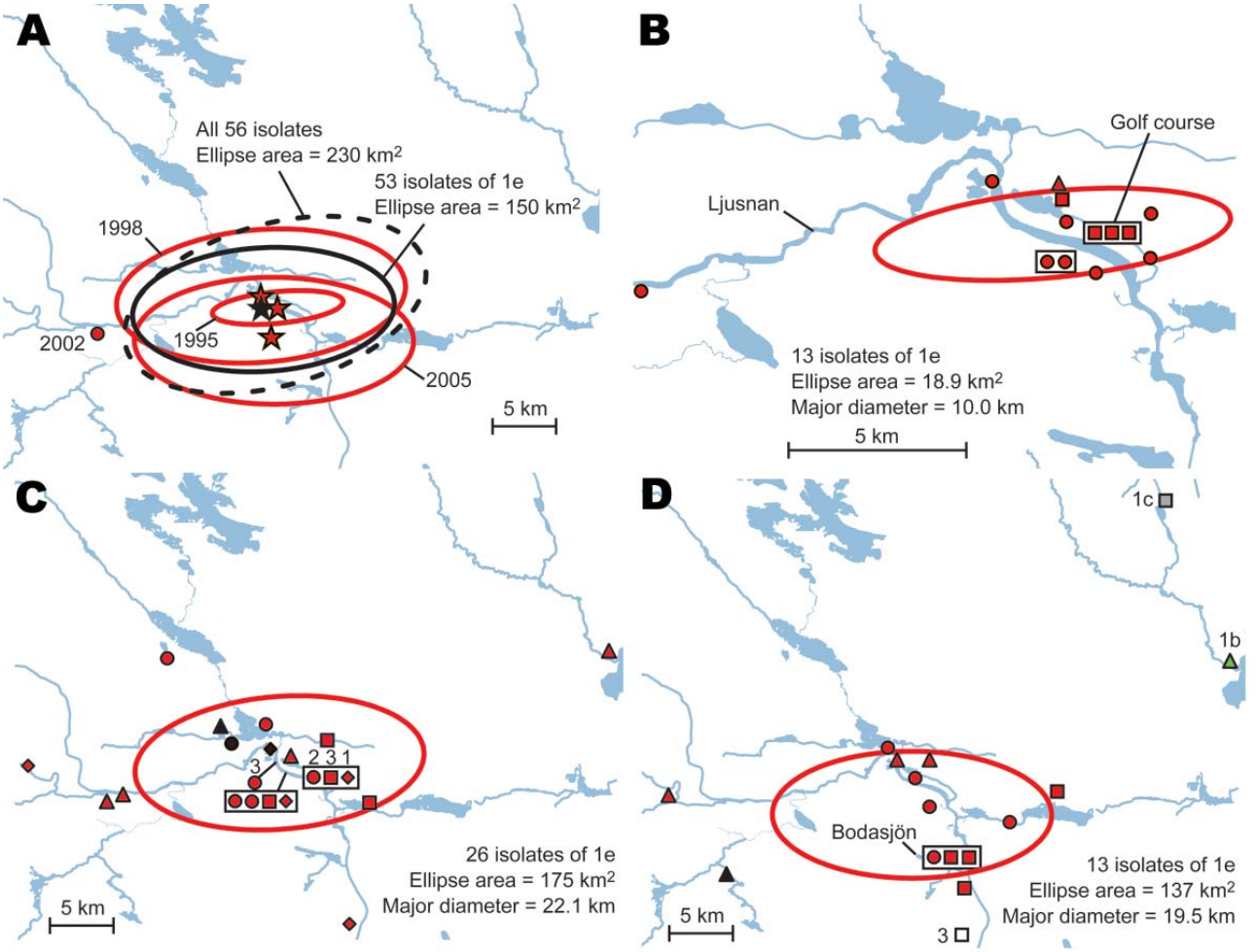

Figure 7. A) Directional distributions of tularemia transmission sites in Ljusdal, Sweden, by outbreak year (red ellipses). The *Francisella tularensis* isolates recovered from patients in Ljusdal were genetically monomorphic, with 53/56 isolates belonging to genetic subgroup 1e (solid black ellipse). The dashed black ellipse represents the distributions of all 56 isolates. Each ellipse represents a 1 standard deviation distribution around the mean centers of occurrence (starred). B) Distributions of 13 isolates of genetic group 1e, genotype identification (ID) 15 (red), Ljusdal, 1995. C) Distributions of 26 isolates of genetic group 1e, genotype ID 15 (red) and genotype ID 16 (black), Ljusdal, 1998. Numbers above symbols indicate multiple data points. D) Distributions of 13 isolates of genetic group 1e, genotype ID 15 (red) and genotype ID 16 (black); genetic group 1b (green); genetic group 1c (gray); and genetic group 3 (white), Ljusdal, 2005. Spatial data quality assessment for each pair of coordinates is shown as certain (circle), probable (square), or possible (diamond); patient residency (triangle) was used when transmission data were unavailable.

Many reasons are likely for a clustering of human tularemia. First, tularemia occurrence depends on the number of persons at risk, i.e., those who visit or live in areas where *F. tularensis* foci exists (Figures 2, 3). Second is the effect of vector ecology. Most of the 134 (91%) patients in Örebro who specified a disease transmission vector reported it to be mosquitoes. Thus, the distance that mosquitoes disperse, 200–2,000 m for most species in Sweden (*28*,*29*), probably strongly influences the infection patterns. Third, local factors affect the persistence and distribution of *F. tularensis* in nature. We found identical genotypes over different years, indicating that tularemia overwinters at the disease cluster sites. Genetic groups also were present during the whole tularemia season from July to September, indicating that no particular temporal patterns were associated with specific bacterial genotypes (Figures 5, 6).

Although uneven distributions of persons at risk and transmission vectors, as well as a general association of tularemia with streaming waters, may explain geographic disease clustering in humans, only different spatial distributions of *F. tularensis* populations can explain clustering of genetic groups and subgroups. Our observations are consistent with Pavlovsky's theory of "natural nidality of transmissible diseases," i.e., a connection of the vector-borne disease with a definite geographic landscape (*30*). In the case of tularemia, a recent study on dog ticks carry-

ing *F. tularensis* subsp. *tularensis* on Martha's Vineyard (Massachusetts, USA) showed persistence in a microfocus in nature over 4 years (*31*). Supporting the existence of a landscape epidemiology of tularemia, we found that different *F. tularensis* subsp. *holarctica* populations are locally present near certain bodies of water where they apparently stably perpetuate.

A major limitation of this study is a retrospective design that may have caused recall bias regarding the locations at which tularemia was contracted. The patient recall time in Ljusdal sometimes was up to 12 years; in Örebro patients were approached at admission to hospital. It is our impression, however, from many patient interviews, that the short incubation time of tularemia (2–5 days), the distinct clinical expression, its occurrence in restricted geographic areas, and a transmission route by blood-feeding arthropods, did facilitate patient recalls.

Ljusdal and Örebro in Sweden have comparatively high tularemia incidence rates. The results of this study suggest that genotyping coupled with global imaging satel-

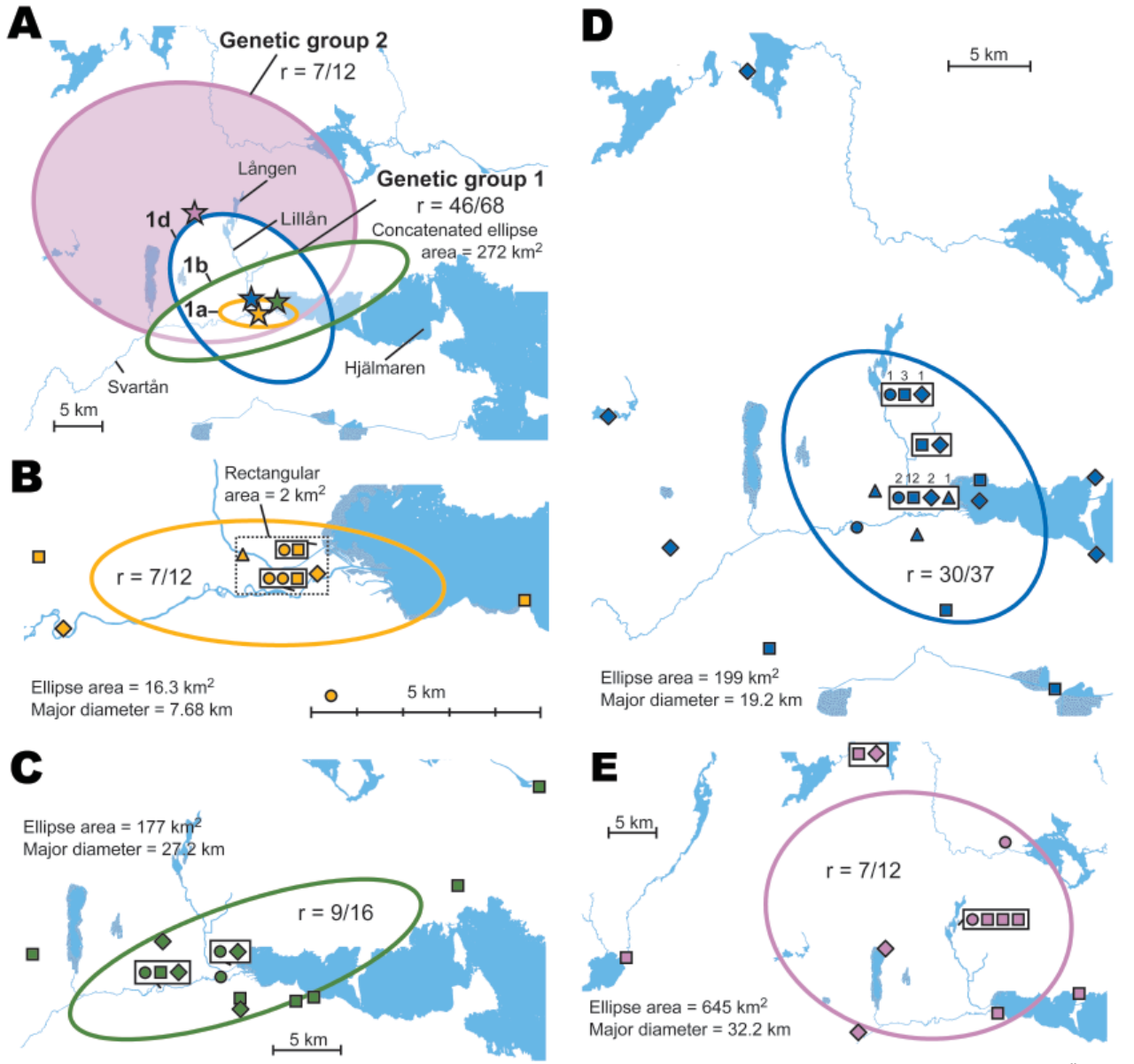

Figure 8. A) Directional distributions and spatial mean centers for 80 *Francisella tularensis* isolates of 4 different genetic groups, Örebro, Sweden. Each colored ellipse represents a 1 standard deviation distribution around the mean centers of occurrence (starred) for a genetic group. B–E) Details on transmission sites in Örebro for genetic groups of *F. tularensis* isolates: B) genetic group 1a; C) genetic group 1b; D) genetic group 1d; E) genetic group 2. Patient self-estimates of the spatial data quality are shown as certain (circle), probable (square), or possible (diamond); patient residency (triangle) was used if no such data were available. Proportions (r) of transmission sites within/outside an ellipse are indicated. Numbers above symbols in panel D indicate multiple data points at the same place.

lite mapping can help identify local environmental sources of tularemia, which is essential for effective infection control. This study also shows that pathogen genome sequencing efforts can contribute to the design of genotyping schemes tailored to a specific outbreak investigation. By combining high-resolution genotyping with patient interviews, we found *F. tularensis* populations to have strong spatial associations in 2 localized tularemia outbreaks. In future investigations, we believe that application of parallel mass-sequencing technologies to *F. tularensis* will be highly valuable for identifying additional genetic markers that, in turn, will facilitate tracking of the zoonotic pathogen through environmental sources, blood-feeding arthropods, and mammals. In addition to a more detailed genetic analysis, we need to identify ecologic correlates to the local areas of *F. tularensis* persistence and replication. Ultimately, the goal is to gain knowledge enabling future focused interventions directed at reducing the risk for tularemia acquisition by humans visiting or living in areas in which tularemia is highly endemic.

Acknowledgments

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Dr Svensson has been a bioinformatician at the Swedish Defense Research Agency in Umeå, Sweden, since 2001. Her research interests focus on improving genetic methods for identifying and tracing the origin of *Francisella* isolates to gain a better understanding of the epidemiology and ecology of tularemia in Scandinavia.

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Delineating *Anaplasma phagocytophilum* Ecotypes in Coexisting, Discrete Enzootic Cycles

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The emerging tick-borne pathogen *Anaplasma phagocytophilum* is under increasing scrutiny for the existence of subpopulations that are adapted to different natural cycles. Here, we characterized the diversity of *A. phagocytophilum* genotypes circulating in a natural system that includes multiple hosts and at least 2 tick species, *Ixodes ricinus* and the small mammal specialist *I. trianguliceps*. We encountered numerous genotypes, but only 1 in rodents, with the remainder limited to deer and host-seeking *I. ricinus* ticks. The absence of the rodent-associated genotype from host-seeking *I. ricinus* ticks was notable because we demonstrated that rodents fed a large proportion of the *I. ricinus* larval population and that these larvae were abundant when infections caused by the rodent-associated genotype were prevalent. These observations are consistent with the conclusion that genotypically distinct subpopulations of *A. phagocytophilum* are restricted to coexisting but separate enzootic cycles and suggest that this restriction may result from specific vector compatibility.

The tick-transmitted bacterium *Anaplasma phagocytophilum* is the causative agent of granulocytic anaplasmosis, an infection of medical and veterinary importance that is widely encountered across the temperate zones of the Northern Hemisphere (*1*–*3*). Although considerable effort has been put into determining the natural diversity of *A. phagocytophilum* (*4*–*6*), our understanding of ecologic and evolutionary processes that underlie this diversity remains far from complete. *A. phagocytophilum* has been detected in the blood of a wide range of mammals and in several different *Ixodes* species, which suggests that it is a generalist parasite with the capacity to exploit multiple hosts and vectors (*1*–*3*,*5*–*9*). However, evidence for the existence of subpopulations that are adapted to specific reservoir host species has recently been forthcoming (*7*,*9*,*10*), and these subpopulations appear to possess differing potential to be a public health threat (*7*,*9*,*10*). This phenomenon has also been described within another tick-borne generalist species complex, *Borrelia burgdorferi* sensu lato (*11*) and, more recently, within the 1 generalist member of this complex, *B. burgdorferi* sensu strictu (*12*,*13*). As yet, no evidence has shown that subpopulations of either *A. phagocytophilum* or *B. burgdorferi* have adapted to different *Ixodes* species as vectors.

Knowledge of the existence of host- or vector-adapted subpopulations is important given the public health consequences of multivector transmission by these pathogens. For example, we and other researchers (*14*–*19*) have hypothesized that pathogen populations maintained in efficient tick-rodent cycles by nidicolous (nest-living and host-specialist) ticks, such as *I. trianguliceps* in the United Kingdom and *I. spinipalpis* and *I. minor* in the United States, could spill over into the human population due to the co-occurrence of sympatric exophilic (and human-biting) tick species such as *I. ricinus* in the United Kingdom and *I. pacificus* and *I. scapularis* in the United States.

The purpose of this study was to characterize the diversity of *A. phagocytophilum* strains circulating in a natural multihost, multivector system and to determine whether the observed diversity had any ecologic basis. We obtained compelling evidence to support the proposition that different subpopulations of *A. phagocytophilum* exploit different tick and mammal species and, as a result, occur in discrete enzootic cycles even though both vectors and hosts are sympatric.

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Materials and Methods

Natural Multihost, Multivector Study System

Kielder Forest is a managed plantation forest dominated by Sitka and Norway spruce that covers 620 km^2 in northern England (55°13′N, 2°33′W). The harvesting of timber generates clearcut areas of 5–12 ha, which are subsequently replanted and progress through grassland and the thicket stage after 12–15 years. The most abundant mammal species encountered within clearcut areas is the field vole (*Microtus agrestis*), which exhibits multiannual population cycles in which densities can reach >700/ha (*20*). Roe deer (*Capreolus capreolus*) are also abundant at an estimated density of 3 deer/km^2 across the forest and are frequent visitors to clearcut areas (*21*). The presence of *I. ricinus* and *I. trianguliceps* ticks in clearcut areas has been documented (*18*,*19*).

Monitoring of Host and Vector Populations

Protocols for the handling and sampling of wild rodents were approved by the University of Liverpool Committee on Research Ethics and were conducted in compliance with the terms and conditions of licenses awarded under the UK Government Animals (Scientific Procedures) Act, 1986. Voles were trapped at 4-week intervals from March 2004 through November 2005 (excepting December 2004 and February 2005) at 4 principal study sites that were 3.5 km–12 km apart. Each animal captured was processed as described previously and a blood sample was taken from the tail tip (*19*). Voles were examined for ticks, with all larvae being removed and stored in 70% ethanol for identification (*22*,*23*) before releasing the animal at the point of capture. Nymph and adult ticks were not removed to minimize any effect on the transmission of tick-borne infections, which were being studied as part of an extensive longitudinal program. Host-seeking *I. ricinus* nymphs and adults were collected at monthly intervals from the principal study sites from March 2004 through September 2005 as previously described (*19*) and from 17 additional sites widely distributed across the Kielder Forest District. Collected ticks were stored and identified as described above. Roe deer blood samples were collected from January 2004 through July 2006 from animals culled throughout the forest and stored in EDTA-containing tubes at –20°C.

Host Bloodmeal Source Identification

The relative importance of different species as hosts for *I. ricinus* larvae was determined as previously described (*24*). Probes for the following taxa were used: *Myodes* spp., *Apodemus* spp., *Microtus agrestis*, *Sciurus* spp., *Sorex araneus*, *Meles meles*, and *C. capreolus,* together with a generic bird probe (*24*).

Monitoring of *A. phagocytophilum* Genotypes

Crude nucleic acid extracts were prepared from blood samples and host-seeking *I. ricinus* nymphs as previously described (*11*). The presence of *A. phagocytophilum* DNA in each extract was assessed by a real-time PCR (*25*).

Genotyping of *A. phagocytophilum* strains exploited sequence variation at 3 genetic loci, 16S rDNA, *msp4,* and DOV1. Fragments of *msp4* and 16SrDNA were amplified and analyzed as described (*18*,*25*). DOV1 is a noncoding region of ≈275 bp lying immediately downstream of a previously described variable number tandem repeat (VNTR) locus (*6*). Amplification of this locus was achieved by using seminested PCR. The first round of amplification contained 10 ρmol of each of the primers DOV1f (5′-GAT CTA TGA ATT GCY RGT GTT ATA-3′) and DOV1r1 (5′-ACA TTG TCA ATT TCT CAC CAT-3′), 12.5 μL of 2× Master Mix (Abgene, Epsom, UK), 1 μL of nucleic acid extract and sterile H_2O to a final volume of 25 μL, which was subjected to a thermal program of 95°C for 3 min, then 35 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 50 s, then a final extension step of 72°C for 5 min. The second round of amplification incorporated 1 μL of first-round product into a reaction containing 10 ρmol of each of the primers DOV1f and DOV1r2 (5′-CAA YRC ACR YAC ATT TCT AGG A-3′), 22.5 μL of Reddymix (ABgene), made up to a final volume of a 25 μL with sterile H_2O. This reaction mix was subjected to the same thermal program as used for the first round of amplification. DOV1 amplicons were sequenced directly by using the second round primers. DNA sequences from all 3 loci studied were verified, assembled and aligned by using Chromas Pro (Technelysium Pty Ltd, Tewantin, Queensland, Australia) and MEGA4 software (*26*). Sequence similarity calculations and phylogenetic inferences were conducted by using MEGA4 software (*26*).

Results

Monitoring of Host and Vector Populations

A total of 2,926 blood samples from 1,503 voles at the 4 study sites was obtained. Similar numbers of voles were encountered at each site and the population size at all sites fluctuated in a broadly synchronous manner, in keeping with the well-documented seasonal and multiannual population cycles (*27*). *A. phagocytophilum* DNA was detected in 183 (6.3%) of the blood samples, representing 157 (10.4%) of individual animals tested. Except for the bacterium being seemingly absent from 1 site in 2004, the seasonal variation in prevalence of infection was similar at all sites, with infections disappearing over winter, before reappearing in the spring and persisting until late autumn (Figure 1, panel A).

Of the 3,378 ticks that were recorded on the surveyed voles, 83.6% (2,823) were larvae, 13.4% (454) were

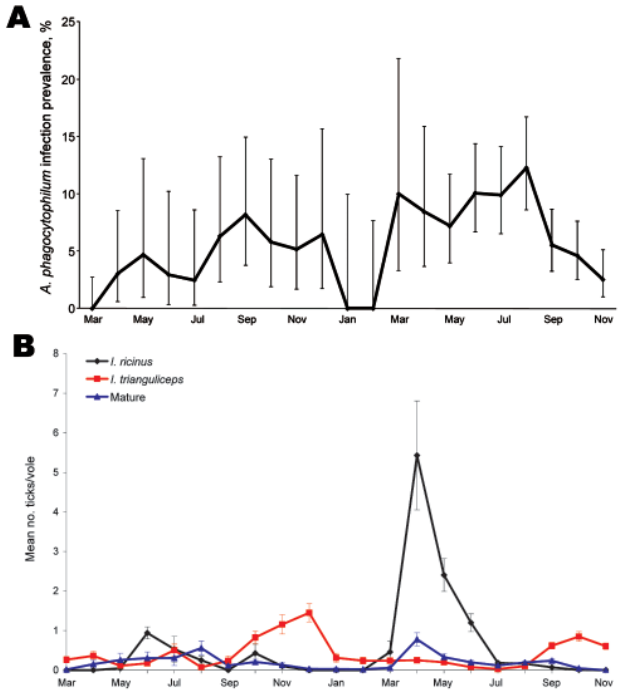

Figure 1. Prevalence of *Anaplasma phagocytophilum* infection in field voles (A) and of infestation of *Ixodes ricinus* tick larvae (black line), *I. trianguliceps* tick larvae (red line), and *I. ricinus*/*I. trianguliceps* adult females and nymphs (blue line) on field voles (B) during March 2004–November 2005. Error bars represent exact binomial 95% confidence intervals (A) or SEM (B).

nymphs, and 2.9% (101) adults. Approximately equal numbers of *I. ricinus* (1,618, 57.3%) and *I. trianguliceps* (1,205, 42.7%) were identified among the larvae, the seasonal dynamics of which are shown in Figure 1, panel B. *I. ricinus* larvae were most abundant in late spring/early summer, whereas *I. trianguliceps* larvae were most abundant in late autumn. The dramatic spike in the number of *I. ricinus* larvae recorded in May 2005 resulted from a small number of voles at one of our principal study sites having an extremely high number of larvae. Although nymph and adult ticks were not removed from voles (so could not be identified to species), their numbers were recorded. Of relevance to this study, virtually no nymphs or adults were observed on voles between November and April (Figure 1, panel B). The absence of the life stages that are capable transmitters of *A. phagocytophilum* underlies the disappearance of infections in voles during winter.

Blood samples were collected from 279 roe deer and *A. phagocytophilum* DNA was detected in 132 (47.3%) of these samples. Infections were detected throughout the year, with infection prevalence consistently high during the late spring/early summer of the years surveyed (Figure 2).

In total, 4,984 nymphs, 680 adult males, and 656 adult female host-seeking *I. ricinus* ticks were collected by dragging. The seasonal dynamics of both life stages have been presented elsewhere (*19*). *A. phagocytophilum* DNA was detected in 30 of 4,256 nymphs tested (0.7%), 9 of 263 adult females (3.4%) and 8 of 321 adult males (2.5%). Infected nymphs were encountered at 10 different sites. Infected host-seeking nymphs were collected during the same dragging session on only 8 occasions, suggesting that, for the most part, infected nymphs had fed on different animals.

Host Bloodmeal Source Identification

Bloodmeal source identification was attempted on 399 host-seeking *I. ricinus* nymphs and unambiguous results were obtained for 105 ticks (26.3%). These ticks were obtained from dragging sessions throughout 2004 (87 ticks) and 2005 (18 ticks) from the 4 principal study sites. Sixty-two (59.0%) showed evidence of having fed on voles as larvae, 18 (17.1%) fed on birds, 15 (14.3%) fed on deer, and the remaining 10 (9.5%) fed on small mammal species other than field voles.

Monitoring of *A. phagocytophilum* Genotypes

Comparison of partial 16S rDNA sequences obtained from 5 infected voles and 5 infected deer showed 4 highly similar (>99%) sequence types. All voles were infected with a sequence type that was identical to one previously associated with various ruminant species (e.g., Old Sourhope, GenBank AY176591). Three 16S rDNA sequence types were obtained from the deer samples, 2 of which had been previously reported associated with a variety of animals and tick species across the Northern Hemisphere (e.g., GenBank AF481850 and AJ242783), but the third sequence type was new. Although comparison of 16S rDNA sequence types was useful in confirming that detected DNAs were derived from strains of *A. phagocytophilum*, the insensitivity of this locus for intraspecies delineation led us to attempt sequence typing on the basis of a more variable locus, *msp4* (*6*,*28*).

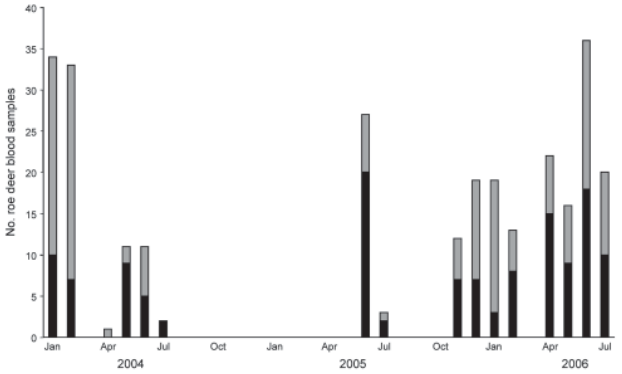

Figure 2. Number of *Anaplasma phagocytophilum*–infected (black bars) and uninfected (gray bars) animals encountered among Kielder Forest District roe deer sampled during January 2004–July 2006.

Unambiguous sequence data were obtained from amplicons derived from 45 infected roe deer, 48 infected voles, and 21 infected host-seeking *I. ricinus* nymphs and adults. For each host or vector, the samples came from across the range of sites, seasons and years of study. Seven *msp4* sequence types were obtained from infected roe deer. One sequence type was detected in most (30) samples. This and a second sequence type had previously been encountered among European deer, while the 5 remaining sequence types were new. Four *msp4* sequence types were encountered among the infected host-seeking *I. ricinus* ticks, all of which were also detected in roe deer. The most commonly encountered sequence type, which infected 17 ticks, was the same as that found most frequently among infected deer. The partial *msp4* sequences obtained from 48 infected field voles were all indistinguishable from one another.

Phylogenetic analysis, based on an alignment of the 50 *A. phagocytophilum msp4* sequence types present in GenBank (as of August 1, 2008), the 6 new alleles reported in this study, and homologous sequences available for the closely related species *A. marginale* and *A. centrale*, was used to infer the relative evolutionary positions of the *A. phagocytophilum* strains encountered in this study. The 5 new sequence types obtained from roe deer and host-seeking *I. ricinus* ticks lay within a cluster of closely related sequence types that also included the 2 other sequence types recovered from roe deer and *I. ricinus* ticks that had been previously encountered elsewhere (Figure 3). This well-supported cluster comprised 50 of the 53 *A. phagocytophilum msp4* sequence types reported to date and was characterized by short intersequence type evolutionary distances and included strains associated with wild and domesticated ruminants, companion animals, and humans.

Three *A. phagocytophilum* sequence types lay outside this cluster (Figure 3) and included types specifically associated with voles in this study, one associated with Chinese rodents (ZJ-China) (*8*), and one obtained from an infected roe deer in Germany ("roe deer") (*5*). The evolutionary distances between these 3 sequence types were markedly longer than those between the sequence types within the large cluster, and although maximum parsimony analysis indicated a shared line of descent for the vole-associated and roe deer–associated sequence types, this branching order was not strongly supported when distance matrix– or minimum evolution–based methods of inference were used, and no approach suggested a clustering of either of these sequence types with ZJ-China.

Examination of DOV1 sequences supported the *msp4*-based analysis. Unambiguous DOV1 sequences were obtained from DNA extracts derived from 8 infected deer, 6 infected field voles, and 14 host-seeking *I. ricinus* ticks. A total of 13 different DOV1 sequence types were obtained; all infected voles yielded the same sequence type, whereas infected deer yielded 5 different sequence types, and infected host-seeking ticks yielded 9 different sequence types. Two sequence types were associated with both deer- and host-seeking *I. ricinus* ticks. Phylogenetic analysis inferred that DOV1 sequence types associated with deer and host-seeking *I. ricinus* ticks were closely related to one another, whereas sequence types associated with voles had markedly diverged (Figure 4). This phylogeny is congruent with that derived from *msp4* data.

Figure 3. Phylogenetic tree inferred from alignment of *Anaplasma phagocytophilum msp4* sequences obtained in this study or available from GenBank. Inference was made by using the neighbor-joining algorithm. The stability of proposed branching order was assessed by bootstrapping (1,000 replicates). At nodes present in >50% of replicates, the percentage of replicates possessing the node is indicated. The GenBank accession numbers of the new *msp4* sequences obtained during this study (in **boldface**) are included in the strain designations. rAP sequence types were detected in questing *Ixodes ricinus* ticks and roe deer, and the tAP sequence type was detected in voles. Scale bar indicates nucleotide substitutions per site.

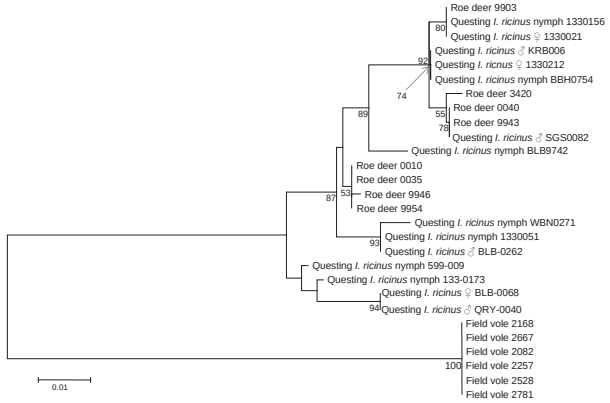

Figure 4. Phylogenetic tree inferred from alignment of *Anaplasma phagocytophilum* DOV1 sequence types obtained in this study. Inference was made by using the neighbor-joining algorithm. The stability of proposed branching order was assessed by bootstrapping (1,000 replicates). At nodes present in >50% of replicates, the percentage of replicates possessing the node is indicated. The DOV1 sequences obtained in this study have been deposited in GenBank under the accession nos. FJ469653–FJ469666. Scale bar indicates nucleotide substitutions per site.

Discussion

Although considerable effort has been put into exploring the genetic diversity of *A. phagocytophilum* (*4*–*6*), the public and veterinary health value of this endeavor has been limited by the failure to identify an ecologic basis for the genotypic variation encountered. Recent studies in the United States have begun to resolve this problem, with the identification of apparent host-preference ecotypes among different 16S rDNA sequence types of *A. phagocytophilum* (*7*,*9*,*10*). One particular sequence type, referred to as Ap-variant 1, appears to exploit only white tailed deer (*Odocoileus virginianus*) as a reservoir host, whereas other variants, including that associated with human granulocytic anaplasmosis (Ap-ha), exploit white-footed mice (*Peromyscus leucopus*), a species that has long been implicated as a important reservoir for *A. phagocytophilum* in North America (*29*). In Europe, the epidemiology of *A. phagocytophilum* infections appears to be quite different from that in the United States. Although far fewer human cases have been reported, infections in livestock are common and represent a large financial burden to the industry (*30*). Surveys of strains infecting European livestock and wild-living ungulates have found numerous 16S rDNA sequence types, including Ap-ha and Ap-variant 1 (*6*,*31*), indicating that this locus is not a marker of the same *A. phagocytophilum* ecotypes present in the USA. In this study we have begun to unravel the ecologic significance of the genetic diversity present among European *A. phagocytophilum* strains by genotyping strains circulating in a natural multihost, multi-vector system and correlating the genotypes we encountered with the provenance of the characterized strains. Our efforts have resulted in the discovery that field voles serve as a reservoir host for a unique genotype of the species that has markedly diverged from those genotypes encountered in wild roe deer and host-seeking *I. ricinus* nymphs and adults. This discovery is incompatible with the hypothesis that voles, *I. ricinus* ticks, and roe deer are all part of the same enzootic cycle but instead provides compelling evidence for at least two co-existing yet distinct enzootic cycles, one involving roe deer as hosts and *I. ricinus* ticks as vectors and another with field voles as hosts. As we have previously reported, *A. phagocytophilum* can be maintained in the absence of *I. ricinus* ticks in a natural cycle involving small mammals and *I. trianguliceps* ticks (*32*), and that, even when present in abundance, *I. ricinus* ticks do not play a major role in this cycle (*19*). *I. trianguliceps* ticks, which occur abundantly in our study system, are almost certainly a component of the enzootic cycle that includes field voles.

The results of our study are not compatible with the hypothesis that pathogen populations maintained in an enzootic rodent–nidicolous tick cycle could spill over into humans or livestock because of the co-occurrence of sympatric exophilic tick species (*14*–*19*). Through the use of host bloodmeal source identification, we demonstrated that *I. ricinus* larvae had ample opportunity to acquire *A. phagocytophilum* infection from voles (over half the questing *I. ricinus* nymphs we tested fed on voles as larvae). However, we found no evidence of the vole-associated genotype in host-seeking *I. ricinus* nymphs. This result suggests that, *I. ricinus* larvae are, at best, inefficient vectors of the vole-associated *A. phagocytophilum* genotype, thereby ostensibly removing the potential "bridge" out of the enzootic cycle that includes voles and *I. trianguliceps*. Notably, we did not detect deer/*I. ricinus* tick–associated *A. phagocytophilum* genotypes in voles despite previously observing *I. ricinus* nymphs feeding on these hosts (*25*). Because we did not remove nymph or adult ticks infesting our surveyed rodents in this study, we were unable to distinguish between *I. ricinus* and *I. trianguliceps* ticks, so were unable to gauge the frequency with which the former were encountered, although we believe that most nymphs on field voles are *I. trianguliceps* (*18*). Thus, this absence may result either from voles not being susceptible to deer/*I. ricinus*–associated genotypes or simply because encounters between infected *I. ricinus* nymphs and voles occur only rarely.

Although vector specialization by arthropod-transmitted pathogens is common (*33*), many of those that are tick-transmitted exploit more than 1 species (*15*–*19*).Also, clear evidence exists for local adaptation, whereby pathogens exhibit greater infectivity in local vector populations than those that are geographically distinct (*34*,*35*), although this phenomenon was not encountered for *A. phagocytophilum*

(*36*). We report evidence for the adaptation of different genotypes of the same pathogen species to transmission by different but co-existing vector species. We are planning laboratory transmission studies to determine the extent to which this adaptation represents complete specialization of genotype to vector. Nonetheless, the data we have already obtained from our field studies provide a clear insight into the ecologic consequences of this adaptation; in other words, of what is, rather than what may be, happening. *A. phagocytophilum* has a wide geographic distribution, and numerous members of the *Ixodes* genus have been implicated in its transmission. Thus, plenty of scope remains for further exploration of vector specificity by subpopulations of the pathogen. The transmission of *A. phagocytophilum* in the laboratory has been reported (*29*) and, subsequently, efforts have been made to examine interstrain variation in the dynamics of this process (*37*). These include a demonstration that strains from the western United States that are naturally transmitted by *I. pacificus* ticks can be transmitted by *I. scapularis* ticks in the laboratory (*35*), which suggests that not all *A. phagocytophilum* strains have adapted to exploit only a single vector species. In addition, *I. scapularis* serves as a vector for both Ap-ha and Ap-variant 1, the 2 *A. phagocytophilum* genotypes that possess different host specificities (*7*,*9*,*10*). These observations, taken together with those made in the current study, provoke the conclusion that although the species as a whole can be considered a generalist, *A. phagocytophilum* embraces a consortium of distinct ecotypes that have evolved a range of strategies to facilitate their own perpetuation. Whether host or vector specialization is the more commonly adopted strategy remains to be explored.

From an infection control perspective, it is important to recognize that cryptic transmission cycles of tick-borne pathogens maintained by nidicolous ticks have substantial human and veterinary health risk implications when sympatric exophilic tick vectors act as a bridge to potentiate human or livestock infections. However, our study shows the value of a more profound understanding of the diversity of the transmission cycles and pathogens on which to base estimation of the environmental health hazard: discrete co-existing transmission cycles can be associated with dilution of the abundance of tick-borne pathogens when, at first sight, an augmentation would be the expected outcome.

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etymologia

Calicivirus

[kə-lis′ ĭ-vi″rəs]

The name of these members of the Caliciviridae family of nonenveloped RNA viruses reflects their structure. They are icosahedral with 32 typical surface depressions that are sometimes described as hollows or cups (from Latin *calyx*, meaning cup). Feline calicivirus, a member of the genus *Vesivirus*, causes respiratory disease in cats. Members of other genera, *Norovirus* and *Sapovirus*, cause gastrointestinal disease in humans.

Source: Mahy BWJ. The dictionary of virology, 4th edition. London: Elsevier; 2009; Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007.

Novel Calicivirus Identified in Rabbits, Michigan, USA

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We report a disease outbreak in a Michigan rabbitry of a rabbit calicivirus distinct from the foreign animal disease agent, rabbit hemorrhagic disease virus (RHDV). The novel virus has been designated Michigan rabbit calicivirus (MRCV). Caliciviruses of the *Lagovirus* genus other than RHDV have not been described in US rabbit populations. The case-fatality rate was 32.5% (65/200). Clinical signs included hemorrhage and sudden death, with hepatic necrosis. Analysis of viral RNA sequence from >95% of the viral genome showed an average similarity of 79% with RHDV. Similarity of the predicted MRCV capsid amino acid sequence ranged from 89.8% to 91.3%, much lower than the 98% amino acid similarity between RHDV strains. Experimentally infected rabbits lacked clinical disease, but MRCV was detected in tissues by PCR. We propose that MRCV primarily causes subclinical infection but may induce overt RHD-like disease under certain field conditions.

Rabbit hemorrhagic disease (RHD) is caused by a calicivirus and is associated with illness and death in up to 90%–100% of susceptible rabbit populations (*1*–*3*). Domestic rabbits (*Oryctolagus cuniculi*) and free-ranging European rabbits (*O. cunicuusi*) are highly susceptible; cottontail rabbits (*Sylvilagus floridanus*) and hares (*Lepus* spp.) are unaffected. Rabbit hemorrhagic disease virus (RHDV) was first detected in China in 1984 (*4*) and subsequently has been described in eastern and western Europe, Asia, South America, Australia, Mexico, and the United Kingdom (*2*,*3*). In the United States, it is considered a foreign animal disease, and outbreaks are of considerable economic concern to the US rabbit industry. In the United States 4 outbreaks of RHD have occurred in domestic, captive rabbits (*O. cuniculi*) since 2000. These cases were confirmed by inoculation study and reverse transcription–PCR (RT-PCR) because the virus is not cultivable in vitro. Subsequent genomic analyses suggested that these incidences resulted from separate viral introductions; however, confirmed points of origin (e.g., imported animal or animal product) were never identified (*3*).

RHDV is classified as a *Lagovirus* within the family *Caliciviridae*. *Caliciviridae* are nonenveloped, positive-sense, single-strand RNA viruses. Within this family are 4 genera: *Lagovirus, Vesivirus, Norovirus*, and *Sapovirus* (*5*). In addition to the highly pathogenic RHDV, the *Lagovirus* genus includes several distinct but related viruses affecting rabbits or hares. These are European Brown hare syndrome virus (EBHSV), which causes disease similar to RHDV in hares only (*Lepus* spp.) (*6*), and the nonpathogenic rabbit calicivirus (RCV), which causes asymptomatic seroconversion in rabbits (*7*). RCV and EBHSV have not been reported and lagoviruses other than RHDV have not been described in US rabbit populations, although a low pathogenicity rabbit *Vesivirus* recently was identified in domestic rabbits from Oregon (*8*). RHDV affects only rabbits of the *Oryctolagus* genus which, in the United States, is limited to domestic rabbit species. Wild rabbit species in the United States are not experimentally susceptible (*9*). Seroprevalence surveys of RHDV or related caliciviruses in US domestic rabbit populations have not, to our knowledge, been published.

Beginning January 1, 2001, and continuing over a 3-week period, a privately owned New Zealand White (*O. cuniculi*) rabbitry in Michigan experienced acute fatalities.

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Before this episode, the farm had operated for 1.5 years without notable disease. Approximately 200 rabbits were kept in a closed barn on a 60-acre farm; new rabbits had not been acquired in 18 months. Inappetence in several animals and vulvar hemorrhage in pregnant does were initially noted. A total of 65 rabbits—consisting of 23 adult does (most pregnant), 2 adult bucks, and 41 young rabbits of both sexes—died over ≈3 weeks, for a case-fatality rate of 32.5%. Clinical signs consisted of vulvar hemorrhage in the does, epistaxis, ataxia, opisthotonos, diarrhea, ocular discharge, vocalization, and death.

Materials and Methods

Biological Samples

Three pregnant does (2 live) with vulvar hemorrhage were submitted for diagnostic evaluation on January 3. Eighteen additional rabbits of either sex ranging from 2 through 9 months of age were submitted near the end of the disease outbreak on January 17 and February 2. Animals submitted live were humanely euthanized by intravenous or intraperitoneal injection of pentobarbital (Fatal-Plus, Vortech, Dearborn, MI, USA) at 1 mL per 10 pounds.

Tissue samples from representative organs of all submitted rabbits were immersion fixed in 10% neutral buffered formalin. We processed tissue samples for histopathology using standard methods. Transmission electron microscopy was performed on a negatively stained clarified pooled liver homogenate from 2 of the originally submitted does using standard procedures.

For immunohistochemistry, liver sections from an affected rabbit were deparaffinized and rehydrated by routine methods. Antigen retrieval was by proteinase K (Dako, Carpinteria, CA, USA) for 10 min at 37°C. Endogenous peroxidase was blocked for 15 min with 3% hydrogen peroxide, and nonspecific immunoglobulin binding was blocked by 10 min incubation with a protein-blocking agent (Dako). Guinea pig anti-RHDV VP60 antibody (dilution 1:1,000) provided by Dr. F. Parra (*10*) was applied for 30 min at room temperature in a Dako autostainer. After incubation with rabbit anti–guinea pig immunoglobulin (Ig) G, detection was by a chain polymer conjugated staining procedure (EnVision, Dako) with visualization by AEC (Dako) and Mayer's hematoxylin counterstaining. Negative controls were homologous (guinea pig) nonimmune serum applied to rabbit liver sections.

In Situ Hybridization

In situ hybridization was performed as described (*11*). Briefly, liver sections from an affected and an experimental rabbit were deparaffinized, digested with 0.25% pepsin and prehybridized. Hybridization was for 5 min at 105°C and 60 min at 37°C with a specific 3′-end digoxigenin-labeled oligoprobe (5′-GAGAGTCGTCTCGGTAGTACCTG-3′, IDT, Coralville, IA, USA) at 5 μL/1 mL using a commercial workstation (Fischer Scientific, Pittsburgh, PA, USA). Detection was by antidigoxigenin (Boehringer Mannheim Biochemica, Indianapolis, IN, USA) conjugated with alkaline phosphatase (dilution 1:500, Boehringer Mannheim Biochemica) and the substrates NBT/X-Phos (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate, Boehringer Mannheim Biochemica). A non-sense probe was applied to liver sections as negative control.

Bacterial Cultures

Representative tissues from the original 3 does and 14 subsequently submitted rabbits were cultured by using standard microbiologic techniques. Virus isolation was attempted by injection of filtered (0.45-μm pore size) 10% homogenate of liver (pooled from 2 of the originally submitted does) in Bovarnik's buffer onto cultures of RK-13, R9ab, or SIRC cells.

Nucleic Acid Extraction and PCR

Total RNA from pooled liver of 2 initially submitted does was extracted with RNeasy Mini Kit (QIAGEN, Inc., Valencia, CA, USA), according to the manufacturer's guidelines. A 398-bp region of the VP60 (capsid) gene was initially targeted using RHDV-specific primers (*12*). RT-PCR was performed on total liver RNA using a One-Step RT-PCR Kit (QIAGEN) with forward and reverse primers, 5′-GTT ACG ACT GTG CAG GCC TAT GAG TT-3′ and 5′-TTG TTG AGC AGT CCA ATT GTC ACT G-3′, respectively, at 0.6 μM in a 50-μL reaction. Cycling conditions were as follows: cDNA synthesis at 50°C for 30 min; then predenaturation at 95°C for 15 min and 43 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and final extension of 72°C for 10 min. Products were visualized in prestained agarose gels by a UV transillumination. The entire capsid gene (≈1.8 kb) was amplified by using primers based on USA Iowa 2000 RHDV strain (spanning nucleotide positions 5273–7065, GenBank accession no. AF258618). Forward and reverse primers, 5′-CGG TAG TAC CTG ACG ACG AAT TTG-3′ and 5′-GCA AGT CCC AGT CCG ATG AAT-3′, respectively, were used as above, with the modification of 35 cycles with 2 min of extension. Further genomic sequencing used primers from conserved RHDV and Michigan rabbit calicivirus (MRCV)–derived sequences (Table) with annealing temperatures 52°C–55°C and extension times 1 min per 1-kb target length. MRCV genomic ends were sequenced using 5′ and 3′ RACE Systems for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA, USA). For 5′ RACE, cDNA was synthesized with gene-specific primer, GSP1, 5′-ACT GTA CTC CCT GGG TGC GAC-3′ (MRCV, 2039–2059, minus sense) with purification and dCTP-tailing with TdT. The

Table. Primers used in genomic sequencing of Michigan rabbit calicivirus*

Genomic region(s) spanned	Forward and reverse primer pairs (5′ → 3′)†	Nucleotide positions in RHDV‡
Polymerase-capsid (VP60)	ATGCCATGACTCCGATGATGGT (+) R	4835–4856
	CTTGTTGGTCCACCTGTTG (−) M	5482–5500
Polymerase	GCGACTTCTTGTGCTTGGACTAC (+) R	4493–4515
	GCACCACTCCAACTGTCTGAGAA (−) M	5027–5049
Polymerase	GTGACCCAGACAGTGACAAGT (+) R	3929–3949
	GGCCTATTTCTGCACATGCTT (−) M	4754–4774
Protease-polymerase	GCGGTGACCARGGTGTTGATG (+) R	2819–2839
	GCCGCAGCACGCTCTATGAAT (−) M	4020–4040
VPg-protease	AACAAAGCCGTTGAAAGTTGG (+) R	1927–1947
	TGGCAGCTCTGTTCTTCATTT (−) M	3119–3139
Helicase	GAGGTTGTTTGACACGTTTGA (+) R	1089–1109
	TGTCATATTCACACAGCCCAG (−) M	2398–2418
Capsid (VP60)–ORF-2	GTTACGACAGTGCAGGCCTATG (+) M	6412–6433
	CTCGCCAGTGGTGTTATAAATC (−) R	7345–7366

*RHDV, rabbit hemorrhagic disease virus; MRCV, Michigan rabbit calicivirus; VP, viral protein; ORF-2, open reading frame 2.
†RHDV-specific primer denoted by R; MRCV-specific primer denoted by M.
‡Positions spanned by primer set in RHDV genome (Italy BS89 strain, GenBank accession no. X87607).

dC-tailed cDNA was PCR amplified by using HotStar Taq DNA Polymerase Kit (QIAGEN) in a 50-μL reaction with 5 μL dC-tailed cDNA, 0.5 μM abridged anchor primer, and gene-specific primer (GSP2) 5′-CAT CGC CGC TGG TGT TAA ACT-3′ (MRCV, 1662–1682, minus sense) at 95°C for 15 min and 35–45 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min 30 sec; with final extension 72°C for 7 min. For 3′ RACE, cDNA was synthesized with an oligo-dT-containing adapter primer according to the kit manufacturer's protocol. PCR was performed with gene-specific primer, 5′-GTT ACG ACT GTG CAG GCC TAT GAG TT-3′ (the same used in the initial RHDV-specific PCR) (*12*) and the kit component abridged universal primer with reaction specifications above.

Sequencing and Sequence Analyses

RT-PCR amplicons were purified from 1.4% agarose gels by QIAquick Gel Extraction Kit (QIAGEN). PCR products were sequenced by the Research Technology Support Facility of Michigan State University by automated DNA sequencing on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Long PCR templates were sequenced by using primer-walking with newly synthesized 5′ and 3′ primers derived with OLIGO 6 primer analysis software (Molecular Biology Insights, Cascade, CO, USA). Similar GenBank sequences were detected by BLAST analysis (*13*). Sequence assembly and analyses, including multiple Clustal W (www.ebi.ac.uk/clustalw) sequence alignments and construction of phylogeny trees, were done with Lasergene software (DNASTAR, Inc., Madison, WI, USA). GenBank accession numbers for complete and partial rabbit caliciviral nucleotide sequences used for phylogenetic analysis are as follows: RHDV strains USA IA00 (AF258618), USA UT01 (EU003582), USA NY01 (EU003581), USA IN05 (EU003579), JX China 97 (DQ205345), China CD (AY523410), China WHNRH (DQ280493), Italy 90 (EU003580), Germany FRG (NC001543), China WX84 (AF402614), Mexico 89 (AF295785), Spain AST89 (Z49271), Ireland 18 (DQ367359), Italy BS89(X87607), EBHSV (NC002615), RCV (X96868), MRCV (GQ166866).

Inoculation Study

Fourteen 16-week-old male and female, specific pathogen–free New Zealand White rabbits (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were each inoculated intramuscularly, orally, and intranasally with 2 mL total of liver homogenate containing 10^4–10^5 PCR detectable units of viral RNA per mL, as determined by PCR assay on serial 10-fold dilutions of RNA extracted from the inoculum. Homogenate was prepared from frozen (−80ºC) liver from 2 of the originally submitted does. Two control rabbits were in containment housing in the same cage bank but not in direct contact with inoculated rabbits and were not sham inoculated. The rabbits were free of *Pasteurella multocida, Bordetella bronchiseptica, Encephalitozoon cuniculi, Treponema cuniculi, Clostridium piliforme,* myxomatosis virus, RHDV, *Toxoplasma* spp., and coccidiosis. Animal husbandry was in keeping with the Animal Welfare Act and US Public Health Service policy, and the infection study protocol was approved by the University Animal Care and Use Committee.

Rabbits were monitored for clinical changes and were humanely euthanized by intravenous pentobarbital injection after 2 (n = 2 control rabbits), 4 (n = 2), or 7 (n = 12) days of infection. Complete necropsies were performed and tissue samples harvested for histology, serology, and RT-PCR. The MRCV capsid encoding region was cloned into a baculovirus expression vector by standard methods, enabling serologic testing by application of serum to

baculovirus-expressing insect cells and immunoperoxidase detection.

Results

Gross and Histologic Findings

The animals submitted for diagnostic evaluation were in good body condition. Several had conjunctival congestion (Figure 1) and mild cyanosis of the lips and ear tips before euthanasia. The pregnant does had small amounts of vulvar hemorrhage and cutaneous hyperemia. The gravid uteri in the does had red to purple serosal discoloration with serosanguinous luminal fluid. Fetuses were in good condition and appeared normal. The livers of all does and 2 of the young adult rabbits were friable and tan and had accentuated lobular pattern (Figure 2). Individual rabbits variably exhibited icterus, opisthotonos, gastric petechiae and ecchymoses (Figure 3), colonic serosal hemorrhage, and multifocal hemorrhage in caudal lung lobes.

In the 3 initially submitted does, the major histologic finding was multifocal random or periportal hepatocellular necrosis (Figure 4). Additionally, we found mild periportal heterophilic (neutrophilic) and lymphoplasmacytic inflammation. There were also pulmonary and uterine hemorrhages with fibrin clots in areas of placental implantation. In the 18 subsequently submitted young adult rabbits, predominant histologic findings were moderate expansion of portal tracts with bile duct proliferation, periductal fibrosis, and mild periportal lymphoplasmacytic inflammation. Five rabbits had concurrent heterophilic and bronchopneumonia, and 1 had suppurative meningitis.

Initial Diagnostic Testing

Caliciviral-like particles were detected in pooled liver homogenate from 2 of the originally affected does by transmission electron microscopy. On request, the US Department of Agriculture Foreign Animal Disease Diagnostic Laboratory further tested tissue samples. Results from inoculation testing and RT-PCR using standard primers (*3*) within the RHDV polymerase region at FADDL were not consistent with RHD, and RHDV was ruled out as the cause of the outbreak.

Uterine cultures from 1 of the initially submitted does grew >1,000 colony-forming units (CFU)/mL *P. multocida*. One young adult rabbit with hepatic necrosis and concurrent bronchopneumonia grew >1,000 CFU/mL *P. multocida* from the lung. Two other rabbits had smaller CFU/mL (<100) *P. multocida* and *B. bronchiseptica* from the lung. Three animals grew low CFU/mL *Escherichia coli* (<100) from the liver. Virus isolation was not successful. Anticoagulants were not detected within liver samples, and no organic toxins were detected by gas chromatography/mass spectrometry.

Immunohistochemistry and In Situ Hybridization

RHDV was immunohistochemically detected within the cytoplasm of approximately 20% of hepatocytes in 1 of the initially submitted does, primarily in the periportal and midzonal areas (Figure 5). MRCV nucleic acid was detected by in situ hybridization in scattered hepatocytes and few Kupffer cells (Figure 6).

Capsid Amplification

Using primers targeting the RHDV capsid gene (VP60), we detected a 398-bp amplicon by RT-PCR on pooled liver samples from 2 originally submitted does. BLAST analysis (*13*) of a unique 344-bp sequence (excluding primer sequences) showed average 79% similarity to GenBank RHDV capsid sequences. This unique sequence translates into 113 amino acids with 86.8% similarity to the partial capsid protein in a representative strain of RHDV (FRG, GenBank NC_001543). For comparison, RHDV capsid sequences typically have 98% identity with one another (*7*).

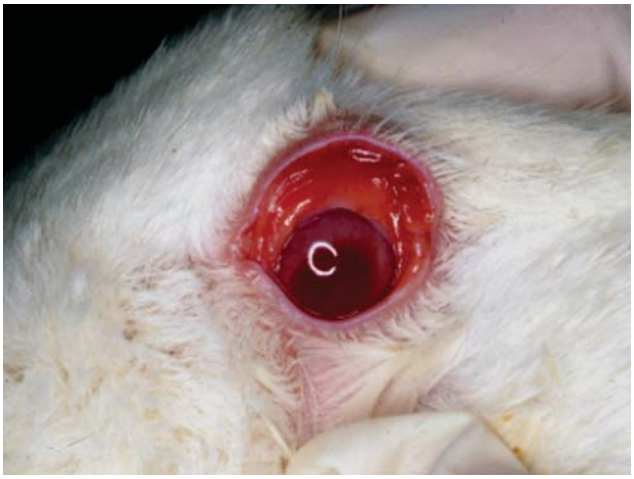

Figure 1. Conjunctival erythema in affected doe.

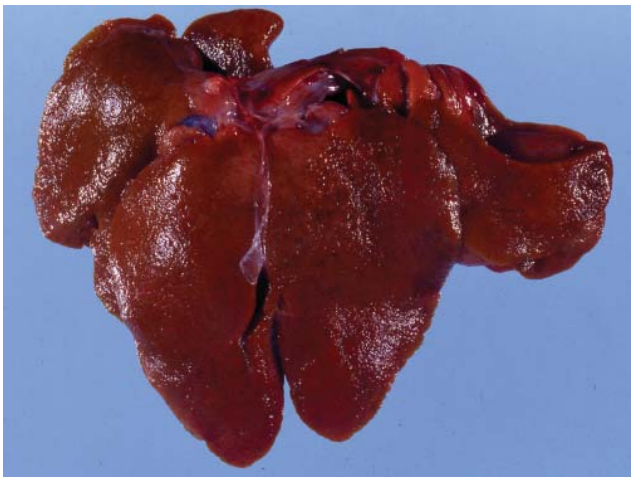

Figure 2. Liver of affected rabbit with granular texture, accentuated lobular pattern, and multifocal capsular petechiae.

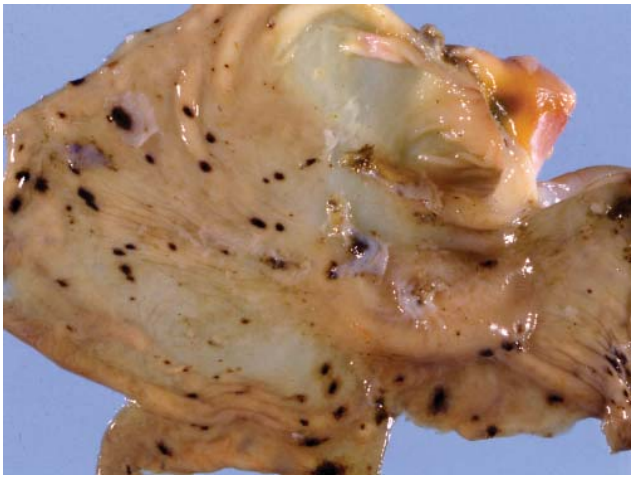

Figure 3. Multifocal gastric hemorrhage in affected rabbit.

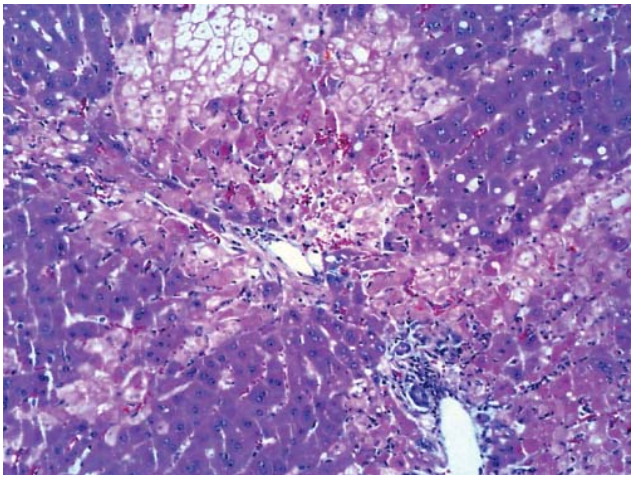

Figure 4. Multifocal periportal and midzonal heptic necrosis in affected rabbit. Hematoxylin and eosin stain. Original magnification ×200.

Subsequent sequencing was performed on the same pooled liver sample; however, the 398-capsid amplicon was detected in liver and spleen samples from 4 additional clinically affected rabbits.

Capsid and Genomic Sequencing and Analysis

Further amplification of the entire VP60 gene and upstream and downstream regions generated a genomic sequence of 7,387 nt, which is similar to the approximately 7.4-kb length of RHDV (*3*). By alignment with other *Lagovirus* sequences, this included all but 32 bases of the 5′ end of the first open reading frame (ORF) of the genome, which could not be sequenced despite multiple attempts. ORF analysis showed a large (2,328 amino acid) ORF-1 and a smaller (113 amino acid) ORF-2 that overlapped ORF-1 by 8 nt (Figure 7). ORF-1 included conserved sequence motifs for nonstructural (helicases, proteases, and polymerases) and structural proteins (VPg and capsid) in the expected order and location for the *Lagovirus* genus within the *Caliciviridae* family (*5*). ORF-2 included conserved sequence motifs for the secondary structural protein VP12. A novel calicivirus related to but distinct from RHDV was tentatively identified. The isolate was termed MRCV, and the genome was further characterized.

Multiple Sequence Alignment and Phylogenetic Analysis

Because most phylogenetic comparisons of RHDV strains are based on capsid sequence, a dendrogram was generated on the basis of MRCV capsid amino acid sequence in comparison with multiple RHDV strains and with the outliers EBSHV and RCV (Figure 8). By this analysis, MRCV appears as an outlier to RHDV strains and is most closely related to the nonpathogenic RCV. MRCV capsid

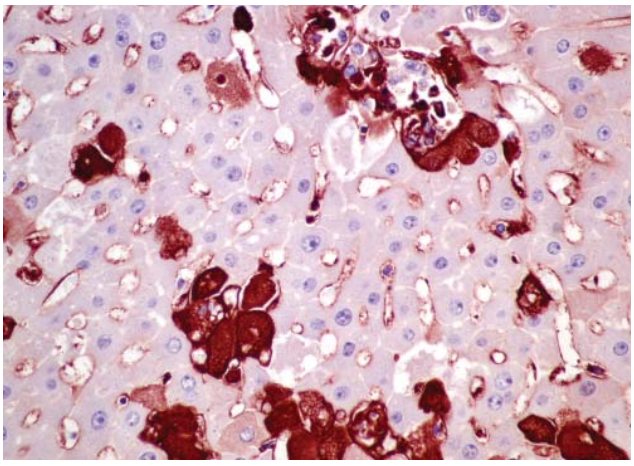

Figure 5. Liver of affected rabbit with positive cytoplasmic immunohistochemical labeling in hepatocytes against rabbit hemorrhagic disease virus capsid. Original magnification ×400.

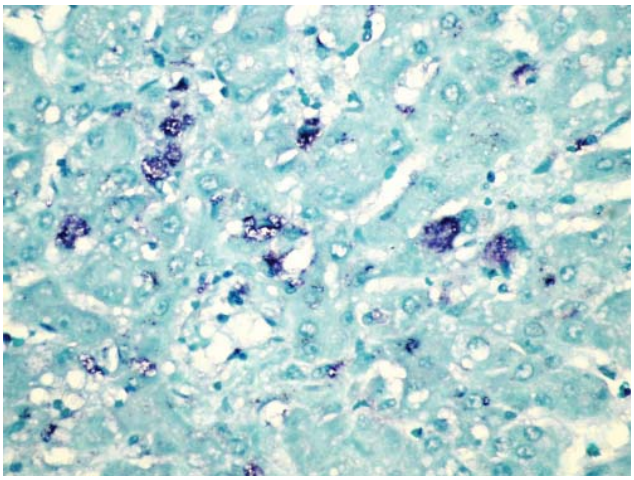

Figure 6. Liver of affected rabbit showing in situ hybridization of a Michigan rabbit calicivirus–specific oligonucleotide probe within scattered hepatocytes. Original magnification ×400.

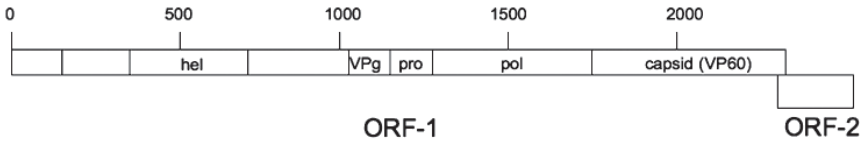

Figure 7. Schematic genomic organization of Michigan rabbit calicivirus consistent with a *Lagovirus* in the family *Caliciviridae*. Lagoviruses contain an initial large open reading frame (ORF), ORF-1 encoding a polypeptide that overlaps with a smaller ORF, ORF-2. Numbering indicates the corresponding amino acid codons predicated from the genomic sequence. hel, helicase; Vpg, virion protein, linked to genome; pro, protease; pol, polymerase; capsid (VP60), capsid protein VP60.

sequence alignments showed 91.7% similarity to RCV and 89.8%–91.3% similarity to RHDV strains. In comparison, RHDV strains shared 95.0%-99.8% similarity with each other and 91.3%–92.7% with RCV. Because the capsid gene may be subject to recombination and positive selection, relatedness predicted solely on capsid sequence is prone to error (*3*). Further analysis compared nucleotide similarities of the ORF-1 polypeptide genomic sequence, excluding the capsid gene (Figure 9). RCV is excluded from this comparison because only capsid sequence is available (*7*). By this analysis, MRCV again appears as an outlier to RHDV, having 77.9%–78.5% similarity to RHDV sequences, which share similarities of 87.8%–98.1% among themselves.

We undertook some additional comparisons of particular genomic regions. The amino acid sequence of MRCV polymerase was only 88.1% homologous to 4 representative RHDV strains, whereas polymerase is typically close to 100% conserved within viral species. Additionally, at the beginning of ORF-2, RHDV strains have 2 initiation codons, and RCV and EBHSV have only 1. MRCV appears to follow the pattern of RCV and EBHSV (Figure 10). These analyses support that MRCV represents a separate, novel caliciviral species.

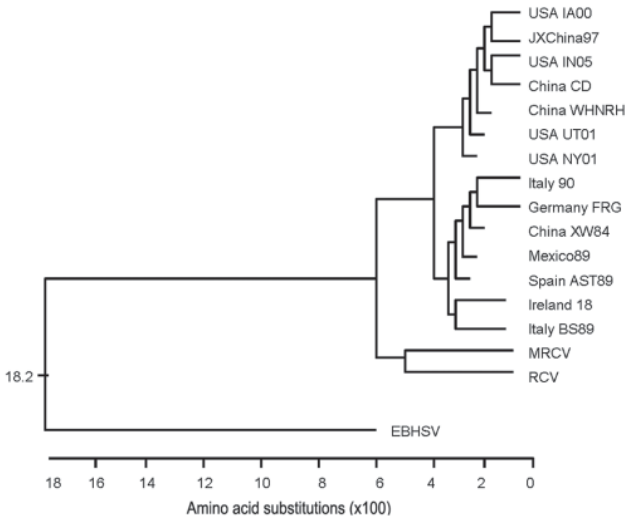

Figure 8. Dendrogram constructed for comparison of capsid (VP60) amino acid sequences. The geographically and numerically named species are strains of rabbit hemorrhagic disease virus. MRCV, Michigan rabbit calicivirus; RCV, rabbit calicivirus.

Inoculation Study

We attempted to reproduce clinical signs of disease with an inoculation study. Clinical signs in inoculated rabbits were limited to decreased activity and inappetence in 2 rabbits on day 4 postinoculation. These rabbits were euthanized, and necropsy indicated they lacked gross or histologic lesions. The other inoculated rabbits remained free of clinical disease through the end of the study and lacked gross postmortem lesions. Histologically there was rare, individual hepatocellular necrosis. Several tissues, including liver, spleen, stomach, jejunum, ileum, cecum, colon, and (in 3 rabbits) trachea/lung were positive for viral RNA by RT-PCR and in situ hybridization was positive in rare hepatocytes within the liver (data not shown).

Depopulation and Necropsy Findings in Remaining Rabbits

Further disease in the affected rabbitry was not reported after February 2, and disease outbreaks in additional rabbitries served by the same rabbit dealer were not identified. For economic reasons, the owner of the premises decided to depopulate the remaining rabbits ≈2 months later. Approximately 130 rabbits were submitted for euthanasia. Twenty-five animals had gross changes in their livers, consisting of a granular capsular texture, firm and collapsed tips of ventral lobes, and firm parenchymal nodules up to 2 cm in diameter (data not shown). Representative tissues from 4 animals were evaluated histologically. The liver showed moderate biliary hyperplasia and periductal to bridging portal-to-portal fibrosis. Although these chronic alterations suggest previous liver insult, they are nonspecific with respect to cause.

Serology

Initial diagnostic evaluations did not include serology because of a lack of reagents, but expression of the viral capsid within a baculovirus system enabled serologic testing by the time of depopulation. Antibody against MRCV was detected in serum harvested from depopulated rab-

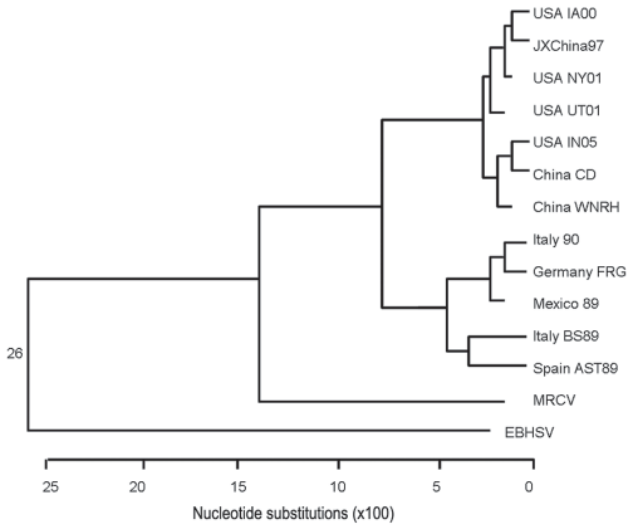

Figure 9. Dendrogram constructed for comparison of open reading frame 1 polypeptide genomic sequence minus the capsid sequence. The geographically and numerically named species are strains of rabbit hemorrhagic disease virus. MRCV, Michigan rabbit calicivirus; EBHSV, European brown hare syndrome virus.

bits (data not shown) but not in any of the experimentally inoculated rabbits that were euthanized as late as 7 days postinoculation.

Discussion

In this study, a naturally occurring disease outbreak with clinical signs and pathologic findings suggestive of RHD was ultimately associated with a novel rabbit calicivirus, designated MRCV. Although other pathogens, principally *P. multocida*, were identified in some animals, these were not consistently present, and the disease lesions and presentation were not typical of pasteurellosis. Other hemorrhage-inducing agents, such as anticoagulants or enterohemorrhagic *E. coli* (*14*), were not identified. In the animals submitted toward the end of the outbreak, the histologic findings were consistent with a reparative response to a previous liver insult. Similar reparative histologic findings have been identified in rabbits surviving experimental challenge with less virulent strains of RHDV (*15*).

On the basis of the percentage of affected animals in the outbreak and the failure to generate clinical disease in inoculated specific pathogen–free (SPF) rabbits, MRCV appears likely to be of low pathogenicity, and clinically evident disease may depend on the health status, age, or individual susceptibility of the host. RHDV is known to be most pathogenic in rabbits >8 weeks of age, possibly because of age-related differences in the expression of enterocyte receptors targeted by the virus (*16–19*). The symptomatic animals in this report were all ≥8 weeks of age. Many were pregnant does, and some had concurrent *P. multocida* infection. Although the inoculated rabbits were also >8 weeks of age, they were nonpregnant, commercially available SPF animals and lacked the apparently endemic *P. multocida* present in the diagnostic cases. RHDV-associated illness and death in laboratory rabbits can be lower than in field situations unless the animals are immune-primed by other disease agents (*3*,*15*). Alternately, the failure to reproduce disease may be due to low viable virus dose (difficult to assess for a noncultivable virus) or premature euthanasia of the inoculated rabbits. Although euthanasia by day 7 was consistent with the anticipated timeframe for calicivirus-induced disease, it may not have been sufficient if a low viral dose was used and was likely too early to expect seroconversion.

The phylogenetic analyses presented here indicate that MRCV is a distinct species from RHDV and the other known lagoviruses, RCV and EBHSV. MRCV is the first lagovirus other than RHDV detected in US rabbits; however, seropositivity to low or nonpathogenic caliciviruses has been demonstrated in rabbit populations in several other countries (*7*,*19–21*). Additional low pathogenicity strains may exist undetected in US rabbit populations. Samples to consider for RT-PCR detection of rabbit lagoviruses include liver, lung, spleen, and intestine. Although MRCV in this case was detected in liver, other low pathogenicity caliciviruses such as RCV replicate in intestine and may not be detected if liver is the sole sample evaluated (*7*). Infection with low or nonpathogenic lagoviruses in other countries has been postulated to mediate some protection against RHDV infection, although whether this is true of MRCV infection is unknown (*20*,*21*). As evidenced by this case study, diagnosticians faced with hemorrhagic disease

6969 GTGGCTTTTCCT**ATG**TCTGA... MRCV

GTGGCTTTTCTT**ATG**TCTGA... RCV (GenBank X96868)

6994 CTGGATATTCCT**ATG**TCTGA... EBHSV (GenBank NC 002615)

7025 **ATG**GCTTTTCTT**ATG**TCTGA... RHDV (FRG strain, GenBank NC 001543)

Figure 10. Alignment of open reading frame 2 sequences showing that Michigan rabbit calicivirus (MRCV) follows the pattern of European brown hare syndrome virus (EBHSV) and rabbit calicirus (RCV) in having 1 initiation codon (ATG, in **boldface**) in comparison with the 2 present in rabbit hemorrhagic disease virus (RHDV).

and sudden death in rabbits should consider the possibility of low pathogenicity lagoviruses once RHDV has been ruled out. The prevalence and effects of low pathogenicity lagoviruses like MRCV within the US rabbit industry and their potential influence on seropositivity and susceptibility to RHDV remain to be determined.

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Pandemic Influenza as 21st Century Urban Public Health Crisis

David M. Bell, Isaac B. Weisfuse, Mauricio Hernandez-Avila, Carlos del Rio, Xinia Bustamante, and Guenael Rodier

The percentage of the world's population living in urban areas will increase from 50% in 2008 to 70% (4.9 billion) in 2025. Crowded urban areas in developing and industrialized countries are uniquely vulnerable to public health crises and face daunting challenges in surveillance, response, and public communication. The revised International Health Regulations require all countries to have core surveillance and response capacity by 2012. Innovative approaches are needed because traditional local-level strategies may not be easily scalable upward to meet the needs of huge, densely populated cities, especially in developing countries. The responses of Mexico City and New York City to the initial appearance of influenza A pandemic (H1N1) 2009 virus during spring 2009 illustrate some of the new challenges and creative response strategies that will increasingly be needed in cities worldwide.

According to United Nations estimates, the percentage of the world's population living in urban areas will increase from 50% in 2008 to 70% (4.9 billion persons) in 2025. During 2007–2025, the number of cities with population 1–5 million will increase from 382 to 524, and the number of megacities (>10 million population, comprising the core city, suburbs, and continuously settled commuter areas) will increase from 19 to 27. Of the 27 megacities, 16 will be in Asia, 4 in Latin America, 3 in Africa, 2 in Europe, and 2 in North America. Currently, 1 in 25 persons lives in a megacity; in Latin America, the ratio is 1 in 7. In central Tokyo, the population density is 5,847 persons/km^2 (*1*). Cities are increasing in developing countries and often have slums that lack basic services (*2*). The accelerating global trend toward megacities is a new paradigm of human existence and poses profound public health challenges. New approaches for surveillance, preparedness, and response will be needed because current strategies may not be easily scalable upward to address huge, densely populated areas, especially in developing countries.

In 2008, the World Health Organization (WHO) International Health Regulations (IHR) Coordination Department, in collaboration with Lyonbiopole (Lyon, France), held a consultation, Cities and Public Health Crises (*1*). Consultants stated that WHO and national guidance does not always adequately address the challenges their cities face, and they could learn much from each other. This article summarizes these challenges, illustrated by the initial appearance of influenza A pandemic (H1N1) 2009 virus during spring 2009 in Mexico City, Mexico, and New York (NYC), New York, USA (metropolitan area populations 20 million and 19 million, respectively). These megacities may not be representative of cities in low-income countries, which face more daunting problems.

A Tale of 2 Megacities: Pandemic (H1N1) 2009, Spring 2009

Mexico City

National surveillance detected an atypical increase in influenza-like illness (ILI) in mid to late February 2009 and a further increase in early to mid April. Anecdotal reports in April of increased hospitalizations of previously healthy young adults with severe pneumonia led to active surveil-

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lance in 23 hospitals in Mexico City and identification of 47 such cases. Patient samples showed nonsubtypeable influenza A, identified on April 23 as a novel influenza A virus of subtype H1N1. The Mexico City response was based on early adaptation of a pandemic influenza preparedness plan that had been developed for a virus originating abroad. After an expert meeting convened by the secretary of health, given the uncertain potential health impact, the president of Mexico invoked emergency powers; on April 24, community mitigation measures were implemented in Mexico City and the neighboring state of Mexico (*3*–7). These measures were announced and coordinated by the federal government, with participation of state authorities. The objective was to decrease transmission; elements included an intensive mass media campaign to inform the population about influenza, promote personal and environmental hygiene, request that sick persons stay home, and implement social distancing measures. Persons with ILI were encouraged to seek prompt medical care. Early in the epidemic, the federal government released antiviral drugs from the national strategic reserve and controlled their distribution. Ill persons and their close contacts had access to this medication free of charge. During the spring outbreak, an estimated 150,000 cases of ILI with 3,312 hospitalizations occurred in metropolitan Mexico City (H. Lopez-Gatell, pers. comm.).

Following the Mexican Pandemic Plan, a program of social mobilization was implemented through a multifaceted mass media saturation campaign featuring visual representations and a previously developed and tested message icon, *Promi*, to address Mexico City's heterogeneous population and literacy rates (Figure 1). The private sector, including pharmacy chains, food stores, and cellular telephone companies, helped deliver health messages. The Mexican telephone company (Telmex) assembled a call center that received >5 million calls. Novel communication strategies included text messaging and mass emails; information from the Ministry of Health was transmitted through >140 million text, 60 million printed, and 18 million email messages. Multilingual health information materials also were provided to all international travelers entering and exiting through Mexican ports, and departing travelers underwent thermal screening.

Frequent hand washing and cough etiquette were promoted, and all government and private facilities open to the public were provided with alcohol gel and other disinfectants. Because of limited water availability in some areas or households, alcohol gel was distributed free. A mass media campaign promoting a healthy distance discouraged greeting others by hugging or kissing, common practice among Mexicans of all social strata. Military personnel distributed disposable surgical masks in public places; their use was recommended primarily for sick persons, but many healthy persons also wore them daily. Compliance with recommendations appeared to be high, although some persons wearing masks may have developed a false sense of security that took priority over cough etiquette and hand washing. When commercially available masks became scarce, some persons made their own, and disposal occasionally was problematic, resulting in littering. Over time, recommendations about cough and sneeze etiquette were followed least frequently.

Figure 1. Sign hung on doorknobs containing information from the Mexican Ministry of Health promoting cough etiquette, using the communications icon *Promi* (*3*). Translation: "Wash your hands. Viruses are not permitted to enter here. When coughing or sneezing, cover your mouth with a disposable handkerchief or use your forearm, never your hands!"

All educational facilities were closed beginning April 24 in Mexico City and, soon after, nationwide. Parents were advised to keep children at home; authorities distributed educational materials for home use. By May 11,

when educational facilities reopened, all schools had been thoroughly cleaned. Parents were requested to keep ill children home; peer pressure among parents to comply was high. Every day, upon arrival at school, children were screened for fever and respiratory symptoms. Ill children were sent home to receive care; return to school required a note from their primary healthcare provider granting medical clearance.

In addition to federal measures, on April 27, the mayor of Mexico City suspended dine-in service in all restaurants and similar establishments, allowing only take-out orders. Many restaurants simply remained closed. When affected businesses were allowed to reopen on May 6, social distancing measures (e.g., avoiding crowding) were encouraged, and hygiene measures were enforced (Figure 2). Grocery stores and supermarkets remained open, with additional cashiers used to keep lines short. Persons in public places were advised to remain separated by at least 2 m. Large gatherings were cancelled or postponed, and entertainment venues, e.g., movie theaters, were closed. Professional sports matches were broadcast, but stadiums were closed to the public. Churches and temples also remained closed, with religious services broadcast over radio and television. When normal services resumed, communion cups and other shared objects were wiped with hand gel after each use. Mass transit operated normally. Masks were provided for drivers and passengers and buses and subway cars were cleaned frequently. Mitigation measures were broadly accepted by the public. Occasional early discrepancies between recommendations from official and academic sources (e.g., regarding mask use) led to a few critical media reports without apparent consequence. Thousands of workplaces of all sizes in Mexico City and the rest of the country were closed for several days, taking a huge toll on the economy. The government provided no financial compensation to businesses or workers. The economic impact of pandemic (H1N1) 2009 virus in Mexico during the spring is estimated as >$2.3 billion (0.3% of gross domestic product) (*8*).

Most important among the many lessons learned in Mexico is that preparation paid off. Although requiring adaptation, the preexisting pandemic plan and planning process facilitated intersectoral work, decision making, and rapid development of a public communications campaign. The availability of a national stockpile of antiviral drugs reassured the public. The participation of the secretary of health as the spokesman demonstrated high-level leadership. Clear and transparent communication was important because Mexico was entering mid-term elections, and some politicians hypothesized that the outbreak was a farce to distract Mexicans.

The outbreak also enabled detection of some weaknesses in the Mexican health system. In Mexico, healthcare is provided by 3 major healthcare systems; thus compilation of epidemiologic information regarding hospitalizations was complex. However, after a few days, a system was devised that provided the necessary consolidated information. Laboratory capacity was inadequate for the challenges posed by the outbreak. At the onset of the outbreak, the Ministry of Health had no state-level laboratories with capabilities for influenza molecular diagnostics; all molecular diagnosis was centralized at the National Epidemiological Reference Laboratory in Mexico City. The Ministry of Health rapidly improved the national laboratory network and Mexico has now 28 laboratories (1 in nearly every state) with PCR molecular diagnostic capabilities. Although having a pandemic plan was useful, operationalizaton of the plan was less smooth. For example, procedures existed to close schools, but criteria for reopening them and the ability to reassure parents that reopened schools were safe did not.

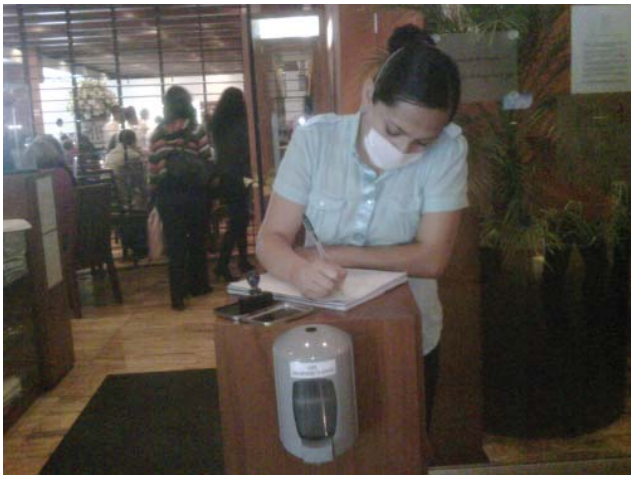

Figure 2. A reopened restaurant in Mexico City, Mexico, illustrating mask use by the person greeting entering customers and a hand hygiene dispenser that all entering customers were required to use, May 2009. Photo courtesy of Carlos del Rio.

NYC

Emergency preparedness planning in NYC accelerated after the World Trade Center and anthrax attacks of 2001 and in anticipation of an influenza pandemic. Novel syndromic surveillance systems monitor visits to hospital emergency departments, calls to emergency medical services, pharmacy sales, worker absenteeism, and outpatient clinic visits. For example, information is collected electronically for ≈90% of daily patient visits from 77% of emergency departments. Patients' age, sex, home postal code, and chief complaint, but not names, are transmitted daily to the NYC Department of Health and Mental Hygiene, where protocols identify and follow up signals that suggest increased

community illness. During spring 2009, these systems were essential for real-time monitoring of the pandemic in NYC, e.g., documenting large increases in children with ILI seeking care at emergency departments) and for tracking its spread throughout the city from 1 school where it apparently was first introduced and amplified (*9*). Additional systems collect etiologic information from, for example, virologic studies on samples of outpatients and hospitalized patients with ILI. However, the first indication to the health department of the outbreak of pandemic (H1N1) 2009 virus came from a school nurse telephoning a report of increased ILI at a single school. Subsequent surveillance and telephone surveys indicated ≈750,000–1 million persons in NYC had ILI during the spring outbreak (*10*).

When the first cases were confirmed, an extensive public communication campaign was implemented through Ready New York, a preexisting program of the NYC Office of Emergency Preparedness (*11*). The program includes outreach to ethnic populations and translation into many languages. The principal messages were 1) wash hands thoroughly and frequently with soap and water; 2) avoid contact with persons who are obviously sick; and 3) if you get sick with any cold or influenza, stay home from work or school, and avoid contact with others as much as possible

During the epidemic peak, the mayor and health commissioner held frequent press conferences in English and Spanish. A NYC government information hotline (311) previously had been established and featured live operators 24/7, with 98% of calls answered within 30 seconds. During the spring outbreak of pandemic (H1N1) 2009, ≈54,000 calls to 311 about influenza and a smaller health department hotline were answered. An electronic health alert network and conference calls provided messages to healthcare providers.

Aside from the public messages, community mitigation measures focused on selective closure of schools. Household contacts of case-patients were not quarantined, businesses were not closed, and public gatherings were not cancelled unless they involved closed schools. School closures were decided on an individual basis (known as "reactive" closures, based on visits for ILI to the school health nurse and on other factors, such as the ability of students to comply with respiratory hygiene) rather than "preemptively" (i.e., before cases in the school but with reports of cases in other schools in the subdistrict or district). Approximately 50 schools closed, for ≈1 week each.

The NYC emergency stockpile of antiviral drugs was not used because normal distribution channels sufficed. Occasional reports of spot shortages required rapid investigation and highlighted the need for close communication with private distributors. If the stockpile had been needed, antiviral drugs would have been distributed to community health centers, public clinics, and hospitals.

Distribution of vaccine for pandemic (H1N1) 2009 in NYC will depend on indications for use, availability, and urgency of administration. Vaccine will be prioritized for high-risk populations (*12*). Mass vaccination campaigns will use 200 point-of-distribution sites developed to meet possible needs for anthrax prophylaxis, e.g., school buildings throughout the city that each could serve ≈40,000 persons.

Problems included basing decisions on a pandemic severity index because, at the pandemic onset, its case-fatality ratio was uncertain. Despite previous planning, several school dismissal issues had never been entirely resolved, including the objectives of closure in a less severe pandemic (i.e., to protect high-risk students, all students, families; to slow community transmission; to allay public fears). The effectiveness of school closure in meeting these objectives was uncertain, as was the extent to which benefits justified the secondary impact, including interrupting the academic program, parental work loss, and disruption of services provided at school (e.g., free breakfast and lunch to children from low-income families, therapy for students with special needs).

Operational questions included criteria for school dismissal and reopening and difficulties in monitoring ILI and even absenteeism rates among students. Absenteeism data were often unavailable to the health department until mid-afternoon, relatively late to notify parents about closure decisions for the next school day. Instructions were not given for children to remain at home, and some may have recongregated elsewhere, such as in public libraries, while their parents were at work (*13*). News of school closures in NYC led to questions from parents in suburban jurisdictions about why their schools remained open, even though they had no known cases. Individual school closings showed the interconnectedness between schools, such as when siblings or neighbors attended different ones. The issue of worker or business compensation for lost time from work to care for ill children remains difficult. On the basis of this experience, in the 2009–10 school year, NYC is urging parents to keep sick children home and emphasizing infection control at school but will close a school only as a last resort. Closure decisions will be made on an individual basis, taking into account whether infection control practices could be improved and whether a high percentage of students have high-risk medical conditions (*10*).

During spring 2009, emergency departments were overcrowded with the worried ill, despite many announcements about indications for persons with ILI to seek medical care. In the fall and winter of 2009 hospitals are prepared to open additional nonemergency ILI care sites (e.g., at primary care clinics). A new 1-stop influenza Web portal provides information, as well as locations of clinical sites, and a call center staffed by nurses accessed through the 311 hotline provides guidance to persons with ILI (*10*).

In the city jail, cases of pandemic (H1N1) 2009 led to screening and control measures. These included isolation and cohorting of ill prisoners, and quarantine of those who had been exposed to them, to limit the spread of infection in the prison and court systems.

Health department staffing to meet surge needs posed challenges, including accessing and training staff from other parts of the health department, especially physicians, and the need to ensure staff time off to prevent burnout. Keeping policies and press releases consistent in the face of changing science and policies required constant attention. Internet survey instruments were effectively used to collect epidemiologic data, as in the initial high school student outbreak (*9*).

Issues for Cities

The experiences from the response to the emergence of pandemic (H1N1) 2009 virus in Mexico City and NYC highlighted several challenges raised at the WHO consultation (*1*). These include response coordination, surveillance and monitoring of illness trends, disease containment and mitigation, delivery of countermeasures, and public communication.

Response Coordination

Multiple government agencies serve large urban areas. Citizens frequently live, work, attend school in, and commute through different jurisdictions. Different political parties may control national, state or provincial, suburban, and city governments. Fringe groups or gangs may effectively control some areas. Incident or unified command systems can be useful approaches to crisis coordination (*14*,*15*). In Mexico City and NYC, advance planning, political leadership at the highest levels, and collaboration among public health and emergency management agencies were particularly important.

Coordination with the private sector often is not well established. Businesses can assist, notably by providing health messages and enabling infectious workers to remain home (*16*). Large companies may have contacts with city leaders, but most are small to medium-sized enterprises with which coordination may be difficult. Multinational corporations, common in cities, may be subject to home-country influences. Response coordination with nongovernment, community, and faith-based organizations also is important. Outbreaks in cities near international borders require coordination with foreign partners.

Surveillance and Monitoring of Illness Trends

Emerging challenges in cities include the vertical dimension (high-rise apartment blocks), travelers, and persons who do not have fixed addresses or who live in slums. Novel approaches for illness reporting and population surveys may include use of cell phones and the Internet. Illness surveillance ultimately depends on the organization and provision of health services; cities with universal health coverage will have important advantages (*17*). Outbreak recognition still often depends on alert clinicians; technology-based systems notwithstanding, a school nurse provided the first indication of the influenza outbreak in NYC.

Disease Containment and Mitigation

Although not generally a problem in Mexico City or NYC during spring 2009, in a larger outbreak in a lower-income country, home isolation or quarantine may be difficult or impossible for large urban families living in 1 or 2 rooms. Contact tracing is problematic in cities, given the frequency of anonymous interactions. Innovative use of nonhealth databases and 3-dimensional mapping, including cell phone records and global positioning technologies, may be helpful but may pose privacy issues.

Decisions regarding school dismissal are problematic because effectiveness for disease mitigation is difficult to quantify, and operational aspects often are uncertain, whereas the potential for societal disruption is considerable. Analysis is pending of the different approaches taken by Mexico City and NYC during spring 2009, but both have kept schools open during the fall, because pandemic severity has remained comparable with that in the spring. This approach is consistent with updated guidance from WHO and the US Centers for Disease Control and Prevention (*18*,*19*). Many questions remain about how to implement social distancing and infection control measures in typical city venues, including schools, institutions of higher education, healthcare institutions, mass transit, workplaces, and marketplaces. These issues are even more difficult in developing countries. Many cities have international airports and may need to assist in health screening of passengers; provide medical care to ill passengers; and accommodate stranded passengers, including those in quarantine. Evacuation of a city poses additional public health challenges (*17*,*20*).

Delivery of Countermeasures

Rapid delivery of countermeasures, e.g., drugs and vaccines, is difficult even for persons with known, fixed addresses, but more so for persons in slums, travelers, undocumented persons, and homeless persons, as well as the elderly and homebound. Measures taken in Mexico City and NYC during spring 2009 appear to have been sufficient, but these systems are being tested again during the 2009–10 winter season.

Public Communication

WHO outbreak communication guidelines emphasize building and maintaining trust, announcing information early, ensuring transparency, listening to the public,

and planning ahead (*21*). The generally successful public communication campaigns of Mexico City and NYC incorporated these approaches. In addition to traditional mass media and the Internet, they also used cell phones and text messaging, which may offer useful models for developing countries. Cell phone networks may need to prioritize health or emergency messages, improved robustness to permit high traffic during emergencies, and redundancy in case transmitting towers are destroyed (e.g., in a storm). Text messages can be targeted geographically, e.g., to phones locked on to a particular transmitting tower at the time of the message. This approach could be useful for broadcasting localized alerts and instructions, such as locations of vaccination clinics.

Discussion

Cities are the norm of global development in the 21st century. As cities become larger and more crowded, traditional guidance for detecting and responding to public health crises requires innovation. Modified guidance may be helpful, but new strategies, technologies, and metrics also will be needed.

Preliminary accounts of response to pandemic (H1N1) 2009 during spring 2009 in 2 world megacities offer grounds for optimism. In each case, advance planning laid the foundation for enhanced surveillance and a generally effective response, made possible by an extensive public communications campaign and effective political leadership. On the other hand, challenges emerged that would have been amplified if the illness had been more severe or the period of societal disruption prolonged. Development of new guidance and approaches requires collaboration among large cities, as well as research and evaluation to identify best practices for cities with different resource levels, particularly for implementing core capacity requirements under the revised IHR in a world where most persons now live in urban environments. The IHR require all countries to have core capacity for disease "surveillance, reporting, notification, verification, response and collaboration activities" by 2012 (*22*,*23*). These requirements must be implemented in urban environments, but they are based on traditional public health levels (local, intermediate, and national), which are less clearly defined for large urban agglomerations. All national governments have committed themselves to IHR implementation; municipalities must play a central role but may not be aware of their obligations or able to meet them. Many partners will be important, including businesses, which may not realize their stake in IHR implementation (*24*). Sharing of experience and research is needed to develop strategies and best practices that can be considered by similar cities worldwide.

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Oseltamivir-Resistant Influenza A Pandemic (H1N1) 2009 Virus, Hong Kong, China

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Resistance to oseltamivir was observed in influenza A pandemic (H1N1) 2009 virus isolated from an untreated person in Hong Kong, China. Investigations showed a resistant virus with the neuraminidase (NA) 274Y genotype in quasi-species from a nasopharyngeal aspirate. Monitoring for the naturally occurring NA 274Y mutation in this virus is necessary.

Emergence of influenza A pandemic (H1N1) 2009 virus, presumably from swine to humans, has spread globally since April 2009 (*1*–*3*). This emergence prompted the World Health Organization to declare a pandemic caused by this virus on June 11, 2009. Although most cases of infection are mild or asymptomatic, 1,462 fatal cases were reported to the World Health Organization as of August 6, 2009 (www.who.int/csr/don/2009_09_11/en/index.html).

Experimental evidence from animal models showed that this virus was able to replicate to high titers in the lungs of infected animals (*4*), unlike seasonal influenza viruses, which mainly infect the upper respiratory tract. Serologic studies found that antibodies induced by current seasonal influenza vaccines show little cross-reactivity to pandemic (H1N1) 2009 virus (*5*).

Therapeutic options are presently limited to 2 neuraminidase (NA) inhibitors, oseltamivir and zanamivir, because this virus has a swine-origin matrix 2 (M2) gene, which contains a mutation associated with resistance to M2 ion channel blockers amantadine and rimantadine. Although oseltamivir has been widely used in persons infected with pandemic (H1N1) 2009 virus, resistance was not observed until recently. Three unrelated cases of resistance to oseltamivir were observed in Denmark, Japan, and Hong Kong (www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708/en/index.html).

Emergence of resistance to oseltamivir by seasonal influenza A virus (H1N1) was detected in Norway in 2007. This virus has evolved into the dominant influenza A virus (H1N1) in humans (*6*). This finding raises strong concerns that the 274Y resistant mutation in pandemic (H1N1) 2009 virus might circulate and become dominant. We report virologic investigation of the emergence of oseltamivir resistance in this virus in a patient from Hong Kong.

The Study

A 16-year-old previously healthy girl had a fever at the Hong Kong International Airport after her arrival from San Francisco, California, USA, on June 11, 2009. Physical examination showed a temperature of 38.3°C, a blood pressure of 117/66 mm Hg, a pulse rate of 94 beats/min, and an oxygen saturation of 99% at room air. Results of a complete blood count and liver and renal function tests were normal. She had a leukocyte count of 4.69×10^9 cells/L, an absolute neutrophil count of 2.36×10^9 cells/L, and a lymphocyte count of 1.74×10^9 cells/L. Findings on her chest radiograph were normal.

A nasopharyngeal aspirate (NPA) was positive for influenza A virus (H1N1) nucleoprotein by immunofluorescence. NPA specimens on days 1 and 5 were positive for influenza A virus (H1N1) M gene and swine-specific specific H1 gene by reverse transcription–PCR (RT-PCR). Samples obtained on days 6–8 were negative. Serum and midstream urine specimens obtained on day 2 were negative for influenza A virus (H1N1) M gene by RT-PCR.

The patient refused antiviral therapy with oseltamivir because of fear of its potential side effects. She was then offered symptomatic treatment. Her clinical condition gradually improved and she was discharged on day 8 uneventfully.

Influenza A pandemic (H1N1) 2009 virus was cultured from NPA. Subsequent drug susceptibility testing showed that this isolate was resistant to oseltamivir (50% inhibitory concentration 197.5 nM), but susceptible to zanamivir, as determined by enzymatic assay (Table).

To confirm whether the virus contained mutations associated with resistance to NA inhibitors, NA sequences from the day 1 NPA specimen and an MDCK cell isolate were examined. Viral RNA was extracted from NPA and MDCK cell supernatants by using reported procedures (*7*). RT-PCR was performed by using primers spanning position 274 of the NA gene (forward: 5′-ACACAAGAGTCTGAATGTGCATGT-3′; reverse: 5′-GTCTCCGAAAATCCCACTGCATAT-3′). Direct sequencing of PCR products was performed by using a BigDye Terminator v3.1 cycle sequencing reaction kit on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

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Table. Quasi-species of 274H and 274 Y pandemic (H1N1) 2009 virus from NPA samples and subsequent virus isolate A/Hong Kong/2369/2009 from MDCK cells conferring resistance to oseltamivir, Hong Kong, China*

Sample	274H, no. samples positive/no. tested (%)	274Y, no. samples positive/no. tested (%)	IC_{50} for oseltamivir, nM	IC_{50} for zanamivir, nM
NPA	45/95 (47.37)	50/95 (52.63)	ND	ND
MDCK cell culture	2/96 (2.08)	94/96 (97.92)	197.5	0.8

*50% inhibitory concentrations (IC_{50s}) for oseltamivir and zanamivir were determined by using NA-Star influenza neuraminidase (NA) inhibitor resistance detection kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Ratios of 274H and 274Y were evaluated by cloning neuraminidase (NA) gene PCR products from nasopharyngeal aspirate (NPA) samples or MDCK cell–cultured virus into the TA vector (Invitrogen, Carlsbad, CA, USA) and sequencing clones containing the NA gene. ND, not done.

Sequences indicated that the NA genes in the NPA and MDCK cell virus isolates contained an H→Y mutation at the NA 274 (H3 numbering, 275 in H1 numbering) residue (GenBank accession no. GQ351316). No other NA mutations known to be associated with oseltamivir resistance were observed. Further examination of sequences showed mixed populations (T/C) in the NA gene from the NPA specimen (Figure, panel A).

Estimation of 274H and 274Y populations in the NPA specimen was performed by cloning and sequencing PCR products. The NPA specimen contained approximately equal proportions of 274Y and 274H (52.63% and 47.37%, respectively). Examination of sequences from the MDCK cell isolate showed predominantly the 274Y type, although a minor 274H peak was also observed (Figure, panel B). Cloning and sequencing of PCR products from the MDCK virus isolate showed that 97.92% of the NA genes were 274Y, which suggests that the 274Y population overtook the 274H population during MDCK cell culture.

Conclusions

Resistance to NA inhibitors among seasonal strains of human influenza viruses (A/H1N1, A/H3N2, and B) has been rare until recently. Development of resistance after oseltamivir treatment has occurred in 0.33%–5.5% of treated patients (*8*). Oseltamivir resistance associated with the NA 274Y genotype was also observed in human infections with avian influenza A virus (H5N1) (*9*,*10*). Low levels of 274Y quasi-species in avian influenza A viruses (H5N1) from avian hosts has been reported (*11*). Oseltamivir-resistant human influenza A viruses (H3N2 and H1N1) have been found to replicate less efficiently than oseltamivir-susceptible strains in cell culture and animal models (*12*–*14*). However, the NA 274Y resistant mutant in highly pathogenic avian influenza A virus (H5N1) retained the high pathogenicity of wild-type virus in mammalian species (*15*).

In 2007, an NA H274Y oseltamivir-resistant variant of seasonal influenza A virus (H1N1) was detected in Norway (*6*). This virus has now become the dominant virus population globally, overtaking oseltamivir-susceptible influenza A virus (H1N1). The molecular basis for the 274Y variant in seasonal influenza A virus (H1N1) virus and the mechanism by which this resistant variant became the dominant population remain unknown.

Lack of general immunity to pandemic (H1N1) 2009 virus in the human population, combined with the inherent adamantane resistance of the virus, indicates that NA inhibitors constitute the primary treatment regimen for susceptible patient groups and those in whom severe diseases develop during the current pandemic. There is great concern that an oseltamivir-resistant variant of pandemic (H1N1) 2009 virus may emerge and circulate in a manner similar to oseltamivir-resistant seasonal influenza A virus (H1N1).

The patient in this study was not treated with oseltamivir. Therefore it is unlikely that the 274Y mutation was drug-induced. Detection of mixed populations of 274Y and 274H in the NPA specimen before antiviral treatment suggests that the mutation occurs naturally, either before or during infection. Although no experimental data exist that show the growth properties of this resistant variant, examination of the quasi-species population in the cell culture–

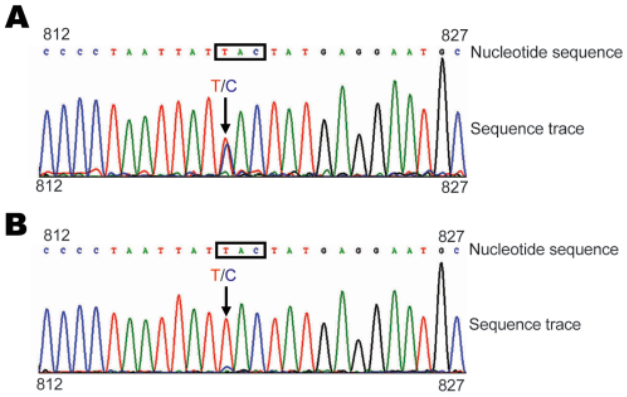

Figure. Neuraminidase (NA) 274Y (H3 subtype numbering) gene mutation in influenza A pandemic (H1N1) 2009 virus A/Hong Kong/2369/2009 isolated from a patient who arrived in Hong Kong, China, from San Francisco, California, USA, on June 11, 2009. A) NA sequence of virus amplified by reverse transcription–PCR and sequenced directly from a day 1 specimen of a nasopharyngeal aspirate from the patient. B) NA sequence of virus grown in MDCK cells. Nucleotide sequence represents identification of nucleotides by the sequencing machine, and the sequence trace represents the signal (peak) of each nucleotide in the sequencing reaction. Nucleotide coordinates (812 and 827) refer to the NA gene sequence of pandemic (H1N1) 2009 virus. Residue 274Y encoded by the 3-nucleotide codon is indicated in boxes and the nucleotide substitution (C→T for amino acid change H→Y) is indicated by arrows Colors of curves match those of specific nucleotides.

propagated virus isolate showed that the 274Y variant has become the dominant population. This finding implies that the 274Y mutation does not compromise replication of pandemic (H1N1) 2009 virus in vitro.

Quarantine procedures adopted by the Hong Kong Special Administrative Region in China during the early containment phase might have limited transmission of this variant virus. Knowledge of this virus is still limited, and characterization of transmission properties of this resistant variant in in vitro and in vivo models is needed. Moreover, pandemic (H1N1) 2009 virus should be closely monitored for emergence of resistant variants.

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Outbreak of Antiviral Drug–Resistant Influenza A in Long-Term Care Facility, Illinois, USA, 2008

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An outbreak of oseltamivir-resistant influenza A (H1N1) occurred in a long-term care facility. Eight (47%) of 17 and 1 (6%) of 16 residents in 2 wards had oseltamivir-resistant influenza A virus (H1N1) infections. Initial outbreak response included treatment and prophylaxis with oseltamivir. The outbreak abated, likely because of infection control measures.

Outbreaks of influenza virus infection cause illness and death, especially among residents of long-term care facilities (LTCFs). In addition to annual vaccination and infection control measures, antiviral agents for treatment and prophylaxis are useful components for control of influenza outbreaks in LTCFs (*1*–*4*), especially in years with vaccine strain mismatches (*4*).

Two classes of antiviral agents are licensed for use in the United States: adamantanes (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). Circulation of influenza A viruses resistant to both classes of antiviral agents, A (H3N2) to adamantanes and A (H1N1) to oseltamivir, was reported during the 2007–08 influenza season (*5*). We describe an outbreak of illness in an LTCF caused by 2 influenza viruses, an oseltamivir-resistant A virus (H1N1) and an adamantane-resistant A virus (H3N2), during January 2008.

The Study

The LTCF in Illinois provides housing, healthcare services, and recreational activities for residents with neurologic and developmental medical conditions. During the outbreak, the LTCF housed 583 residents. Building A, the main site of the influenza outbreak, housed 108 residents in 6 wards; 104 (96%) received the 2007–08 influenza vaccine. Of the 685 LTCF employees involved in direct patient care, 385 (56%) received the 2007–08 influenza vaccine on site.

We defined a confirmed case as a positive rapid or reverse transcription–PCR result for influenza virus from January 20 through February 8, 2008, in a resident of the LTCF. Surveillance for new case-patients included obtaining a nasopharyngeal specimen from all residents with new onset of fever or respiratory symptoms or any unusual behavior within 24 hours after illness onset. All specimens were tested by using the QuickVue A and B Influenza Test (Quidel, San Diego, CA, USA). A second specimen was obtained from all persons with positive rapid test results and some (57%) from persons with negative results for confirmation of influenza virus infection and virus subtyping by reverse transcription–PCR. Medical records, vaccination records, resident activity, and visitor logs were reviewed.

Testing for antiviral drug resistance was conducted directly on clinical specimens by pyrosequencing as described (*6*,*7*), including identification of the oseltamivir resistance–conferring H274Y mutation in the neuraminidase gene of influenza viruses (H1N1) (H275Y in N1 numbering) and the adamantane resistance–conferring mutations in the matrix 2 protein (*7*,*8*). The HA1 portion of the hemagglutinin (HA) gene of the outbreak viruses was sequenced and compared with those of epidemiologically relevant viruses.

Phylogenetic analysis of HA1 was performed by using MEGA version 4.0.1 software (*9*). A phylogenetic tree was inferred by using maximum composite likelihood available in MEGA version 4.0.1. The outbreak investigation was considered a public health response and granted exemption from review by the Institutional Review Board of the Centers for Disease Control and Prevention.

On January 27, the first 3 residents with fever or respiratory symptoms in ward 1 within building A were positive for influenza A virus infection by rapid test (Figure 1). On January 28, outbreak infection control measures were initiated in all 6 wards, including surveillance for new cases, 5 days of treatment with oseltamivir for confirmed cases, and 14 days of prophylaxis with oseltamivir for all healthy residents in wards with confirmed case-patients (*2*). Confirmed case-patients were quarantined in their rooms for 10 days; all residents in all 6 wards were quarantined for 10 days, and visitor movement was restricted. Staff and

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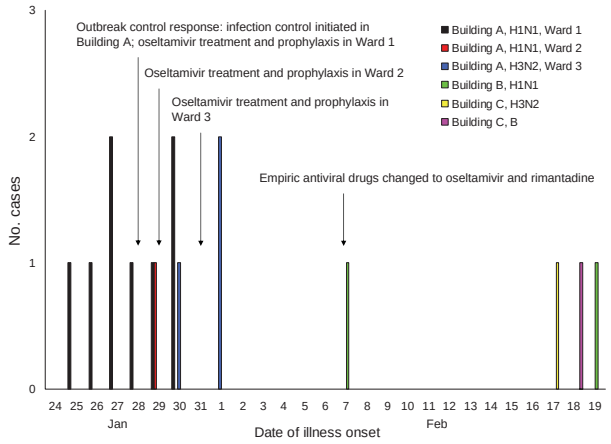

Figure 1. Number of cases of influenza by date of symptom onset and outbreak control protocol during an influenza A outbreak in a long-term care facility, Illinois, USA, 2008. Retrospective medical chart review of all nontested building A residents identified 1 potential missed case-patient with influenza who had symptom onset on January 29. Additional cases were detected in 2 other residential buildings in the long-term care facility (buildings B and C). Building B housed 53 residents in 4 wards and building C housed 16 residents in 1 ward. All (100%) of residents in both buildings had received the 2007–08 influenza vaccine. Of the 16 rapid test specimens with negative results from all 3 buildings that were subjected to confirmatory testing, 5 (31%) were positive by reverse transcription–PCR for influenza A virus (H1N1).

visitors were required to use personal protective equipment and practice respiratory and hand hygiene. Prescriptions for prophylactic courses of oseltamivir and influenza vaccinations were offered to all staff of building A; uptake was not recorded.

From January 28 through January 31, 2008, a total of 6 additional confirmed case-patients were identified. Eight (47%) of 17 residents in ward 1 and 1 (6%) of 16 residents in ward 2 were infected with influenza A viruses (H1N1) that contained the H274Y mutation but did not have markers of resistance to adamantanes or zanamivir.

On January 30, high fever developed in a male resident in ward 3 while on the first day of a home visit (Figure 1). He returned to building A on January 31, was positive for influenza by rapid test, and was placed in ward 2 in an attempt to group him with other already ill residents. Because of an ongoing outbreak in other nearby wards, oseltamivir prophylaxis was initiated for all residents in ward 3 who were not ill. On February 1, symptoms developed in 2 other residents in ward 3 who were positive for influenza by rapid test. Three (18%) confirmed cases of influenza A virus (H3N2) resistant to adamantanes but sensitive to oseltamivir were detected among 17 residents in ward 3. Additional cases, but no clusters, were detected in other buildings 1–2 weeks later.

Characteristics of case-patients are shown in the Table. Establishing a firm epidemiologic link between cases, other than ward of residency, was not possible. Antiviral drug resistance results became available on February 7 when all case-patients had completed their treatment courses. Ongoing prophylaxis courses were changed: oseltamivir was replaced with rimantadine in ward 1, and rimantadine was added to oseltamivir in ward 2. Prophylaxis with oseltamivir alone was continued in ward 3. Zanamivir could not be used by most residents because of underlying conditions.

Sequence analysis of the HA1 gene in outbreak influenza A viruses (H1N1) showed identical or nearly identical sequences, differing by only 1 or 2 nt (Figure 2). These viruses were phylogenetically more closely related to A/Brisbane/59/2007 (H1N1) than to the A/Solomon Islands/3/2006, the influenza A virus (H1N1) strain in the 2007–08 influenza vaccine. GenBank accession numbers of HA (HA1) sequences for the 9 oseltamivir-resistant influenza A viruses (H1N1) are FJ231752–FJ231760.

Conclusions

The attack rate of illness caused by oseltamivir-resistant influenza A viruses (H1N1) in ward 1 was within the range (20%–80%) reported for other facility influenza outbreaks (*1*,*10*,*11*), indicating effective person-to-person transmission of oseltamivir-resistant influenza A viruses (H1N1). Nosocomial transmission of oseltamivir-resistant influenza A viruses (H1N1), with possible healthcare worker involvement, has been described (*12*). We were un-

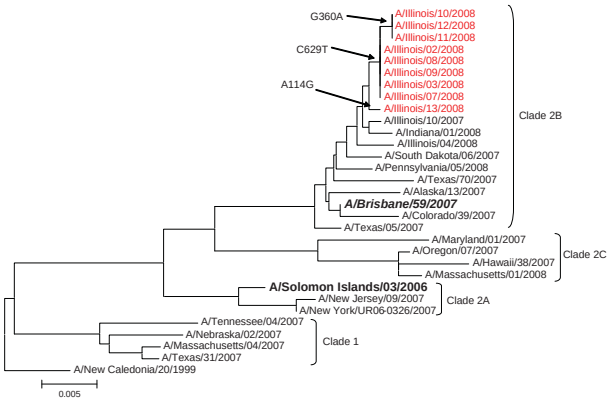

Figure 2. Phylogenetic analysis of the hemagglutinin gene (HA1 portion) of influenza A viruses (H1N1) isolated during an influenza A outbreak in a long-term care facility, Illinois, USA, 2008. Viruses from buildings A and B shared nearly identical sequences. One of the viruses from building B was more similar in sequence to 1 virus from building A. However, this finding could reflect natural variance in circulating viruses. Red indicates outbreak viruses, ***boldface italics*** indicates vaccine strain for 2008–09, **boldface** indicates vaccine strain for 2007–08, and arrows indicate nucleotide differences in HA1 subunit. Scale bar indicates nucleotide substitutions per site.

Table. Characteristics of 12 confirmed influenza case-patients in building A, long-term care facility, Illinois, USA, 2008*

Characteristic	Influenza virus subtype	
	A (H1) (n = 9)	A (H3) (n = 3)
Age, y, median (range)	29 (14–47)	32 (21–37)
Any underlying medical conditions	9 (100)	3 (100)
Neurologic disorders	9 (100)	3 (100)
Gastrointestinal disorders	8 (89)	3 (100)
Pulmonary disease	2 (22)	2 (67)
Fever >100.5°F	9 (100)	3 (100)
Cough	7 (78)	2 (67)
Desaturation	4 (44)	1 (33)
Lowest % oxygen saturation, median (range)	88.5 (88–92)	92 (NA)
Elevated or new oxygen requirement	2 (22)	0
Difficulty breathing	2 (22)	1 (33)
Increased secretions	3 (33)	2 (67)
Increased respiratory rate	3 (33)	0
Lethargy	1 (11)	0
Distress	3 (33)	0
Elevated level of care	4 (44)	1 (33)
Hospitalized†	1 (11)	0
Length of stay, d	1	NA
Clinical treatment		
Antimicrobial drugs	3 (33)	0
Antipyretics	9 (100)	3 (100)
Nebulizer (albuterol)	3 (33)	1 (33)
Received 2007–08 influenza vaccine	8 (89)	3 (100)
Died‡	1 (11)	0

*All values are no. (%) case-patients except as indicated. NA, not applicable.
†One case-patient was hospitalized for parotitis to rule out infection with mumps and was discharged in stable condition after 1 day.
‡One case-patient who had a diagnosis of end-stage lung disease and a do not resuscitate/do not intubate directive died.

able to assess staff illness in this investigation. Before the 2007–08 influenza season, transmission of neuraminidase-resistant influenza viruses had rarely been reported (*13*).

Although we documented a relatively high attack rate in 1 ward (ward 1), and despite resistance to the antiviral agent initially used, the outbreak abated quickly. High annual vaccination rates among residents and relatively high rates among employees (*2*) may have played a role in limiting the spread of the outbreak viruses. However, the A/Brisbane/59/2007 (H1N1)–like outbreak viruses were not optimally matched to the A/Solomon Islands/3/2006 (H1N1) vaccine strain (*14*). Also, infection control measures, such as isolation and quarantine, likely played a role in controlling this outbreak.

The proportion of circulating influenza viruses resistant to oseltamivir increased from 12% during the 2007–08 season to 99% during the 2008–09 season in the United States, and new interim guidelines for use of antiviral agents were released in December 2008 (*15*). These guidelines were updated for the 2009–10 season to account for the emergence of pandemic (H1N1) 2009 virus in September 2009 (www.cdc.gov/h1n1flu/recommendations.htm). This outbreak underscores the possibility of 2 influenza A viruses, with different antiviral susceptibilities, in the same facility. During a facility outbreak of influenza, providers should consult antiviral recommendations of the Centers for Disease Control and Prevention and obtain influenza virus typing and subtyping to guide appropriate antiviral drug choices.

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At the time of this study, Dr Dharan was an Epidemic Intelligence Service Officer in the Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. She is currently an infectious diseases fellow at New York University School of Medicine. Her research interests are clinical characteristics and epidemiology of respiratory viruses.

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Sympatry of 2 Hantavirus Strains, Paraguay, 2003–2007

Yong-Kyu Chu, Douglas Goodin, Robert D. Owen,[1] David Koch, and Colleen B. Jonsson

To explore geographic and host-taxonomic patterns of hantaviruses in Paraguay, we established sampling sites in the Mbaracayú Biosphere Reserve. We detected Jaborá virus and Itapúa37/Juquitiba–related virus in locations ≈20 m apart in different years, which suggested sympatry of 2 distinct hantaviruses.

Hantaviruses are rodent-borne viruses that may cause hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome in humans, although some strains do not cause disease (*1*,*2*). In Paraguay in 1995, Laguna Negra virus carried by *Calomys laucha* (little laucha) caused an outbreak of hantavirus pulmonary syndrome in western Paraguay (*3*).

We have identified 4 additional strains in Paraguay: Alto Paraguay virus harbored by *Holochilus chacarius* (Chacoan marsh rat) in western Paraguay; and Ape Aime virus (AAIV) harbored by *Akodon montensis* (Montane akodont), Itapúa virus strain 37 (IPV37) harbored by *Oligoryzomys nigripes* (black-footed colilargo), and Bermejo virus strain Ñeembucu harbored by *O. chacoensis* (Chacoan colilargo) in eastern Paraguay (*4*,*5*). We have continued our surveillance of hantaviruses in the interior Atlantic forests within and near Reserva Natural del Bosque Mbaracayú (RNBM), a World Biosphere Reserve located within Departamento Canindeyú in eastern Paraguay (Figure 1).

The Study

We established and sampled 10 mark-recapture sites within and adjacent to RNBM during 2003–2007. Sampling of grids depended upon weather conditions, the purpose of the grid, and transitory human settlements. Each mark-recapture grid consisted of an 11 × 11 array of trap stations spaced 10 m apart, each of which had 1 standard live trap (H.B. Sherman Traps, Tallahassee, FL, USA) placed on the ground, and another in branches or vines 2–3 m above ground to capture arboreal species. Grids were sampled for 8 nights, with at least 2 months between sampling sessions. Rodents captured in the mark-recapture grids were individually marked with passive integrated transponder tags, and ≈100 μL of blood was collected from the retroorbital sinus (once per trapping session). Animals were identified to species, age class, reproductive condition, sex, and weight and released.

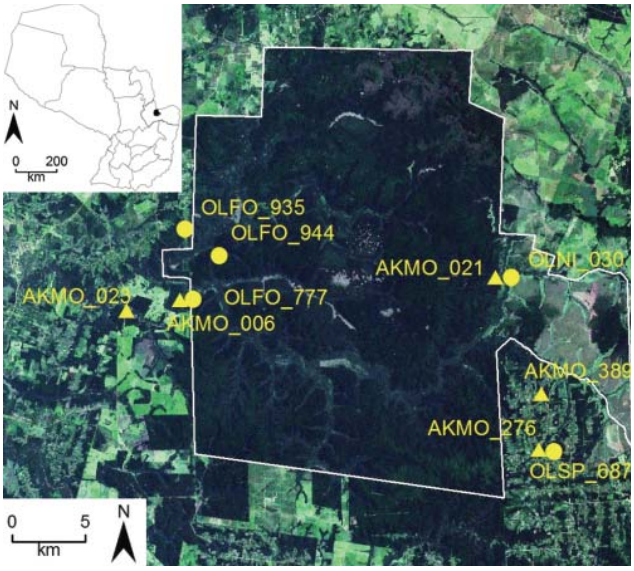

Figure 1. Satellite image of collection sites of hantavirus RNA–positive rodents, including selected Juquitiba virus (circles) and Jaborá virus (triangles) samples, Paraguay, 2003–2007. Inset shows location of study site in Paraguay. OLFO, *Oligoryzomys fornesi*; OLNI, *O. nigripes*; OLSP, *Oligoryzomys* sp.; AKMO, *Akodon montensis*.

Rodents were also collected in a series of traplines, each of which contained 50 traps placed ≈10 m apart. Animals collected in traplines were killed, standard collecting information was recorded, and liver, lung, heart, kidney, muscle tissues, and blood specimens were collected. All samples were snap-frozen in liquid nitrogen, transported to the Museum of Texas Tech University (TTU), and stored at –80°C. Standard voucher specimens were prepared from these animals, and have or will be deposited in the Museum of TTU or the Museo Nacional de Historia Natural del Paraguay. All field protocols followed American Society of Mammologists guidelines for the use of wild mammals in research (*6*), and were reviewed and approved by the TTU Animal Care and Use Committee.

A total of 1,150 small mammals from ≥20 species were captured, including 13 sigmodontine rodent species

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(1,140 animals) (online Technical Appendix, available from www.cdc.gov/EID/content/15/12/1977-Techapp.pdf). The dominant rodent species in Mbaracayú were *A. montensis* (55.7%), *Necromys lasiurus* (hairy-tailed akodont) (10.8%), *C. callosus* (big laucha) (6.5%), and *O. fornesi* (Fornes' colilargo) (6.3%).

Antibodies to hantavirus antigens were detected in blood specimens by using an indirect immunofluorescent antibody assay and irradiation-sterilized slides of Vero E6 cells infected with Andes virus as described (*4*). Seven species were antibody positive: *A. montensis*, *N. lasiurus*, *O. fornesi*, *O. nigripes*, *Oligoryzomys* sp., *Oryzomys megacephalus* (Azara's broad-headed *Oryzomys* sp.), and *Oxymycterus delator* (Paraguayan hocicudo). Antibodies to hantavirus antigens were 3× more abundant in blood samples from males than females (online Technical Appendix).

Total RNA was extracted from antibody-positive blood clots from mark-recapture samples or lung tissue from killed animals. Nested reverse transcription–PCR was performed to amplify a 371-nt small (S) hantavirus RNA segment (*4*). Hantavirus RNA was detected in 23 *A. montensis*, 5 *O. fornesi*, 1 *O. nigripes*, and 1 *Oligoryzomys* sp. Of these animals, all but 2 *A. montensis* were males, which indicated that male rodents play the primary role in maintenance and transmission of hantavirus.

A representative sample (15 *A. montensis*, 3 *O. fornesi*, 1 *O. nigripes*, and 1 *Oligoryzomys* sp.) were selected for additional PCR, sequencing, and phylogenetic analysis. PCR-amplified cDNAs of a 1,014-nt amino terminus region of the S segment were purified by agarose gel electrophoresis and cloned into pCRII (Invitrogen, Carlsbad, CA, USA) (*4*,*5*). M13 forward and reverse primers were used for sequencing. For sequence comparison and phylogenetic analysis, sequences of representative New and Old World hantaviruses were obtained from GenBank. Phylogeny reconstruction was conducted by using Modeltest version 3.6 analysis (http://darwin.uvigo.es/software/modeltest.html.), maximum-likelihood estimation, and Bayesian inference (Figure 2).

Bayesian analysis based on the 1,014-nt sequence showed that all sequences from *A. montensis* formed a strongly supported clade, which included AAIV-related hantaviruses from Itapúa Department, Jaborá virus (JABV) from southern Brazil, and strains from RNBM in Paraguay (Figure 2, clade C1). Phylogenetic analyses identified 3 subclades representing virus sequences from animals in the RNBM, Itapúa, and southern Brazil. This type of geographic clustering is similar to Sin Nombre–related viruses in deer mice in North America (*7*). All S segment sequences from *A. montensis* were closely related, with nucleotide sequence differences between RNBM strains and AAIV and JABV of 4% and 12%, respectively, and derived amino acid differences of 0% or 1%, respectively (Table 1).

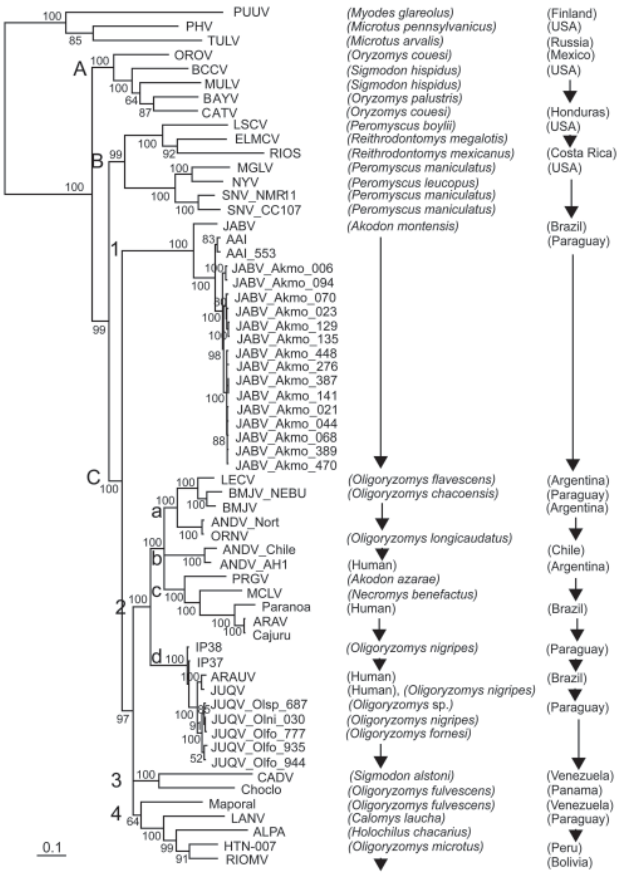

Figure 2. Phylogenetic tree based on Bayesian analysis of the small (S) segment of American hantaviruses, Paraguay, 2003–2007. The tree is based on 1,014 nucleotides of partial S segment of North and South American hantaviruses. Clades (upper case letters), subclades (numbers), and groups (lower case letters) are indicated on the left. Numerical values at the nodes indicate posterior probabilities that supported each interior branch. Scale bar indicates mean number of nucleotide substitutions per site. Alignment and editing of nucleotide sequences were conducted by using Vector NTI version 10.3.1 (Invitrogen, Carlsbad, CA, USA). Sample identifier numbers are the same as in Figure 1. Complete sequencing and abbreviation information is available online at www.cdc.gov/EID/content/15/12/1977-F2.htm.

In contrast, all virus sequences from *O. fornesi*, *O. nigripes*, and *Oryzomys* sp. at RNBM formed a strongly supported clade with viruses related to Juquitiba virus (JUQV) from Brazil and Itapúa virus strain 37, which was originally detected in *O. nigripes* from Itapúa Department in eastern Paraguay (Figure 2, clade C2d). Nucleotide sequence differences between JUQV strains from RNBM were 0%–2%. Nucleotide sequence differences between JUQV strains from RNBM and Itapúa virus strain 37 or JUQV (Brazil) were 5% or 4%, respectively, and derived amino acid differences were 0% (Table 1). This clade is phylogenetically

Table 1. Nucleotide and amino acid sequence similarities of small gene segments among hantaviruses identified in Paraguay and nearby countries, 2003–2007*

Virus	LANV	RIOMV	ALPA	JABV	JABV Akmo_006	AAI	ANDV	BMJV-NEBU	IPV37	JUQV Olfo_777	JUQV	PRGV	ARAV
LANV	–	83	78	75	75	76	79	80	80	80	80	78	78
RIOMV	93	–	81	77	78	77	80	80	79	79	78	79	79
ALPA	92	96	–	77	77	78	78	78	78	77	77	77	78
JABV	85	89	88	–	88	89	76	77	78	77	78	75	77
JABV Akmo_006	86	88	88	99	–	96	76	75	77	76	76	75	76
AAIV	88	90	90	99	99	–	77	76	77	77	77	76	76
ANDV	90	90	89	86	86	88	–	83	82	81	82	82	82
BMJV-NEBU	89	90	88	86	85	88	98	–	84	83	84	82	81
IPV37	90	90	89	86	86	88	96	95	–	95	95	83	81
JUQV_Olfo_777	89	88	87	86	86	87	96	94	100	–	96	82	80
JUQV	90	90	89	86	85	88	96	95	100	100	–	82	81
PRGV	90	90	89	85	84	87	96	95	93	92	93	–	83
ARAV	91	91	90	90	89	90	96	94	94	94	94	96	–

*Values above the diagonal are percentage nucleotide sequence similarities, and values below the diagonal are percentage amino acid sequence similarities. LANV, Laguna Negra virus; RIOMV, Rio Mamore virus; ALPA, Alto Paraguay virus; JABV, Jaborá virus; AAIV, Ape Aime virus; ANDV, Andes virus; BMJV-NEBU, Bermejo virus from Ñeembucú; IPV37, Itapúa virus strain 37; JUQV, Juquitiba virus; PRGV, Pergamino virus; ARAV, Araraquara virus.

distinct from viruses that form the *Akodon montensis* clade at RNBM and more closely related to Andes (clade C2b) and Bermejo-Ñeembucú (clade C2a) viruses. This finding suggests that spillover infection of JUQV-related viruses is actively occurring among oryzomyine rodent species at RNBM, as reported for other hantaviruses in oryzomyines (*8*) and other rodent hosts of Old World hantaviruses (*9*,*10*). Additional data are needed to determine the primary oryzomyine reservoir of JUQV and to better understand mechanisms by which spillover occurs.

In addition to spillover infection of JUQV among oryzomyine rodents, we identified 2 virus strains (JUQV and JABV) in close proximity (collected ≈20 m apart on the same grid in the same sampling session) on 2 occasions, in sites separated by ≈30 km (Figure 1; Table 2). Thus, these 2 distinct hantaviruses appear to be maintaining a sympatric status across a considerable expanse of landscape, rather than reflecting a temporary or localized phenomenon. We use the term sympatric to underscore that these viruses are in the same community and are near enough (their rodent reservoirs) to interact.

Conclusions

Coexistence of hantaviruses in 2 rodent species at mark-recapture sites has been observed (*11*–*13*). Serologic analyses in these studies would not have differentiated whether distinct strains of hantaviruses were co-circulating or active spillover infection was occurring among sympatric rodents at collection sites. Recently, Raboni et al. reported JUQV circulating in 3 sympatric rodent species in southern Brazil and 2 distinct hantaviruses (Jabora and JUQV) in 1 rodent species (*A. montensis*) (*14*). We have not detected JUQV in *A. montensis* in Paraguay. To address host-jumping of hantaviruses among sympatric rodent species in RNBM and other regions in South America, future longitudinal studies are warranted. Such studies are critical to understanding evolutionary adaptation of hantaviruses in rodents in South America, the ability of these viruses to adapt to new rodent reservoirs, and their emergence and maintenance in the environment.

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Table 2. Incidence of sympatry of 2 hantaviruses and their presumed reservoirs, Paraguay, 2003–2007*

ID no.	Species	Collection date	Collection site	Virus antibody	Virus RNA
JAB_Akmo_006	*Akodon montensis*	2005 Sep 15–18	Mark-recapture	–	–
		2005 Nov 12, 15, 17		+	+
		2006 Feb 27–Mar 6		+	+
JUQV_Olfo_777	*Oligoryzomys fornesi*	2005 Feb 14–16		–	–
		2005 Sep 12		+	+
JAB_Akmo_276	*A. montensis*	2007 Jun 12	Trapline	+	+
JUQV_Olsp_687	*Oligoryzomys* sp.	2006 Aug 18		+	+
JAB_Akmo_021	*Akodon montensis*	2003 Sep 12	Trapline	+	+
JUQV_Olni_030	*O. nigripes*	2003 Sep 13		+	+

*ID, identification; JAB, Jaborá virus; JUQV, Juquitiba virus.

and tissues; and the field crew, led by Ismael Mora, for dedication to their work, regardless of circumstances.

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Cross-sectional Survey of Hantavirus Infection, Brazil

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A cross-sectional serosurvey was conducted to assess the proportion of persons exposed to hantaviruses in a virus-endemic area of the state of Minas Gerais, Brazil. Findings of this study suggested the presence of ≥1 hantaviruses circulating in this region causing hantavirus pulmonary syndrome, mild disease, or asymptomatic infection.

In Brazil, >1,080 cases of hantavirus pulmonary syndrome (HPS) have been confirmed since 1993 (case-fatality rate 40%). More HPS cases (209) are reported in the state of Minas Gerais than in any other state in Brazil (M.L. Nunes, pers. comm.). In Minas Gerais, molecular studies identified a hantavirus called *Araraquara* virus associated with HPS cases. The wild rodent *Necromys lasiurus* (the hairy-tailed bolo mouse, also named *Bolomys lasiurus*) was implicated as a reservoir of this virus (*1*). Because asymptomatic infection with hantaviruses also has been detected in Minas Gerais, we conducted a cross-sectional survey to assess the proportion of persons exposed to hantaviruses and to identify associated risk factors.

The Study

The hantavirus cross-sectional survey was carried out April through May 2006 in the municipality of Uberlândia, Minas Gerais, at an average altitude of 863 m (18º55′S,48º16′W) (Figure). A randomized and stratified (sex and age) sample was collected from the entire rural area and from the south sector of the municipality's periurban area. The term periurban refers to a residential area on the outskirts of the city that is in close contact with the rural area. The participants answered a questionnaire that included demographic information (sex, age, place of birth, and address) and questions relating to HPS risk factors (type of dwelling, exposure to rodents at home or work, labor activity, risk activities, history of severe pneumonia, and direct contact with HPS patients). Blood samples were collected by venipuncture, centrifuged, and sent to the Laboratory of Hantaviruses and Rickettsioses at the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, for analysis. The ethics review board of the Federal University of Uberlândia approved the study.

Figure. A) Location of the study area in Brazil (box). B) Detail of study area showing municipalities.

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We screened serum samples by ELISA for hantavirus-specific immunoglobulin G using a recombinant antigen of the nucleocapsid protein of *Araraquara* virus, produced in *Escherichia coli* and supplied by the Virus Research Unit of the University of São Paulo, Brazil, according to the procedure previously described (*2*). All positive serum samples were retested; only those that had 3 ELISA-positive results at ≥1:400 dilutions were considered positive.

The Mann-Whitney U and Fisher exact/binomial tests for 2 proportions were applied for comparison among medians and proportions, respectively, using EPI INFO 3.3.2 (www.cdc.gov/epiinfo) and BIOSTAT 5.0 (www.biostat.org) software. Fisher exact test was used to estimate the odds ratio and 95% confidence intervals.

The 400 study participants comprised 200 rural and 200 periurban residents ranging in age from 12 to 76 years (mean = 41 years). Twelve (3%) samples were hantavirus antibody-positive by ELISA. The 8 rural area antibody-positive samples were from male farmers (Table 1). Presence of antibody was significantly associated with male sex, older age class, and potential risk activities (Table 1). Although all case-patients reported exposure to rodents or their excreta, this exposure was not statistically significant (Table 1). In the periurban area, the presence of antibody was associated with age but not with sex, risk activity, or exposure to rodents (Table 1). The mean age of seropositive persons from periurban and rural areas was similar (p = 1.0). The relationship between antibody and sex depended on urban vs. rural residence (p = 0.02). Three antibody-positive persons in the rural zone and 2 in the urban zone reported a history of pneumonia, albeit without complications.

We examined data from an HPS outbreak in Uberlândia during 1998–2005. The largest number of cases occurred among periurban residents, but the highest cumulative incidence was among rural residents (Table 2). Nevertheless, rural and periurban areas did not differ significantly in either prevalence or incidence. We found higher prevalence among rural residents (Table 2).

Conclusions

Overall hantavirus antibody prevalence among periurban residents was 2.0%, with a higher prevalence among women (2.6%). In previous studies, the prevalence of hantavirus antibodies was higher in men (*4*–*6*). All the positive samples in the rural area came from male farm workers. This finding is similar to a situation reported in Colombia, where all positive samples came from men engaged in rural activities (*6*). These activities involve a high risk for infection by hantaviruses (*7*).

In this study, hantavirus positivity was found only in persons >39 years of age, and the difference in the mean age of the participants in relation to positivity was significant. This fact might suggest a historic high-risk event to which the older age class, but not the younger age class, was exposed.

High hantavirus antibody prevalence has been found in studies of some human populations in Latin America (*5*,*8*,*9*). The prevalence of Araraquara virus–reactive antibodies among the volunteers in this study demonstrates that transmission is not rare, reinforcing the hypothesis of the existence of mild disease or asymptomatic infections (*10*). Two hypotheses have been proposed: clinically mild disease or inapparent infections might result from differences in the nature of exposure (e.g., low inoculum or inefficient mechanism of transmission) or genetic differences in immune response to infection, or they might indicate the circulation of ≥1 hantavirus genotypes of greatly reduced virulence (*10*,*11*).

Table 1. Relationship between independent variables and antibody to hantaviruses in the municipality of Uberlândia, Minas Gerais, Brazil, 2006

	Rural		Periurban	
Variable	No. antibody positive (no. tested)	p value*	No. antibody positive (no. tested)	p value*
Sex				
M	8 (130)	0.03	1 (84)	0.44
F	0 (70)		3 (116)	
Age, y				
12–39	0 (82)	0.01	0 (112)	0.04
>39	8 (118)		4 (88)	
Risk activity or labor†				
No activity	0 (79)	0.02	2 (92)	0.63
With activity	8 (121)		2 (108)	
Exposure to rodents				
Yes	8 (168)	0.24	3 (152)	0.67
No	0 (32)		1 (48)	

*Determined by using 2-tailed Fisher exact test.
†Clearing land, farming, working in pastures or cellars, or cleaning sheds barns, or other outbuildings.

Table 2. Incidence of hantavirus pulmonary syndrome and hantavirus antibody prevalence in the municipality of Uberlândia, Brazil, according to geographic area, 2006*

Variable	Area			p value†	OR (95% CI)
	Overall	Rural	Periurban		
Disease					
Cases‡	13	5	8	0.24	1.92 (0.63–5.90)
Population	71,122	17,406	53,716§		
Cumulative incidence, 1998–2005 ($\times 10^4$)	1.83	2.87	1.50		
Infection					
Antibody positive	12	8	4	0.38	0.49 (0.14–1.65)
Sample	400	200	200		
Prevalence, % (95% CI)†	3.0 (1.3–4.7)	4.0 (1.3–6.7)	2.0 (0.1–3.9)		

*OR, odds ratio; CI, confidence interval.
†Rural versus periurban. Determined by using 2-tailed Fisher exact or binomial tests for 2 proportions.
‡Limongi et al. (*3*).
§Total population of the southern part of the periurban area.

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Mr Limongi is chief biologist of the Regional Center of Zoonotic Diseases of Uberlândia. His research interests focus on ecoepidemiology and of zoonotic disease control.

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Bartonella rochalimae in Raccoons, Coyotes, and Red Foxes

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To determine additional reservoirs for *Bartonella rochalimae*, we examined samples from several wildlife species. We isolated *B. rochalimae* from 1 red fox near Paris, France, and from 11 raccoons and 2 coyotes from California, USA. Co-infection with *B. vinsonii* subsp. *berkhoffii* was documented in 1 of the coyotes.

Twelve *Bartonella* species/subspecies have been recognized as zoonotic agents (*1*,*2*), including *B. rochalimae* isolated from a woman who had traveled from the United States to Peru before becoming ill with fever, splenomegaly, mild anemia, and rash (*3*). *B. rochalimae*, previously described as a *B. clarridgeiae*–like organism, has also been isolated from rural domestic dogs and gray foxes (*Urocyon cinereoargenteus*) from northern California (*4*,*5*). A case of fatal endocarditis in a domestic dog was associated with a *B. clarridgeiae*–like strain (*6*), later determined to be identical to *B. rochalimae* (*5*). Recently, *B. rochalimae* DNA was detected in a dog from Greece (*7*). High (43%) prevalence of bacteremia observed in gray foxes in California suggests that they might act as a wildlife reservoir for this newly identified species. Furthermore, several *B. clarridgeiae*–like and *B. rochalimae* genes have been detected in fleas collected from humans (*8*), rodents (*9*,*10*), red foxes (*Vulpes vulpes*) (*11*), and the environment in the Democratic Republic of Congo (*12*) during a plague outbreak. To determine whether other wildlife reservoirs exist, we tested samples from 3 additional wildlife species: coyotes, raccoons, and red foxes.

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The Study

From 1996 through 1999 in central coastal California, 21 *Canis latrans* coyotes (3 juveniles [<1 year of age] and 18 adults) and 42 *Procyon lotor* raccoons (11 juveniles and 31 adults) were trapped. In 2002, a blood sample was collected from a road-killed red fox near Paris, France. All samples were collected in EDTA tubes and frozen at −70°C until plated on heart infusion agar containing 5% rabbit blood and incubated in 5% CO_2 at 35°C for up to 4 weeks (*13*); subsequently, extracted DNA was tested for *Bartonella* spp. by PCR. In addition, from May 2003 through September 2004, blood was collected from 42 red foxes (23 females and 19 males; 2 kits [<1 year] and 40 adults) in Israel, and extracted DNA was tested for *Bartonella* spp. by PCR.

Bartonella isolates from 2 (9.5%) coyotes (coyote 004 [7-month-old male] and coyote 22 [adult female captured in central California], which yielded 2 different-size colonies: coyote 22/sub1, large size; coyote 22/sub2, small size), 11 (26%) of the raccoons (7 adult females and 4 adult males), the 1 (100%) red fox from France, and DNA from the blood of 2 (5%) foxes from Israel were compared with *B. rochalimae* strains isolated from a human, rural dogs, and gray foxes. *Bartonella* isolates were analyzed by PCR restriction fragment length polymorphism (RFLP) of the 16S–23S intergenic transcribed spacer (ITS) region (all strains) and the *glt*A, *rpo*B and *fts*Z genes (raccoons, gray foxes, coyotes, and dogs), as previously described (*5*). For the isolate from the red fox from France, extracted DNA was also amplified for fragments of the *groEL* gene by using the primer sets HSPps1, HSPps2, and HSPsp4 (*11*,*14*). Sequencing was done in both directions by using a fluorescence-based automated sequencing system (Davis Sequencing, Davis, CA, USA). Sequences were imported into Vector NTI Suite 9.0 software (Invitrogen, Carlsbad, CA, USA) to obtain a consensus sequence. Align X in Vector NTI was used for aligning sequence variants with each other and other known *Bartonella* spp. for each of the 4 genes. A neighbor-joining tree was constructed in MEGA version 3.0 (www.megasoftware.net) by concatenating the 4 sequences. Bootstrap replicates were performed to estimate node reliability of the phylogenetic tree; values were obtained from 1,000 randomly selected samples of the aligned sequence data. Sequence data for the *groEL* gene of the isolate from the fox in France (GenBank accession no. FJ545656**)** was compared with sequences of DNA extracted from fleas collected on 4 foxes from Hungary (*11*) and deposited in GenBank under accession no. DQ522300.

Amplified PCR products were obtained from the ITS region and the *glt*A, *rpo*B, and *fts*Z genes of all isolates. Isolates from coyote 004 and coyote 22/sub2, the red fox from France, and the 11 raccoons had identical RFLP profiles, also identical to those observed in the rural dogs and

Table 1. Percent similarity based on comparisons of the combined *glt*A, *rpo*B, *fts*Z, from 7 *Bartonella rochalimae* isolates

Isolate source	Red fox, France	Coyote 004	Coyote 22/sub2	Raccoon 60	Dog 318006	Dogs/gray foxes	Human
Red fox (Paris suburb, France)	100	99.5	99.8	99.6	99.5	99.9	99.8
Coyote 004 (San Mateo County, CA, USA)		100	99.6	100	100	99.6	99.6
Coyote 22/sub2 (Santa Clara County, CA, USA)			100	99.6	99.6	100	99.8
Raccoon 60 (San Jose, Santa Clara County, CA, USA)				100	100	99.6	99.6
Dog 318006 (San Mateo County, CA, USA)					100	99.6	99.6
Dogs/gray foxes (Humboldt County, CA, USA)						100	99.8
Human (Peru)							100

gray foxes from California (*5*). Isolate sub1 from coyote 22 had banding patterns identical to those of *Bartonella vinsonii* subsp. *berkhoffii* (American Type Culture Collection 51672), indicating co-infection with *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*. Consensus sequences were obtained for isolates from all coyotes and the red fox from France. Because the RFLP profiles for the 11 raccoon isolates were identical, only 2 isolates (from raccoons 60 and 75, adult females from central California) were selected for sequencing and were identical for all genes. Partial sequences from the 4 genes were identical for the isolates from coyote 004 and the 2 raccoons. These isolates were 100% similar to a strain recovered from the dog with endocarditis (GenBank accession nos. DQ676488–DQ676491) (*5*,*6*). The *B. rochalimae* isolate sub2 from coyote 22 was identical to isolates from rural dogs and gray foxes from northern California (accession nos. DQ676484–DQ676487). Similarity of isolates from these regions ranged from 99.5% to 100% (Tables 1,2). A tree constructed from the merged set of concatenated sequences (Figure 1) demonstrates that isolate sub2 from coyote 22 clustered with isolates from the dog and gray fox from northern California; those from coyote 004 and raccoon 60 grouped with those from the dog with endocarditis.

According to PCR results and comparison of a 571-bp sequence amplified from the ITS region, the sequences from 2 foxes (1 male, 1 female) from 2 villages in northern Israel were identical to each other and to that from the fox from France (Figure 2). Differences of 2–5 bp were observed among ITS region sequences when comparing those from the foxes from Israel and France with those from *B. rochalimae* from gray foxes and raccoons from California. When the *groEL* partial sequence FJ545656 from the red fox from France was compared with sequence DQ522300 from a *Pulex irritans* flea collected from foxes from Hungary, the 156-bp fragment (based on the consensus sequence from both directions) from the red fox from France was 100% identical to that of the flea.

Conclusions

We report the isolation or detection of *B. rochalimae* from red foxes, raccoons, and coyotes from North America, Europe, and the Middle East. Sequence analysis of 4 genes identified small variations in the *B. rochalimae* isolates from these different geographic regions. A relatively high percentage (26%) of raccoons had *B. rochalimae* bacteremia compared with only 9.5% (2/21) coyotes. A previous study found that of 109 coyotes, none were infected with *B. rochalimae*, but 31 (28%) harbored *B. vinsonii* subsp.

Table 2. Percent similarity based on comparisons of the intergenic transcribed spacer sequence alignment from 7 *Bartonella rochalimae* isolates

Isolate source	Red fox, France	Coyote 004	Coyote 22 sub2	Raccoon 60	Dog 318006	Dogs/gray foxes	Human
Red fox (Paris suburb, France)	100	99.5	99.8	99.6	99.5	99.9	99.8
Coyote 004 (San Mateo County, CA, USA)		100	99.6	100	100	99.6	99.6
Coyote 22/sub2 (Santa Clara County, CA, USA)			100	99.6	99.6	100	99.8
Raccoon 60 (San Jose, Santa Clara County, CA, USA)				100	100	99.6	99.6
Dog 318006 (San Mateo County, CA, USA)					100	99.6	99.6
Dogs/gray foxes (Humboldt County, CA, USA)						100	99.8
Human (Peru)							100

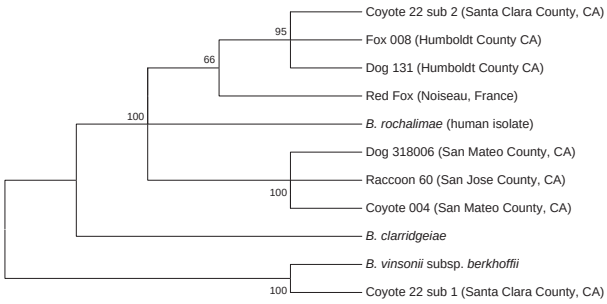

Figure 1. Phylogenetic tree of *Bartonella* species based on the combined *glt*A, *rpo*B, *fts*Z, and intergenic transcribed spacer sequence alignment. The tree shown is a neighbor-joining tree based on the Kimura two-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. The analysis provided tree topology only; the lengths of the vertical and horizontal lines are not significant.

berkhoffii (*13*). In raccoons, bacteremia was found in adults only, which is surprising because for all animals in general, *Bartonella* spp. bacteremia is detected more frequently in younger animals (*1*). Gray foxes from northern California had *B. rochalimae* bacteremia prevalence of 42% (*4*), suggesting that gray foxes and raccoons could be natural reservoirs of *B. rochalimae* in California and that infection of coyotes and domestic dogs could result from occasional spillover. Co-infection of a coyote also illustrates that wild canids can simultaneously harbor >1 species of *Bartonella*. Co-infection of humans with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* has also been reported (*15*). Additionally, co-infection with 2 zoonotic *Bartonella* species in this coyote raises the possibility that humans and domestic dogs could also be co-infected with these species, making appropriate diagnosis more difficult. *Pulex* fleas collected on red foxes from Hungary (*11*) were indeed infected with a strain of *Bartonella* that was identical, at least for the *groEL* partial sequence, with that of the isolate from the red fox from France, suggesting that red foxes from central Europe may also be infected with *B. rochalimae*. Future studies with larger sample sizes will be needed to better define the role of these wild carnivores—red foxes, raccoons, and coyotes—in maintaining *B. rochalimae* in the environment.

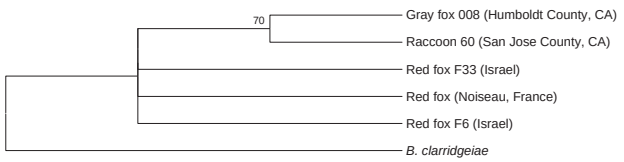

Figure 2. Phylogenetic tree of *Bartonella* species based on intergenic transcribed spacer sequence alignment for the isolates from the gray foxes, red foxes, and raccoons. Raccoon and gray fox isolates are shown for comparison. The tree shown is a neighbor-joining tree based on the Kimura 2-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. The analysis provided tree topology only; the lengths of the vertical and horizontal lines are not significant.

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Dr Henn is an epidemiologist with Napa County Health and Human Services and conducted this study for her PhD thesis. She has a strong interest in the epidemiology of emerging zoonoses.

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CTX-M β-Lactamase Production and Virulence of *Escherichia coli* K1

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We report a patient with neonatal meningitis caused by a CTX-M-1–producing *Escherichia coli* K1 strain. The influence of CTX-M production on virulence was investigated in cell culture and a newborn mouse model of meningitis. CTX-M production had no influence on virulence but was a major factor in clinical outcome.

Escherichia coli is the second most common cause of neonatal meningitis. Neonatal meningitis *E. coli* (NMEC) belong mainly to phylogenetic group B2 and harbor numerous virulence factors (*1*).

Since the beginning of the 21st century, an explosive spread of CTX-M–type extended-spectrum β-lactamases (ESBLs) in *E. coli* has occurred (*2*). These enzymes confer resistance to nearly all β-lactam antimicrobial drugs, including third-generation cephalosporins, the first-line treatment for patients with serious *E. coli* infections. However, CTX-M–type ESBLs have been observed mainly in *E. coli* strains with few virulence factors or in strains causing minor infections (*3*–*5*). In addition, bacterial resistance to antimicrobial drugs is frequently reported as difficult to reconcile with bacterial virulence (*6*). Highly pathogenic *E. coli* such as NMEC are therefore considered susceptible to antimicrobial drugs (*7*). We report a clinical case of neonatal meningitis caused by CTX-M–producing NMEC and the influence of CTX-M production on virulence.

The Study

In April 2007, a 39-year-old pregnant woman with amniotic sac rupture was admitted to a hospital in Orleans, France, at 28 weeks and 4 days of gestation. Treatment was started with betamethasone (12 mg 1×/d) for fetal lung maturation and amoxicillin (1g 3×/d) for 4 days. Because of a high serum level of C-reactive protein, antimicrobial drug therapy was switched to amoxicillin with clavulanic acid (1g 3×/d) for 1 day. A cesarean delivery was performed at 29 weeks and 2 days of gestation. A lumbar puncture sample of the low-weight (1,560 g) newborn female was tinged with blood. Cerebrospinal fluid (CSF) protein and glucose values were 4.00 g/L and 3.5 mmol/L, respectively. Results of CSF Gram staining were negative.

The infant was admitted to the neonatal critical care unit and received amoxicillin (150 mg), cefotaxime (120 mg), and netilmicin (8 mg) 2×/d for 2 days. Culture of placenta, maternal and infant blood, and infant gastric fluid yielded *E. coli*. The isolate was resistant to antimicrobial drugs, including third-generation cephalosporins. Imipenem/cilastatin (25 mg 4×/d) and amikacin (15 mg 2×/d) were given for 2 days. Treatment with imipenem/cilastatin was given for 15 days and then stopped because of the infant's clinical improvement and return of C-reactive protein to the reference level. Similar drug treatment was administrated to the mother.

One week after drug treatment was discontinued, the infant showed signs of septicemia. A second lumbar puncture sample had protein and glucose levels of 4.56 g/L and 0.1 mmol/L, respectively, and a leukocyte count of 4,700 cells/μL (54% polymorphonuclear cells). *E. coli* were isolated from blood and CSF cultures and showed a resistance pattern identical to that of the previous isolate. Meningitis was a complication of the initial sepsis or a relapse of initial unapparent meningitis (*8*).

Treatment was started with imipenem/cilastatin (30 mg 4×/d) for 25 days and amikacin (15 mg 2×/d) for 5 days. Because the infant had a seizure, phenobarbital (22.5 mg) and ciprofloxacin (15 mg 2×/d) were prescribed for 5 additional days. Her condition gradually improved and blood and CSF values returned to reference levels. The infant was discharged from the hospital 1 month later and treatment with the anticonvulsant was discontinued. She showed normal psychomotor development at a regular follow-up pediatric visit.

E. coli strains isolated from the mother and infant were indistinguishable by enterobacterial repetitive intergenic consensus sequence 2 PCR, random amplified polymorphic DNA analysis, and typing with a MALDI BioTyper (Bruker Daltonique, Wissembourg, France) (*9*). Thus, the isolates corresponded to the same strain, designated Orl-1. PCR-based phylogenetic analysis and serotyping showed that the strain belonged to group B2 and serotype O7:K1:H7, a major O antigen encountered worldwide in NMEC (*1*).

Resistance of the Orl-1 strain (MICs 128 μg/mL for cefotaxime and 8 μg/mL for ceftazidime) was caused by

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the gene encoding CTX-M-1. This strain was also resistant to tetracycline, trimethoprim, and sulfamethoxazole and susceptible to cefoxitin, imipenem, aminoglycosides, quinolones, chloramphenicol, and fosfomycin. It harbored the major *E. coli* genes associated with neonatal meningitis (Table 1) (*1*,*10*).

Plasmids from Orl-1 were used to transform *E. coli* K-12 DH5α. Three resistance profiles that enabled detection of 3 plasmids were obtained. On the basis of screening of plasmid transformants and Orl-1, most virulence factors genes were presumably chromosomally encoded. Three virulence factors (*aer*, *iss*, and a second copy of *iroN*) were mediated by a tetracycline-resistant, large (≈180 kb), conjugative plasmid (pOrl-1-Te). The 2 other plasmids were pOrl-1-CTX-M-1, the CTX-M-1–encoding large (≈150 kb) conjugative plasmid carrying resistance to trimethoprim and sulfamethoxazole, and pOrl-1-TEM-1, a TEM–1-encoding small (<40 kb) plasmid.

A derivative strain that did not harbor the 3 plasmids (Orl-c) was obtained by plasmid elimination with ethidium bromide. Orl-1, Orl-c, and *E. coli* DH5α harboring pOrl-1-CTX-M-1 were tested for invasiveness in human brain microvascular endothelial cells (*10*) and in a newborn mouse (C57BL/6 wild-type) model of meningitis (R. Mittal et al., unpub. data) to investigate the influence of CTX-M-1 production on virulence. *E. coli* strain E44, a rifampicin-resistant mutant of archetypical NMEC K1 strain RS218, was used as a positive control (*10*).

Orl-1 and Orl-c strains exhibited 3.5× lower invasiveness than strain E44. However, their ability to invade human brain microvascular endothelial cells was 400× higher than that of strain DH5α–CTX-M1. In the mouse model, DH5α–CTX-M1 did not cause bacteremia or meningitis. In contrast, Orl-1, Orl-c, and E44 induced meningitis (prevalences of 100%, 84%, and 85%, respectively) (Table 2).

The difference between Orl-1 and Orl-c in the mouse model may be explained by loss of plasmid pOrl-1-Te from Orl-c. Plasmid pOrl-1-Te is likely similar to pS88-related plasmids of NMEC because they share 3 virulence factor genes (*iss*, *aer*, and *iroN*) and are large and conjugative. These plasmids contribute to virulence of NMEC (*11*). Orl-1 and Orl-c showed similar behaviors, which suggested that the CTX-M-1–encoding plasmid pOrl-1-CTX-M-1 does not alter virulence of the strain.

Table 1. Virulence factors of Orl-1 *Escherichia coli* K1 strain, France

Integrative elements* (*1*)	Virulence genes	Present
PAI III_{536}–like	*iroN*	Yes
	sfa/foc	Yes
GimA-like	*ibeA* (*gimA4*), *ptnC* (*gimA1*)	Yes
PAI II_{J96}–like	*hra*	Yes
	hlyC, cnf1	No
PAI I_{CFT073}–like	*hlyC*	No
	aer (*iucC*)	Yes
HPI-like	*fyuA, irp-2*	Yes
GimB-like	*gimB*	Yes
pks island†	*clbA, clbK-J, clbP, clbQ*	Yes
Others	*chuA*	Yes
	ompA	Yes
	hek	Yes
	iss	Yes
	malX	Yes
	cdtB-I to *-V*	No

*PAI, pathogenicity island; HPI, high-pathogenicity island.
†Positive cytopathogen effect with transient infection of HeLa cells.

Mice with E44- and Orl-1–induced neonatal meningitis were treated with the third-generation cephalosporin cefotaxime, as recommended for humans. Despite antimicrobial drug treatment, Orl-1, but not strain E44, caused meningitis, suggesting that drug resistance is a major factor in clinical outcomes.

Conclusions

Studies have reported emergence of *E. coli* as the predominant organisms responsible for sepsis at any gestational age and for increased rates of drug-resistant *E. coli* caused by intrapartum drug prophylaxis (*12*). Spread of ESBLs in *E. coli* and intrapartum exposure to antimicrobial drugs may favor emergence of NMEC strains resistant to third-generation cephalosporins.

Two other well-characterized *E. coli* K1 strains producing ESBLs have been isolated from patients with neonatal meningitis in Algeria and France. The ESBL was identified as CTX-M-15 in both patients, and 1 infection was lethal (*13*,*14*). Other putative ESBL-producing *E. coli* K1 have been recently isolated, especially in developing countries (*15*).

Emergence of ESBL-producing *E. coli* strains, which are frequently resistant to fluoroquinolone (*2*), highlights the need for possible alternatives to third-generation ce-

Table 2. Incidence of meningitis in a newborn mouse model by *Escherichia coli* strain, France*

Bacterial strain	No. animals	Mean ± SD bacteremia, log CFU/mL blood	No. positive CSF cultures (% meningitis)
E44	20	6.95 ± 0.6	17 (85)†
Orl-1	16	6.75 ± 0.8	16 (100)†
Orl-c	17	6.60 ± 0.5	14 (82)†
DH5α-CTX-M1	10	0.10 ± 0.1	0

*CSF, cerebrospinal fluid.
†$p<0.005$, significantly higher than the incidence of meningitis by DH5α-CTX-M1 by χ^2 test.

phalosporins for treatment of patients with infected with NMEC. Carbapenems are usually recommended for treatment of infections with ESBL-producing *E. coli* (*2*). However, this case report shows the role of treatment duration and the need for additional pharmacokinetic and safety studies in neonates and for adjunctive therapies (*8*).

This characterization of a CTX-M-1–producing NMEC strain highlights the emergence of CTX-M–type ESBL in highly virulent *E. coli*. Because of worldwide spread of CTX-Ms, caution should be exercised in the management of patients with NMEC, and first-line treatment for neonatal meningitis may need to be reconsidered.

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Ehrlichia chaffeensis Infection of Sika Deer, Japan

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To determine whether *Ehrlichia chaffeensis* exists in Japan, we used PCR to examine blood from sika deer in Nara, Japan. Of 117 deer, 36 (31%) were infected with *E. chaffeensis*. The *E. chaffeensis* 16S rRNA base and GroEL amino acid sequences from Japan were most closely related to those of *E. chaffeensis* Arkansas.

Human infection with *Ehrlichia chaffeensis* causes human monocytic ehrlichiosis (HME), an influenza-like illness. Severity of the disease varies from mild to severe and can even cause death (*1*). HME cases have been reported primarily in the southeastern and south–central regions of the United States (*1*).

The organism, *E. chaffeensis*, until recently has been reported only in the United States; however, numerous reports now indicate that *Ehrlichiae* spp. closely related or identical to *E. chaffeensis* exist throughout the world (*1*,*2*). In the United States, *E. chaffeensis* has been most frequently identified in the lone star tick (*Amblyomma americanum*) (*3*). *E. chaffeensis* DNA has also been detected in *A. testudinarium* and *Haemaphysalis yeni* ticks from southern People's Republic of China (*4*), in *H. longicornis* ticks from South Korea (*5*), and in *A. parvum* ticks in Argentina (*6*). Other than *A. americanum*, the role of these tick species as *E. chaffeensis* vectors has not been investigated. The white-tailed deer (*Odocoileus virginianus*) is the only vertebrate species currently recognized as a complete and sufficient host for maintaining the transmission cycle of *E. chaffeensis* (*3*,*7*). To look for molecular evidence of *E. chaffeensis* in sika deer (*Cervus nippon*) in Japan, we examined blood specimens by using PCR amplification of the 16S rRNA and *groEL* genes.

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The Study

In Nara, Japan, to prevent injuries to park visitors, the Foundation for the Protection of Sika Deer in Nara Park captured 97 pregnant female sika deer in April 2005 and 20 male sika deer with antlers in November 2005. Blood specimens were collected from all 117 deer, and buffy coat fractions were stored at –80°C for further analysis. Genomic DNA was extracted from the buffy coat fractions by using a QIAamp tissue kit (QIAGEN, Valencia, CA, USA). Nested PCR amplification of genomic DNA was performed by using primer pairs designed to amplify the *E. chaffeensis* 16S ribosomal RNA (rRNA) gene and the *E. chaffeensis groEL* gene (Table).

Of the 117 specimens, 36 (31%) yielded *E. chaffeensis* 16S rRNA amplification products and 35 (30%) yielded *E. chaffeensis groEL* amplification products. Of 36 16S rRNA–positive specimens, 33 were positive for *groEL* (92% concordance rate). Of 35 *groEL*-positive specimens, 33 were positive for 16S rRNA (94% concordance rate). The *E. chaffeensis* sequences were conserved in that all sequences obtained from sika deer in Nara were nearly identical to those of a representative strain that we named NS101 and submitted to GenBank (accession no. AB454074). The sequence of the 16S rRNA gene from *E. chaffeensis* NS101 was most closely related (99.6% identity; 5 bases of 1,333 bp that can be aligned for comparison differed) to that of 5 human isolates: Arkansas (GenBank accession no. M73222) (*8*), Sapulpa (U60476) (*9*), 91HE17 (U23503) (*10*), St. Vincent (U86665) (*11*), and Jax (U86664) (*11*); the next closest sequence was from an *E. chaffeensis* isolate from *A. testudinarium* ticks in China (GenBank accession no. AF147752) (99.2% identity; 10 bases of 1,333 bp that can be aligned for comparison differed).

When *E. chaffeensis* NS101 was compared with *Ehrlichia* spp. previously identified in Japan, the sequence of the 16S rRNA gene (a 1,328-bp segment that can be aligned for comparison) of *E. chaffeensis* NS101 was 98.9%, 98.9%, and 98.6% identical to that of *E. muris* AS145 strain, *Ehrlichia* sp. HF565 (the HF strain, *Ixodes ovatus* Ehrlichia) and *Candidatus* Ehrlichia shimanensis TS37, respectively. Phylogenic analysis concurred with the observation that *E. chaffeensis* from Nara sika deer has the highest identity to the *E. chaffeensis* human isolates from the United States (Figure 1).

Although longer 16S rRNA gene sequences are desirable for strain comparison, the sequence of the 16S rRNA gene of *E. chaffeensis* from *H. longicornis* ticks in Korea (GenBank accession no. AY350424, 390 bp) and from *A. parvum* ticks in Argentina (GenBank accession no. EU826516, 470 bp) were identical to a corresponding, but incomplete, segment (358 bp and 402 bp, respectively) of the 16S rRNA gene of *E. chaffeensis* NS101 and Arkansas strains. An *Ehrlichia* sp. was detected in blood samples

Table. Detection of 16S rRNA gene and *groEL* gene of *Ehrlichia chaffeensisin* in sika deer, Japan

Target gene	1st PCR or nested PCR	Primer ID	Product size, bp	Sequence of primers (5′ → 3′)	No. positive
16S rRNA	1st	NS16SCH1F	1195	ACGGACAATTGCTTATAGCCTT	7
		NS16SCH1R		ACAACTTTTATGGATTAGCTAAAT	
	Nested	NS16SCH2F	443	GGGCACGTAGGTGGACTAG	36
		NS16SCH2R		CCTGTTAGGAGGGATACGAC	
groEL gene	1st	NSgroCH1F	849	GTTGTAACTGGTGAACAACTC	4
		NSgroCH1R		CTTTTCTTCTATCACCAAACCC	
	Nested	NSgroCH2F	469	GTTCGTATTTTGGAAGATGCTG	35
		NSgroCH2R		ACTGTGATAACTCCATCCTTAC	

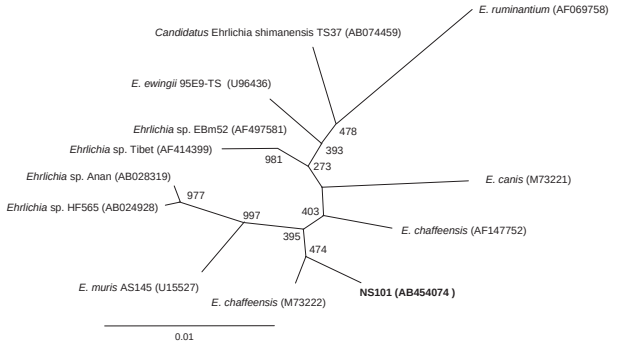

Figure 1. Phylogenetic relationship between *Ehrlichia chaffeensis* NS101 (in **boldface**) and other *Ehrlichia* spp. 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. Numbers above internal nodes indicate the number of bootstrap replicates of 1,000 that supported the branch. Scale bar indicates percent sequence divergence.

from marsh deer (*Blastocerus dichotomus*) captured near the Parana River in southeast Brazil in 1998 (*12*). However, the 16S rRNA sequence from these marsh deer (GenBank accession no. DQ345720, 383 bp) shares 98.7% identity to a corresponding, but incomplete, segment (381 bp) of the *E. chaffeensis* NS101 and Arkansas strains. Because the sequences of the corresponding segments of *Ehrlichia* sp. HF565 and '*Candidatus* Ehrlichia shimanensis' TS37 both had 99.0% identity to that of *E. chaffeensis* Arkansas strain, higher than the 98.7%, the *Ehrlichia* sp. in marsh deer from Brazil might not be *E. chaffeensis.*

Among the *E. chaffeensis groEL* sequences available in current databases, only that from *E. chaffeensis* Arkansas has >1,000 bp for reliable comparison. The *groEL* DNA sequence (1,208 bp that can be aligned) of the NS101 strain (GenBank accession no. AB454077, 1,311 bp) was most closely related to that of *E. chaffeensis* Arkansas (GenBank accession no. L10917), followed by the *Ehrlichia* sp. HF565 strain (GenBank accession no. AB032712), and the *E. muris* AS145 strain (GenBank accession no. AF210459) (Figure 2). The deduced *E. chaffeensis* NS101 GroEL amino acid sequence (402 residues) was most closely related (99.5% identity) to that of *E. chaffeensis* Arkansas, followed by the *Ehrlichia* sp. HF565 strain (99.2% identity) and *E. muris* AS145 strain (99.0%).

Conclusions

On the basis of the long 16S rRNA and the *groEL* DNA sequences, our study demonstrates the presence of *E. chaffeensis* in sika deer from Nara, Japan. The genetic similarity of *E. chaffeensis* in the sika deer in Japan to strains isolated from HME patients in the United States raises the possibility that HME may exist in Japan. Of 1,803 serum samples collected from persons in metropolitan Tokyo from 1991 through 1995, when *E. muris* was used as antigen, 20 were seropositive (*13*). Because *E. chaffeensis* and *E. muris* antigens are highly cross-reactive (*14*), some of these persons might have been infected with *E. chaffeensis*. Of the 10 tick species found on sika deer (*15*), the primary tick species found on sika deer in Nara is *H. longicornis*, which is known to bite humans in Japan and to be infected with *E. chaffeensis* in South Korea (*5*). Because sika deer are abundant and increasing throughout Japan (*16*), this finding highlights need to survey sika deer and humans in Japan for *E. chaffeensis* infection.

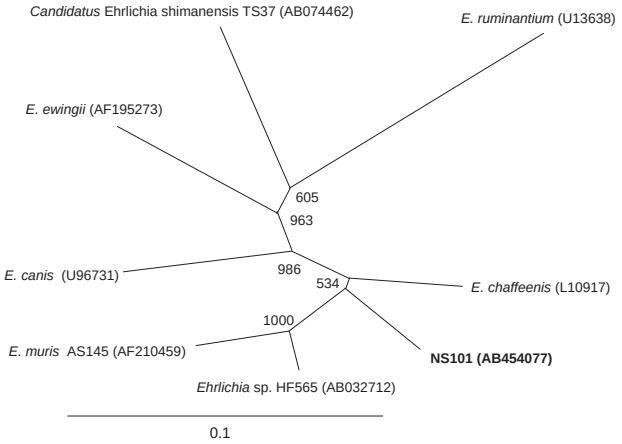

Figure 2. Phylogenetic relationship between the *Ehrlichia chaffeensis* NS101 *groEL* sequence (1,208 bp) (in **boldface**) and other *Ehrlichia* spp. *groEL* sequences. GenBank accession numbers are shown in parentheses. Numbers above internal nodes indicate the number of bootstrap replicates of 1,000 that supported the branch. Scale bar indicates percent sequence divergence.

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This work is dedicated to the late Masayoshi Tsuji; without his cooperation, this work would not have been possible. We are grateful to the Foundation for the Protection of Sika Deer in Nara Park for collection of blood samples.

Dr Kawahara is a researcher at the Nagoya City Public Health Research Institute. His research focuses on the *Ehrlichia* and *Anaplasma* species in Japan. He discovered *Ehrlichia muris* and *Candidatus* Neoehrlichia mikurensis and characterized the HF strain and *Mycoplasma haemomuris*.

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Diagnostic Assay for *Rickettsia japonica*

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We developed a specific and rapid detection system for *Rickettsia japonica* and *R. heilongjiangensis*, the causative agents of spotted fever, using a TaqMan minor groove binder probe for a particular open reading frame (ORF) identified by the *R. japonica* genome project. The target ORF was present only in *R. japonica*–related strains.

Rickettsia, a genus that includes the causative agents for spotted fever rickettsioses and typhus fever, comprises obligate intracellular bacteria (*1*). The first case of Japanese spotted fever (JSF), caused by *R. japonica* (*2*), was reported in 1984 in Japan (*3*). According to the national surveillance system in Japan (http://idsc.nih.go.jp/idwr/ybata/report-Ea.html), JSF cases, including sporadic cases resulting in death, have been gradually increasing. Rapid diagnosis of rickettsial infections is important because rickettsioses can be cured when appropriate antimicrobial drug treatment is given during the early clinical stages of the disease. Furthermore, development of a rapid and specific diagnostic system for *R. japonica* is now a matter of increasing urgency (*4*) because JSF has also been reported in several other countries in Asia (*1*).

The Study

Since 1998, thirteen complete *Rickettsia* genome sequences have been reported (www.ncbi.nlm.nih.gov/Genbank/index.html). In addition, the National Center for Biotechnology Information (NCBI) genome project for *R. japonica* strain YH has recently concluded (project ID 38487). Results of this project show specific DNA regions for *R. japonica* in the *Rickettsia* genome. One of these regions includes a 216-bp open reading frame (ORF) (GenBank accession no. AB437281). On the basis of information from this genome project, we performed DNA sequencing for this 216-bp ORF to determine whether the specific DNA sequences are conserved in all *R. japonica* strains and other closely related strains, including *R. heilongjiangensis* (*5*) and *Rickettsia* sp. LON (*6*).

R. heilongjiangensis is also a causative agent of spotted fever in northeastern Asia and has been classified within the *R. japonica* group (*5*). Several studies have reported that *Rickettsia*. sp. LON strains also have similar sequences to *R. japonica*. Our PCR can easily distinguish *Rickettsia* sp. LON strains (LON-2, LON-9, and LON-13) from *R. japonica* strains. This test can help in the diagnosis because *Rickettsia* sp. LON strains have only been isolated from ticks and may not be pathogenic in humans (*6*).

DNA sequencing was performed by using an ABI PRISM BigDye Terminator version 3.1 Kit (Applied Biosystems, Foster City, CA, USA) with an ABI 3130 sequence detector. DNA sequences were aligned by ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-e.html) with an open gap penalty of 15, a gap extension penalty of 6.66, a gap distance of 8, and a maximum division penalty of 40. For determination of the DNA sequence for the 216-bp ORF, the primer pair of JapoSP5′ (5′-ACAACATCAATATTATAATTAGTATCC-3′) and JapoSP3′ (5′-TTCACGTATGTCTATATATGCTGCAG CG-3′) was used to amplify a 564-bp section, including this ORF, because this unique DNA sequence was located as the inserted sequence of the homolog for *R. conorii* RC1338 (Figure, panel A).

The nucleotide sequence of this ORF was identical among 5 of the *R. japonica* strains: DT-1, YH, FLA-1, HH-8, and HH-9 (100%); the sequence was highly conserved with significant identity in *R. heilongjiangensis* (99.5%), except for the *Rickettsia* sp. LON strains (92.1%; Figure, panel B). This 216-bp ORF had been previously applied to BLAST searches with NCBI blast (nblast) for humans, mice, and others (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results showed that no similar ORF had been reported to date. Therefore, we focused on this conserved region of the 216-bp ORF to develop a TaqMan minor groove binder (MGB) probe (Applied Biosystems) that could detect the pathogenic *R. japonica* group, including *R. heilongjiangensis*, with a high degree of specificity.

Oligonucleotide primers (SpRija5′ and SPRija3′) and the TaqMan MGB probe (SpRijaMGB) were designed by using Primer Express software version 2.0 (Applied Biosystems Figure, panel B). The detection probe was labeled with the fluorescent reporter FAM (carboxyfluorescein labeling) at the 5′ end; the nonfluorescent quencher and MGB were labeled at the 3′ end (Figure, panel B). The detection sequence (shown as a bold line in Figure 1, panel B) was registered in GenBank (accession no. AB437281).

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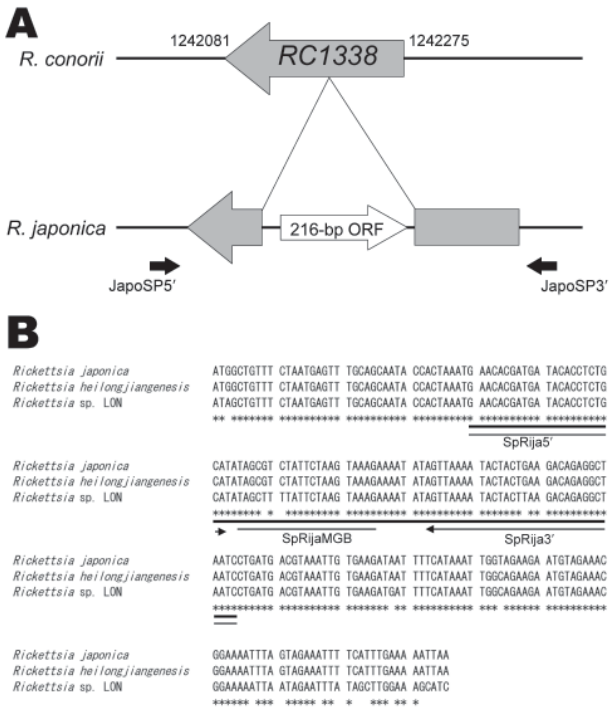

Figure. Unique DNA sequence in the *Rickettsia japonica* genome that analyzed PCR in this study. A) Comparative genome map of the 216-bp open reading frame (ORF). The *R. japonica*–specific sequence region (AB437281) in the *R. japonica* genome and the complete genome sequence of *R. conorii* strain Malish 7 were compared. The *RC1338* DNA sequence and the mapping position data for *R. conorii* were obtained from the *Rickettsia* genome database (www.igs.cnrs-mrs.fr/mgdb/Rickettsia). Two solid black arrows indicate primer positions; this region was amplified and sequenced with the same primers. B) Alignments of *R. japonica*–specific 216-bp ORF (AB437281) between *R. japonica* YH, *R. heilongjiangensis* CH8-1, and *Rickettsia* sp. LON, as performed by the program ClustalW (www.ebi.ac.uk/clustalw), and the positions of primers and probe in real-time PCR. Primer positions and directions (black arrows) and the TaqMan minor groove binder (MGB) (line) probe position are shown. The 216-bp ORF of *R. heilongjiangensis* and *Rickettsia* sp. LON were registered on GenBank with accession nos. AB512783 and AB512784, respectively. DNA sequences are identical among *R. japonica* strains and *Rickettsia* sp. LON strains (astericks). The alignment was edited with BioEdit version 7.0.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Real-time PCR was performed by using an ABI 7500 system (Applied Biosystems). DNA polymerase (perfect real-time PCR) for the PCR was obtained from Takara Bio (Kyoto, Japan). A 20-μL sample was added to each well of a 96-well microplate (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Thermal cycle protocol was performed as follows: first incubation stage, 20 s at 95°C; second stage, 5 s at 95°C and 34 s at 60°C. The second stage was repeated 45 times. For analysis of real-time PCR, the threshold line was fixed at 0.2 to avoid detection of nonspecific fluorescence. This detection procedure can be completed within 1 h.

The reactivity of this assay was examined by using various copy numbers of synthetic *R. japonica* DNA fragments that were amplified by the primer pairs JapoSP5′ and JapoSP3′ within the *R. japonica* genome. Genomic DNA of *R. japonica* strain YH was prepared from cultivated bacteria according to methods proposed by Furuya et al. (*7*). A calibration curve was generated with 5 calibrators, ranging from 10^2 to 10^9 copies/well in triplicate. We found a linear correlation (R>0.99) between the detection cycle numbers and *R. japonica* DNA copy numbers from 10^2 to 10^9 copies/reaction (data not shown).

A total of 26 rickettsial strains, classified into 11 species, were used in this study (Table 1). Specificities for this TaqMan PCR are also summarized in Table 1. Genomic DNA of *R. prowazekii* and *R. rickettsii* were prepared from antigen slides (Panbio Inc., Sinnamon Park, Queensland, Australia) by using a Gentra Puregene kit (QIAGEN, Valencia, CA, USA). Genomic DNA of other *Rickettsia* strains was also prepared from cultivated bacteria according to methods proposed by Furuya et al. (*7*).

Our results showed that this novel assay could detect all 5 *R. japonica* strains and 1 *R. heilongjiangensis* strain used in this study. However, it could not detect *R. rickettsii*, *R. prowazekii*, or other *Rickettsia* strains. These results indicate that the combination of probes and primers in this study had high specificity for the pathogenic *R. japonica* group. Nonspecific reactions were not observed when genomic DNA from human or murine fibroblasts was used in any of the assays (data not shown).

The detection limits of this PCR were compared to those of conventional PCRs by using serially diluted genomic DNA. The conventional PCRs, designated as Rj5-Rj10 and R1-R2 assays, were designed to detect the 17-kDa antigen gene of *Rickettsia*, by using a primer set of Rj5 (5′-CGCCATTCTACGTTACTACC-3′) and Rj10 (5′-ATTCTAAAAACCATATACTG-3′) (*7*) and R1 (5′-TCAATTCACAACTTGCCATT-3′) and R2 (5′-TTTACAAAATTCTAAAAACC-3′) (*15*), respectively. Since 1996, these assays have been used for molecular diagnosis of JSF in clinical laboratories in Japan. Recent studies have suggested that a TaqMan PCR assay may be as much as 100× more sensitive than these assays (data not shown). Therefore, in our study, the TaqMan PCR was assumed to be much more sensitive than the conventional assays that are known to show false-negative results, even for a patient with acute-stage JSF.

We requested clinical samples to verify the validity of our assay. Eighteen DNA templates were extracted from blood clots collected from 18 patients in the acute stages of illness (male:female ratio 1:1; average age 64.1 years [range 27–88 years]); average number of days after onset

Table 1. Reactivity of the real-time PCR for *Rickettsia* strains used in this study*

Species	Strain	Isolation source	Reference	Real-time PCR
Rickettsia asiatica	IO-1	*Ixodes ovatus*	ATCC VR-1593 (*8*)	–
	IO-2	*I. ovatus*	(*8*)	–
	IO-3	*I. ovatus*	(*8*)	–
	IO-25	*I. ovatus*	(*8*)	–
	IO-38	*I. ovatus*	(*8*)	–
R. conorii	Malish 7	Human	ATCC VR 613T	–
R. heilongjiangensis	CH8-1	*Haemaphysalis concinna*	(*6*)	+
R. helvetica	IM-1	*Ixodes monospinosus*	(*9*)	–
	IP-1	*I. persulcatus*	(*9*)	–
	IP-2	*I. persulcatus*	(*10*)	–
	IP-6	*I. persulcatus*	This study	–
R. honei	TT-118	*Ixodes* sp.	(*11*)	–
R. japonica	DT-1	*Dermacentor taiwanensis*	(*12*)	+
	YH	Human	ATCC VR-1363	+
	FLA-1	*Haemaphysalis flava*	(*9*)	+
	HH-8	*H. hystricis*	This study	+
	HH-9	*H. hystricis*	This study	+
R. prowazekii	breinl	Human	(*13*)	–
R. rickettsii	Sheila Smith	Human	(*14*)	–
R. sibirica	246	Human	ATCC VR-151	–
R. tamurae	AT-1	*Amblyomma testudinarium*	ATCC VR-1594 (*12*)	–
	AT-4	*A. testudinarium*	(*6*)	–
	AT-13	*A. testudinarium*	(*6*)	–
R. typhi	Wilmington	Human	ATCC VR-144	–
Rickettsia sp. LON	LON-2	*Haemaphysalis longicornis*	(*6*)	–
	LON-9	*H. longicornis*	(*6*)	–
	LON-13	*H. longicornis*	(*6*)	–

*+, positive; –, not detected.

Table 2. Application of PCRs for blood clot specimens derived from acute-stage Japanese spotted fever patients*

Patient no.	Age, y/sex	Days after onset of fever†	Laboratory examinations				
			Rickettsia isolation‡	Serodiagnosis§	Conventional PCR		Real-time PCR¶
					Rj5–Rj10 assay	R1–R2 assay	
C1	83/F	5	+	+	–	–	38.2 ± 0.6
C2	35/F	6	+	+	–	–	–
C3	70/M	6	+	+	–	–	37.6 ± 1.3
C4	71/M	3	+	+	–	–	–
C5	66/F	6	+	+	–	–	38.4 ± 1.0
C6	49/M	3	NT	+	–	–	–
C7	88/F	5	NT	+	–	–	40.7 ± 0.4
C8	49/M	7	NT	+	–	–	31.4 ± 1.1
C9	65/F	3	NT	+	–	–	36.0 ± 0.5
C10	78/M	4	NT	+	–	–	39.0 ± 0.8
C11	72/F	4	NT	+	–	–	–
C12	68/F	5	NT	+	–	–	–
C13	27/M	5	NT	+	–	–	–
C14	45/M	7	NT	+	–	–	–
C15	76/F	5	NT	+	–	–	39.0 ± 0.5
C16	79/M	3	NT	+	–	–	39.9 ± 1.0
C17	69/M	2	NT	+	–	–	–
C18	64/F	3	NT	+	–	–	–

*+, positive; NT, not tested; –, not detected.
†Blood clot was prepared from whole blood at indicated days after onset of fever.
‡Isolation from whole blood specimens.
§Seroconversion was identified by the indirect immunofluorescent test with paired serum specimens.
¶Cycle threshold values are given as means ± standard errors of the means for 3 independent assays.

of fever 4.6 [range 2–7 days; Table 2]). These templates were reexamined by using our TaqMan PCR. Although the conventional assays could not detect the presence of any *Rickettsia* DNA, 9 of 18 samples displayed positive results with the TaqMan PCR (Table 2). The blood clot from the patient in whom Scrub typhus disease was previously diagnosed was used as a negative control in this real-time PCR assay, resulted were not detected. Our TaqMan PCR is currently available to clinical laboratories that need to rule out false-negative results in molecular diagnoses.

Conclusions

JSF is a threat to public health in Japan. Our results suggest that an *R. japonica*–specific 216-bp ORF may have been conserved throughout the *R. japonica* species and closely related *Rickettsia* spp. The newly developed real-time PCR system, which demonstrated a high level of sensitivity and specificity, may be a useful tool for laboratory diagnosis.

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Novel Lineage of Methicillin-Resistant *Staphylococcus aureus*, Hong Kong

Luca Guardabassi, Margie O'Donoghue, Arshnee Moodley, Jeff Ho, and Maureen Boost

To determine whether *spa* type of methicillin-resistant *Staphylococcus aureus* in pigs belonged to sequence type (ST) 398, we analyzed nasal swabs from pig carcasses at Hong Kong markets in 2008. ST9 belonging to *spa* type t899 was found for 16/100 samples, which indicates that a distinct lineage has emerged in pigs.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been recognized as an important hospital pathogen and in recent years has emerged in the community. Increasing numbers of reports have concerned MRSA in animals. Multilocus sequence typing (MLST) has shown widespread dissemination of sequence type (ST) 398 among pigs in the Netherlands (*1*). This MRSA lineage has subsequently been reported in several other countries and animal species. Compelling microbiologic and epidemiologic evidence indicates that persons living or working on farms, especially pig farms, have an increased risk for colonization or infection with ST398 (*2*). In the present study, MRSA isolates obtained from slaughtered pigs in Hong Kong were characterized genotypically and compared with ST398.

The Study

Nasal swab specimens collected by using Transwabs (Medical Wire Ltd, Corsham, UK) were collected from 100 pig carcasses at 2 wet markets in Hong Kong on 5 separate days over a 7-week period in 2008. Cross-contamination was minimized by selecting carcasses with intact nasopharyngeal tracts and by instructing the butchers taking part in the project to avoid causing damage to the nares when cutting up the heads. The frontal section of each snout was cleaned with 75% alcohol before swabbing the nasal mucosa up to 8 cm into the nares. Nasal swabs were enriched in brain–heart infusion broth (Oxoid Ltd, Basingstoke, UK) with 7% NaCl at 37°C for 48 h and then injected into mannitol salt agar (Oxoid) supplemented with 6 μg/mL oxacillin, for 24 h. Presumptive *S. aureus* colonies were tested for heat-stable nuclease (DNase) and coagulase production, and isolates positive for both were confirmed to the species level by latex agglutination (Staphaurex Plus, Murex Diagnostics Ltd, Dartford, UK). Antimicrobial drug sensitivity testing was performed by using disk diffusion following Clinical and Laboratory Standards Institute recommendations (*3*). Methicillin resistance was confirmed by disk diffusion using cefoxitin (30 μg) and *mec*A PCR detection. All MRSA isolates were characterized by pulsed-field gel electrophoresis (PFGE) by using *Sma*I *(4*), staphylococcal chromosome cassette (SCC) *mec* typing (*5*), and *spa* typing (*6*) with Ridom StaphType 1.4.1 software (www.ridom.de/staphtype). Two isolates representative of distinct PFGE patterns and SCC*mec* types were analyzed by MLST (*7*), and the remaining isolates were characterized by single-locus (*aroE*) sequencing.

MRSA was isolated from 16 samples collected on 4 of the 5 sampling days. In contrast to ST398, which has the characteristic of being nontypeable by PFGE using *Sma*I (*1*,*2*), the 16 MRSA isolates were typeable. They displayed 6 PFGE patterns; 2 predominant types (A1 and B1) were associated with SCC*mec* types IV and V, respectively (Table 1). Both PFGE types were ST9 according to MLST analysis. The 4 remaining patterns were either closely related to A1 (A2, A3, and A4) or possibly related to B1 (B2) according to the criteria of Tenover et al. (*8*). All isolates belonged to *spa* type t899 and harbored the ST9-associated *aroE* allele 3, which differs from that in ST398 (allele 35) by multiple mutations. Porcine MRSA ST9 isolates were negative for Panton-Valentine leukocidin genes and resistant to a broader range of antimicrobial agents than that previously described for MRSA ST398 isolated from pigs in the Netherlands (*1*). Twelve isolates displayed a typical multiple resistance pattern, including resistance to chloramphenicol, ciprofloxacin, clindamycin, cotrimoxazole, erythromycin, gentamicin, and tetracyline. The remaining 4 isolates were additionally resistant to fusidic acid (Table 1). All isolates were negative for Panton-Valentine leukocidin and susceptible to vancomycin and linezolid.

A search of the scientific literature and the Internet for information about the frequency of *S. aureus* ST9 in humans and animals indicated that ST9 is a clone of porcine origin. In 2005, Armand-Lefevre et al. (*9*) reported that ST9 was the most prevalent ST of methicillin-susceptible *S. aureus* (MSSA) isolated from pig farmers and infected pigs in France but not from a control group of persons without occupational contact with pigs. In 2007, an erythromycin-resistant MSSA ST9 clone belonging to *spa* type 337 was found to be endemic on a farm in Denmark (*10*). A clinical ST9 isolate of porcine origin carrying the multidrug resistance gene *cfr*, associated with linezolid resistance, has

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Table 1. Characteristics of methicillin-resistant *Staphylococcus aureus* isolates from pig carcasses in Hong Kong, 2008*

Isolate	Sampling date	Resistance pattern	*spa* type	SCC*mec* type	PFGE pattern†	*aroE* allele	MLST type
K1	Feb 22	3	t899	IVb	A1	3	ST9
G29	Mar 11	4	t899	IVb	A2	3	ST9‡
55	Mar 27	1	t899	IVb	A1	3	ST9‡
56		1	t899	V	B1	3	ST9
57		1	t899	V	B1	3	ST9‡
61		1	t899	V	B1	3	ST9‡
54		1	t899	V	B2	3	ST9‡
B40	Apr 15	1	t899	IVb	A1	3	ST9‡
B46		1	t899	IVb	A1	3	ST9‡
B50		1	t899	IVb	A1	3	ST9‡
B51		1	t899	IVb	A1	3	ST9‡
B52		1	t899	IVb	A1	3	ST9‡
B22		1	t899	V	B1	3	ST9‡
B39		1	t899	IVb	A3	3	ST9‡
B37		2	t899	IVb	A2	3	ST9‡
B36		2	t899	IVb	A4	3	ST9‡

*SCC, staphylococcal chromosome cassette; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing. Resistance patterns: 1, oxacillin, penicillin, tetracycline, chloramphenicol, gentamicin, clindamycin, erythromycin, ciprofloxacin, cotrimoxazole; 2, oxacillin, penicillin, tetracycline, chloramphenicol, clindamycin , erythromycin, ciprofloxacin, fusidic acid; 3, oxacillin, penicillin, tetracycline, chloramphenicol, gentamicin, clindamycin, erythromycin, ciprofloxacin, cotrimoxazole, fusidic acid, 4, oxacillin, penicillin, tetracycline, chloramphenicol, clindamycin, ciprofloxacin, cotrimoxazole, fusidic acid.
†Closely or possibly related PFGE patterns according to the Tenover criteria (*8*) are designated by the same letter and different numbers.
‡ST9 was predicted on the basis of PFGE and *spa* and *aroE* typing.

recently been described (*11*). Six ST9 sequences have been submitted to the MLST database (http://www.mlst.net), including 5 for MSSA isolates from bloodstream infections in the United Kingdom and 1 for a MRSA isolate from the nose of a pig in China (ID2357). Single cases of human infection with MRSA ST9 t899 have been reported in the Netherlands (*2*) and in Guangzhou, China (*12*). Eighteen *spa*-type t899 isolates have been submitted to the Ridom SpaServer (http://spaserver2.ridom.de): 10 from Germany, 7 from the Netherlands, and 1 from Belgium. All submissions were recorded as MRSA, but unfortunately, the origins of the isolates and the MLST types were not reported. Although MRSA t899 has been previously associated with ST398 isolates from pigs (*13*) and from participants at a conference on pig health (*14*), the repeat succession of this *spa* type is completely different from those of other ST398-related *spa* types and similar to those of *spa* types related to ST9 (Table 2). That the same *spa* type occurs in both ST9 and ST398 is surprising because the MLST allelic sequences of these 2 *S. aureus* lineages are unrelated (Table 2). However, the occurrence of the same *spa* types in distant lineages has been previously reported (*15*) and could have resulted from either convergent evolution or genetic recombination.

Conclusions

Our study of MRSA colonization of commercial pigs in Asia provides evidence that methicillin resistance has emerged in a porcine *S. aureus* lineage other than ST398. It appears that ST9 has achieved methicillin resistance through multiple acquisitions of SCC*mec*, as indicated by the recovery of distinct PFGE and SCC*mec* types. A combination of the results of literature and database searches indicates that ST9 is associated with pig farming and, although it is found infrequently, this ST has been isolated from infected persons worldwide.

Several studies have previously investigated the prevalence of MRSA nasal carriage in pigs sampled immediately after slaughter (*2*) or at the farm of origin (*13*). In our study, samples were collected at wet markets, because it not possible to access pigs at the single slaughterhouse in Hong Kong or at the farm sites of origin because >90% of slaugh-

Table 2. Tandem repeat successions in *spa* types previously associated with *Staphylococcus aureus* ST9 and ST398*

spa type	Tandem repeat sequence	MLST allelic profile	ST
t899	07-16-23----------02-34	3-3-1-1-1-1-10	9
t337	07-16-23-23-02-12-23-02-34	3-3-1-1-1-1-10	9
t3198	07-16-16-23-23-02-02-12-23-02-34	3-3-1-1-1-1-10	9
t011	08-16-02---------25-34-24-25	3-35-19-2-20-26-39	398
t034	08-16-02-25-02-25-34-24-25	3-35-19-2-20-26-39	398
t0108	08-16-02---------25-----24-25	3-35-19-2-20-26-39	398
t567	08----------02-25-----24-25	3-35-19-2-20-26-39	398
t571	08-16-02-25-02-25-34-----25	3-35-19-2-20-26-39	398

*MLST, multilocus sequence typing; ST, sequence type.

ter pigs are raised in mainland China and delivered by train directly to the slaughterhouse in Hong Kong. Notably, the previously reported human infection in China with MRSA-ST9 occurred in Guangzhou, the province closest to Hong Kong, where most of the pigs originate. The colonization rate determined in our study represents the level of contamination immediately prior to sale of pig meat to consumers. Although pig heads are rarely available in European and North American markets, because these parts of the animal are generally centrally processed, homemade soup using the pig's nose is commonly consumed in Hong Kong; this gastronomic tradition may increase the risk for zoonotic transmission of MRSA. Further epidemiologic studies are needed to determine the rates of colonization and infection with MRSA and MSSA ST9 both in personnel exposed to pigs and in the community.

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Respiratory Infection in Institutions during Early Stages of Pandemic (H1N1) 2009, Canada

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Outbreaks of respiratory infection in institutions in Ontario, Canada were studied from April 20 to June 12, 2009, during the early stages of the emergance of influenza A pandemic (H1N1) 2009. Despite widespread presence of influenza in the general population, only 2 of 83 outbreaks evaluated by molecular methods were associated with pandemic (H1N1) 2009.

Respiratory infection outbreaks in institutions housing large numbers of residents create an ideal environment for disease transmission (*1*). Patients in long-term care facilities (LTCFs) for the elderly are more susceptible to respiratory infections and have a higher risk for complications (*2*,*3*).

The Study

We reviewed respiratory infection outbreaks registered with the Public Health Laboratory (PHL), Ontario Agency for Health Protection and Promotion dating back to October 2007 (Table 1). Molecular detection methods were used for a subset of outbreaks registered during October 1, 2008–April 19, 2009. After emergence of severe respiratory illness clusters in Mexico in early April, intensified tracking of respiratory infection outbreaks in Ontario was undertaken. Consequently, more information was available on outbreaks registered during the spring (April 20 to June 12, 2009); these data comprise the bulk of the study.

Respiratory infection outbreaks in LTCFs were defined as any of the following: 2 cases of acute respiratory tract illness, 1 of which was laboratory-confirmed; 3 cases of acute respiratory tract illness within 48 hours in a geographic area (e.g., unit, floor); and >2 units having a case of acute respiratory illness within a 48-hour period. Influenza-like-illness was defined as acute onset of respiratory illness with fever and cough with ≥1 of the following: sore throat, arthralgia, myalgia, or prostration.

From April 20 through June 12, 2009, a total of 112 respiratory infection outbreaks were registered. Molecular testing was not used in 29 outbreaks (e.g., insufficient/inappropriate sample). Most of the remaining 83 outbreaks submitted for molecular testing originated from LTCFs (91%); hospitals (2%), child care centers (2%), and psychiatric care facilities (1%) comprised the remainder. Facility type was not known for 4% of outbreaks tested. Mean age of persons tested as part of an outbreak investigation was 82 years (SD 13.96 years) and median age was 85 years; 95% were >57 years of age.

Testing on the 589 specimens received from 161 outbreaks registered from October 1, 2008 through June 12, 2009 was performed by real-time reverse transcription–PCR (RT-PCR) for the influenza A virus matrix gene and the Luminex Respiratory Viral Panel (RVP) (Luminex Molecular Diagnostics, Toronto, Ontario, Canada) for other respiratory viruses.

An etiologic agent was identified in 89% of the 161 outbreaks tested by molecular methods. One-hundred-eleven (69%) were caused by 1 etiologic agent. Two and 3 different pathogens were identified in 24 (15%) and 6 (4%) outbreaks, respectively. Four pathogens were identified in 2 (1%) outbreaks. No etiologic agent was identified in 18 (11%) of the outbreaks tested by molecular methods, which includes 1 specimen in which the result was indeterminate for coronavirus OC43. A wide range of causative etiologic agents were detected for outbreaks by the RVP assay (Table 2). Specimens from most patients were positive for enterovirus/rhinovirus (114 patients) followed by metapneumovirus (85), parainfluenza virus type 3 (55), and human influenza virus A (H3) (41). No virus was identified in 186 patients.

Table 1. Respiratory outbreak submissions to Ontario, Canada, public health laboratories by geographic location and season*

	Influenza season submissions		Spring submissions	
Region	2007–08	2008–09	2008	2009
Ontario	671	543	117	112†
Greater Toronto area‡	139	101§	34	21

*Influenza season is delineated as October 1–April 19; spring season is delineated as April 20–June 12.
†Specimens from 83 of the 112 outbreaks were tested by the RVP assay.
‡Greater Toronto Area includes submissions by Peel, York, and Toronto Public Health Units.
§Specimens from 78 of the 101 outbreaks were tested by the Luminex xTAG Respiratory Viral Panel (Luminex Molecular Diagnostics, Toronto, Ontario, Canada).

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Table 2. Etiologic agents identified by the Luminex Respiratory Virus Panel* from samples submitted by regional health units during outbreaks, Canada†

Etiologic agent	2009 spring outbreaks, Ontario, no. (%)	2009 spring outbreaks GTA,‡ no. (%)	2008–2009 influenza season outbreaks, GTA,‡ no. (%)
Coronavirus OC43	1 (1)	0	18 (23)
Coronavirus NL63	0	0	6 (8)
Coronavirus 229E	4 (5)	0	9 (12)
Metapneumovirus	17 (20)	2 (12)	21 (27)
Respiratory syncytial virus A	0	0	5 (6)
Respiratory syncytial virus B	1 (1)	0	17 (22)
Influenza A (H3, human)	11 (13)	6 (35)	4 (5)
Parainfluenza 1	1 (1)	0 (0)	1 (1)
Parainfluenza 3	22 (27)	7 (41)	3 (4)
Enterovirus/rhinovirus	31 (37)	3 (18)	15§ (19)
Pandemic (H1N1) 2009 virus	1 (1)	1 (6)	0
Invalid test¶	0	0	1 (1)
None	6 (7)	0	11 (14)
Outbreaks tested	83	17	78

*Luminex Molecular Diagnostics, Toronto, Ontario, Canada.
†GTA, Greater Toronto area. Spring season is delineated as April 20–June 12; influenza season is delineated as October 1–April 19.
‡Iincludes submissions by Peel, York, and Toronto Public Health Units only.
§Seven of the 15 outbreaks were confirmed as rhinovirus by the Seeplex RV12 detection kit (Seegene, Inc., Seoul, South Korea).
¶Reported when the internal control is not detected during a run.

Co-infections were noted in 22 of the patients tested by the RVP assay. In 1 LTCF outbreak, 2 patients had co-infection of an untypeable influenza A and enterovirus/rhinovirus on testing by RVP. An influenza A real-time RT-PCR result was negative in both patients; 1 patient had a co-infection with respiratory syncytial virus B and enterovirus/rhinovirus. Co-infections with coronavirus subtypes 229E and NL63 were the most common, observed in 10 of the 22 patients (45%) infected with multiple pathogens. Isolates from 1 patient were positive for 3 viruses (coronavirus subtypes 229E and NL63 and enterovirus/rhinovirus).

One of the 2 outbreaks identified as caused by pandemic (H1N1) 2009 originated from a LTCF was not observed until June 3, 2009, six weeks into the evolving pandemic, despite widespread community prevalence. The second pandemic (H1N1) 2009 outbreak, registered on June 11, 2009, originated from a hospital treating patients with influenza-like illness. Seasonal influenza (H1N1 and H3N2) or pandemic (H1N1 2009) was detected in 2,966 (25.5%), and pandemic (H1N1) 2009 in 2,203 (19%) of 11,612 persons tested at PHL for influenza A by real-time RT-PCR during April 20–June 12, 2009. Seasonal influenza A (H3N2) was only identified in 273 specimens (11.0%) of the 2,476 influenza A positive samples subtyped. However, it was the strain responsible for 15 (88%) of the typeable influenza A outbreaks at the same time. Seasonal influenza A (H1N1) was absent from institutional outbreaks and only detected in 41 (2%) of subtyped influenza A–positive samples from the general population.

Persons with laboratory-confirmed pandemic (H1N1) 2009 infection tested at the PHL, Ontario Agency for Health Protection and Promotion, were younger than those tested as part of outbreak investigations. Mean and median ages were 21.5 and 16 years, respectively; only 10% were >46 years of age.

Conclusions

The number of respiratory infection outbreaks in institutions submitted to PHL may reflect disease impact caused by respiratory viruses during the influenza season. Respiratory viruses during the 2007–08 season may have been more active than those of the 2008–09 season because the number of outbreaks registered with PHL decreased from 1 year to the next. Declaration of pandemic status for the novel (H1N1) virus has not influenced the reporting of respiratory infection outbreaks from institutions in Ontario because submission rates for the corresponding period in 2007–08 and 2008–09 are similar. Variation would not be expected because reporting is required by Ontario law (*4*).

Respiratory viruses detected in outbreaks in institutions reflect those known to be major causes of acute respiratory disease in the community; prevalence varies based on geographic location, season, and detection methods (*5*–*7*). Free access to such institutions by members of the community (staff or visitors), in conjunction with communal close quarters of residents, creates an ideal environment for propagation of viral respiratory outbreaks (*8*).

Current guidelines for isolation during viral respiratory outbreaks are not tailored for the specific virus. As shown in this study, multiplex molecular testing makes it possible to identify the virus causing most LTCF respiratory infection outbreaks. Infection control guidelines for a specific outbreak could be modified based on the incubation period and duration of viral shedding for the identified virus (*9*).

The most commonly identified virus in our study was enterovirus/rhinovirus. Clinicians should be reminded that

rhinovirus can cause severe lower respiratory tract infection, including death, as documented in several LTCF outbreaks (*10*,*11*). These data highlight the need for molecular capacity to diagnose rhinovirus infection because detection is otherwise limited to less sensitive viral culture systems.

This review of outbreaks predominantly involving elderly persons in LTCFs highlights the sparing of older persons by pandemic (H1N1) 2009. Possible explanations include cross-protective antibodies from previous exposure to influenza A (H1N1) strains circulating before the antigenic shift of influenza A to subtype H2N2 in 1957 or minimal contact with those most likely to have imported the pandemic strain into Canada (young travelers) (*12*). In addition, older persons may have less contact with the age group (children 10–19 years of age), with most cases being in Ontario. Our findings support Centers for Disease Control and Prevention guidelines for vaccination with monovalent pandemic (H1N1) 2009 virus vaccine. These guidelines have not placed older persons in a high priority group for vaccination because increased rates of hospitalization and severe disease caused by pandemic (H1N1) 2009 have not been observed (*13*,*14*). Investment in multiplex technologies to investigate respiratory outbreaks in LTCFs shortens time for pathogen detection, helps guide infection control and vaccination policies, and can potentially save resources spent on other investigations.

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Mr Marchand-Austin is a Liaison Technical Officer for the province of Ontario, employed by the Public Health Agency of Canada. This recently created position serves to facilitate communication between provincial and federal public health laboratories.

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Estimates of the Prevalence of Pandemic (H1N1) 2009, United States, April–July 2009

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Through July 2009, a total of 43,677 laboratory-confirmed cases of influenza A pandemic (H1N1) 2009 were reported in the United States, which is likely a substantial underestimate of the true number. Correcting for under-ascertainment using a multiplier model, we estimate that 1.8 million–5.7 million cases occurred, including 9,000–21,000 hospitalizations.

Human cases of influenza A pandemic (H1N1) 2009 were first identified in the United States in April 2009 (*1*,*2*). By the end of July, >40,000 laboratory-confirmed infections had been reported, representing only a fraction of total cases. Persons with influenza may not be included in reported counts for a variety of reasons, including the following: not all ill persons seek medical care and have a specimen collected, not all specimens are sent to a public health laboratory for confirmatory testing with reverse transcription–PCR (RT-PCR; rapid point-of-care testing cannot differentiate pandemic [H1N1] 2009 from other strains), and not all specimens will give positive results because of the timing of collection or the quality of the specimen. To better estimate the prevalence of pandemic (H1N1) 2009 during April–July 2009 in the United States, we created a simple multiplier model that adjusts for these sources of under-ascertainment.

The Study

Through July 23, 2009, a total of 43,677 laboratory-confirmed infections with pandemic (H1N1) 2009 had been reported in the United States by the 50 states and the District of Columbia, including 5,009 hospitalizations and 302 deaths. To estimate the total number of cases of pandemic (H1N1) 2009, we built a probabilistic multiplier model that adjusts the count of laboratory-confirmed cases for each of the following steps: medical care seeking (A), specimen collection (B), submission of specimens for confirmation (C), laboratory detection of pandemic (H1N1) 2009 (D), and reporting of confirmed cases (E) (Figure). This approach has been used to calculate the underrecognized impact of foodborne illness in the United States (*3*).

At each step, we identified a range of proportions observed in prior published studies and recent surveys and investigations of pandemic (H1N1) 2009. These include 2 unpublished community surveys on influenza-like illness (ILI) and health-seeking behavior, the 2007 Behavioral Risk Factor Surveillance Survey conducted in 10

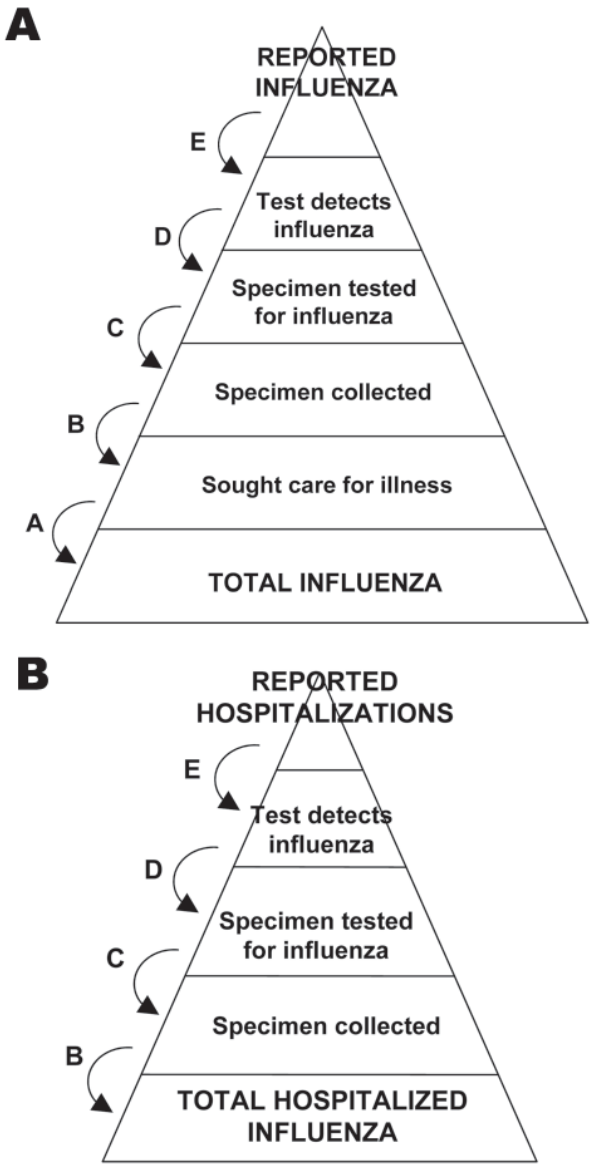

Figure. Schematic of the steps involved in adjusting counts of reported cases of pandemic (H1N1) 2009 to estimate total cases.

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states and repeated in the same states during May 2009, and field investigations conducted during early outbreaks of pandemic (H1N1) 2009 in Chicago and Delaware (online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2004-Techapp.pdf; [*4*]). We theorized that, given recommendations for testing, patients hospitalized with pandemic (H1N1) 2009 would more likely have been tested and their cases reported than would outpatients. We therefore stratified our model between hospitalized and nonhospitalized cases (Figure). For hospitalized patients, we used larger estimates of the proportion of specimens collected, tested, and reported, which resulted in smaller multiplier values (Table 1). We also adjusted for the fact that early in the epidemic physicians and health departments were encouraged to collect clinical specimens from all suspect case-patients with ILI and forward them for confirmatory testing with RT-PCR. By May 12, due to the increasing number of cases and the demands on public health laboratories, guidance for confirmatory testing was revised to focus on hospitalized patients. We therefore used a lower estimate for the proportion of specimens collected from patients with mild illness after that date, effectively increasing the multiplier for those patients (Table 1).

Multipliers were calculated as the simple inverses of the proportions at each step. We accounted for variability and uncertainty in model parameters by using a probabilistic (Monte Carlo) approach (built by using SAS version 9.2; SAS Institute, Cary, NC, USA). For each parameter included in the model, we used uniform probability distributions that covered a range of minimum to maximum values, from which the model randomly sampled 10,000 iterations (online Technical Appendix). We generated median, upper, and lower 90% values for the number of total illnesses and hospitalizations.

To further divide estimated cases into age groups, we applied the age distribution of confirmed cases and hospitalizations as reported to the US Centers for Disease Control and Prevention through July 23, 2009 (online Technical Appendix), and calculated overall and age-specific incidence of illness and hospitalization, based on the US Census monthly population estimates for May 2009. We did not have age-specific parameter estimates, and thus did not stratify by age group within the model. This approach may not fully capture differences in the probability of ascertainment by age.

Using this approach, between April and July 2009, we estimate that the median multiplier of reported to estimated cases was 79; that is, every reported case of pandemic (H1N1) 2009 may represent 79 total cases, with a 90% probability range of 47–148, for a median estimate of 3.0 million (range 1.8–5.7 million) symptomatic cases of pandemic (H1N1) 2009 in the United States. Likewise, we estimate that every hospitalized case of pandemic (H1N1) 2009 that was reported may represent a median of 2.7 total hospitalized persons (90% range 1.9–4.3). This represents a median estimate of 14,000 (range 9,000–21,000) hospitalizations (Table 2) and thus an estimated ratio of hospitalizations to total symptomatic cases of 0.45% (range 0.16%–1.2%).

We also estimate that incidence of pandemic (H1N1) 2009 over the first 4 months of the pandemic in the United States ranged from a median of 107/100,000 in persons ≥65 years of age, to 2,196/100,000 in persons 5–24 years of age (Table 2). The incidence of hospitalization was estimated to be highest in young children <5 years of age (median 13.0/100,000, 90% range 8.8–20.2).

Table 1. Model parameters and sources of data included in the model estimating prevalence of pandemic (H1N1) 2009, United States, April–July 2009*

	Parameter	Observed value	Source	Ranges included in the model, %	
				Not hospitalized	Hospitalized
A	Proportion of persons with influenza who seek medical care, %	42	2007 BRFSS, 9 states†	42–58	100
		52–55	2009 ILI survey, 10 states†		
		49–58	Delaware university survey		
		52	Chicago community survey		
B	Proportion of persons seeking care with a specimen collected, %	25	2007 BRFSS, 9 states†	19–34	40–75
		22–28	2009 ILI survey, 10 states†		
		19–34	Delaware university survey		
C	Proportion of specimens collected that are sent for confirmatory testing, %	26 (through May 3)	Delaware university survey	20–30 (through May 12); 5–15 (after May 12)	50–90
D	Test detects influenza		Published studies	90–100	90–100
E	Proportion of confirmed cases reported to CDC		Assumption	95–100	95–100
	No. reported cases	43,677	Reports to CDC through July 23, 2009	4,759 (through May 12); 33,909 (after May 12)	5,009

*BFRSS, Behavioral Risk Factor Surveillance Survey; ILI, Influenza-like illness; CDC, Centers for Disease Control and Prevention. States include California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee.
†Parameter estimates and sources are described in further detail in the online Technical Appendix; available from www.cdc.gov/EID/content/15/12/2004-Techapp.pdf.

Table 2. Estimates of pandemic (H1N1) 2009–related cases and rates of illness and hospitalization by age distribution of confirmed case-patients, United States, April–July 2009

Parameter	Estimated no. case-patients		Estimated rate/100,000*	
	Median	90% range	Median	90% range
Total no. case-patients by age group, y†	3,052,768	1,831,115–5,720,928	997	598–1,868
0–4	397,033	238,149–744,045	1,870	1,122–3,505
5–24	1,820,284	1,091,845–3,411,237	2,196	1,317–4,115
25–49	612,862	367,608–1,148,511	577	346–1,081
50–64	180,297	108,146–337,879	319	192–599
≥65	42,292	25,368–79,256	107	64–201
No. hospitalized case-patients by age group, y	13,764	9,278–21,305	4.5	3.0–7.0
0–4	2,768	1,866–4,285	13.0	8.8–20.2
5–24	4,991	3,364–7,725	6.0	4.1–9.3
25–49	3,440	2,319–5,324	3.2	2.2–5.0
50–64	1,912	1,289–2,959	3.4	2.3–5.2
≥65	654	441–1,012	1.7	1.1–2.6
Multiplier				
Hospitalized	2.7	1.7–4.5	–	–
Nonhospitalized	79	47–148	–	–
Through May 12	33	23–49	–	–
After May 12	84	50–163	–	–

*United States Population Estimates, 2009.
†Age distributions from line list and aggregate reports of laboratory-confirmed cases and hospitalizations to the Centers for Disease Control and Prevention through July 23, 2009.

Conclusions

We demonstrate that the reported cases of laboratory confirmed pandemic (H1N1) 2009 are likely a substantial underestimation of the total number of actual illnesses that occurred in the community during the spring of 2009. We estimate that through July 23, 2009, from 1.8 million to 5.7 million symptomatic cases of pandemic (H1N1) 2009 occurred in the United States, resulting in 9,000–21,000 hospitalizations. We did not estimate the number of deaths directly from our model, but among reports of laboratory-confirmed cases though July 23, the ratio of deaths to hospitalizations was 6%. When applying this fraction to the number of hospitalizations calculated from the model—that is, by assuming that deaths and hospitalizations are underreported to the same extent—we obtain a median estimate of 800 deaths (90% range 550–1,300) during this same period. Because this assumption has several limitations (*5*), more sophisticated models are also being developed to better understand the severity of the US epidemic in the spring of 2009, including intensive care unit admissions and deaths (*6*).

Our analysis involves several assumptions. Data for parameter estimates were collected in limited periods and areas and thus may not be fully representative of the entire United States. To account for some of this uncertainty, a range of values was included for each proportion. Additional data from surveys of health-seeking behavior, physician testing practices, and policies for confirmatory testing at public health laboratories could help refine the parameter estimates. In addition, parameters were obtained from studies of persons with ILI, defined as fever with cough or sore throat. Persons with milder illness may be less likely to seek care or be tested, and thus may not be fully captured in these estimates. Likewise, in some heavily affected areas, the size of the outbreak quickly exceeded the capacity to ascertain and test case-patients. Thus, our results may reflect a conservative estimate of total cases.

As pandemic (H1N1) 2009 continues to spread through the United States and the world, laboratory-confirmed cases will continue to greatly underestimate the number of actual cases that occur. Surveillance for influenza does not traditionally rely on complete case ascertainment, which would be impractical, but on focused case ascertainment with well-characterized surveillance systems and special studies. Unfortunately, relying on laboratory-confirmed cases limits the ability to understand the full impact and severity of the epidemic, especially when severe cases are more likely to be recognized (*5*).

This model provides a relatively quick and simple approach to estimate the human health impact of the epidemic in advance of more rigorous analysis of surveillance and health care data that will be available over the next few years. Health systems and infrastructure may be unprepared in the short-term if plans are based on a number of confirmed cases that substantially underestimates the impact of the epidemic. We estimate that the total number of pandemic (H1N1) 2009 cases in the United States during April–July 2009 may have been up to 140× greater than the reported number of laboratory confirmed cases. A spreadsheet version of the model has been developed and is available online (www.cdc.gov/h1n1flu/tools). Using this tool, health officials and policy makers could adjust

the model parameters to represent their local experience, which may provide useful estimates of the prevalence of pandemic (H1N1) 2009 in their areas and help plan for a subsequent wave of the epidemic in the fall and winter months of 2009–2010.

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Mopeia Virus–related Arenavirus in Natal Multimammate Mice, Morogoro, Tanzania

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A serosurvey involving 2,520 small mammals from Tanzania identified a hot spot of arenavirus circulation in Morogoro. Molecular screening detected a new arenavirus in Natal multimammate mice (*Mastomys natalensis)*, Morogoro virus, related to Mopeia virus. Only a small percentage of mice carry Morogoro virus, although a large proportion shows specific antibodies.

Arenaviruses are segmented negative-strand RNA viruses. Their natural hosts are various rodent species. The virus family comprises several human pathogens causing hemorrhagic fever, namely Machupo, Guanarito, Junin, Sabia, and Chapare viruses in South America, and Lassa and Lujo viruses in Africa (*1*–*3*). In addition, Africa harbors arenaviruses that are not linked with human disease: Mobala, Ippy, Mopeia, and Kodoko viruses (*4*–*7*). We conducted a systematic search in wildlife in Tanzania to identify new African arenaviruses.

The Study

During 1985 through 1989, a total of 2,520 small mammals were live-trapped in different regions of Tanzania. After species determination, they were measured and bled by orbital puncture. Serum samples were tested by indirect immunofluorescent antibody (IFA) assay (*8*). Lassa virus was used as antigen due to its cross-reactivity with immune sera from animals infected with other arenaviruses (*4*,*6*). Clusters of seropositivity were found in *Arvicanthis* spp. rodents from the Iringa region (20%) and in Natal multimammate mice (*Mastomys natalensis*) from Arusha (18%) and Morogoro (17%) (Table 1), which suggests that these animals are reservoirs of arenaviruses. Titers ranged from 16 to 512 and 16 to 4,096 in *Arvicanthis* spp. rodents and *M. natalensis* mice, respectively. Peak prevalence in *M. natalensis* mice was found on the campus of the Sokoine University in Morogoro (23.7% of 746 animals collected over several seasons).

In 2004, *M. natalensis* mice were trapped in a mosaic of maize fields and fallow grassland at the university campus in the city of Morogoro (6°50′34.9794″S; 37°38′8.232″E) to identify the virus. The animal voucher specimens were deposited at the Royal Museum of Central Africa, Tervuren, Belgium. RNA was prepared from 10 μL of rodent serum by using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA), and screening was performed by using a pan–Old World arenavirus reverse transcription–PCR (RT-PCR) specific for the large (L) gene (*9*). One of 96 serum samples was positive (no. 3017/2004) (Table 2), and sequencing of the PCR fragment showed a new arenavirus sequence. The virus was isolated in Vero cells and called Morogoro virus (strain 3017/2004).

For sequencing, the isolate was propagated in T75 flasks, virus particles in supernatant were pelleted by ultracentrifugation, and RNA was isolated by using the QIAamp Viral RNA kit (QIAGEN). The entire 3.5-kb small (S) RNA segment was amplified by RT-PCR as described previously (*10*). The 7-kb L RNA segment was amplified in 2 fragments by using a long-range RT-PCR protocol and primers targeting the conserved termini of L RNA and Morogoro virus–specific primers designed on the basis of the sequence of the fragment detected by RT-PCR screening. By using the PCR products as a template, short overlapping fragments were amplified and sequenced with a set of consensus primers for Old World arenaviruses, and S and L RNA sequences were assembled (GenBank accession nos. EU914103 and EU914104). (Sequences reported in this article have been submitted to GenBank and assigned the following accession numbers: full-length S and L RNA sequences of Morogoro virus, EU914103–04; partial L gene sequences of Morogoro virus, EU914107–22; cytochrome B gene of Morogoro virus-positive *Mastomys natalensis*, EU914105–06.)

Full-length amino acid sequences of glycoprotein precursor (GPC), nucleoprotein (NP), and L protein of Morogoro virus were aligned with published Old World arenavirus sequences and pairwise p distances were calculated. Morogoro virus showed genetic similarity to strains

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Table 1. Detection of African arenavirus-specific antibodies in small mammals in Tanzania, 1985–1989*

Genus	Antibody detection† by region (no. positive/no. tested)									Total
	Arusha	**Iringa**	**Lindi**	Mbeya	**Morogoro**	Mtwara	Ruvuma	Songea	Tanga	
Acomys	–	0/3	0/2	0/2	0/57	0/2	–	–	–	0/66
Aethomys	–	0/3	0/4	–	0/23	0/11	0/7	0/8	–	0/56
Arvicanthis	0/13	**6/30**	–	–	–	–	–	–	0/87	**6/130**
Cricetomys	–	–	–	–	0/35	–	–	–	–	0/35
Lemniscomys	0/5	**1/2**	**1/2**	–	**1/30**	0/2	0/1	–	–	**3/42**
Lophuromys	0/3	0/1	–	–	0/3	–	–	–	0/7	0/14
Mastomys	**7/39**	0/17	**1/120**	0/12	**181/1,054‡**	0/81	0/8	0/25	0/82	**189/1,438**
Mus	–	0/1	–	0/1	**1/47**	–	–	–	–	**1/49**
Praomys	–	0/3	–	0/1	0/1	–	–	–	0/1	0/6
Rattus	–	–	0/24	0/1	0/49	0/20	0/3	0/15	0/196	0/308
Tatera	0/1	0/1	0/32	–	0/127	0/69	0/11	0/3	–	0/244
Uranomys	–	–	–	–	0/11	–	–	–	–	0/11
Sciuridae	–	–	0/13	–	0/2	–	0/2	–	0/10	0/27
Crocidura	–	–	–	–	**1/14**	–	–	–	–	**1/14**
Petrodomus	–	–	0/9	–	–	0/18	–	–	–	0/27
13 other genera	–	0/1	0/2	0/7	0/21	0/20	–	–	0/2	0/53
Total	**7/61**	**7/62**	**2/208**	0/24	**184/1,474**	0/223	0/32	0/51	0/385	**200/2,520**

*Positive samples as well as the respective sampling sites and animals are indicated in **boldface**.
†Immunofluorescent antibody (IFA) assay was performed with Lassa virus–infected cells (cut-off titer 16).
‡Fifty IFA assay–positive serum samples were randomly selected and tested by immunoblotting. Presence of African arenavirus–specific antibodies, as defined by reactivity with Lassa virus nucleoprotein and glycoprotein 2, was confirmed in 47 serum specimens.

of Mopeia virus that were circulating in Mozambique (*4*) and Zimbabwe (*5*). A close relationship between both viruses was also demonstrated by phylogenetic analysis using GPC, NP, and L gene sequences (Figure 1, panel D, and data not shown). Both viruses are sister taxa, sharing a common ancestor with Mobala virus.

Although the distances between Morogoro and Mopeia virus in the amino acid sequence of GPC (12%), NP (12%–13%), and L gene (26%) were higher than intraspecies differences among known African arenaviruses (i.e., pairwise differences between strains of the same species; <11% in GPC and NP; <21% in L), they did not reach the level of interspecies distances (>20% in GPC and NP; >37% in L) (Figure 1, panels A–C). Therefore, we currently consider Morogoro virus a subspecies of Mopeia virus rather than a new arenavirus species. This classification is supported by the fact that both viruses share the same host. Sequencing of the mitochondrial cytochrome b gene of rodent liver samples positive for Morogoro virus confirmed that its natural host is *M. natalensis* mice (GenBank accession nos. EU914105 and EU914106).

An additional 303 ethanol-preserved liver samples and 63 serum samples were collected in 2004 and 2007, respectively. Liver tissue (≈3 mg) was homogenized by using a bead mill. Cell debris was pelleted by centrifugation, and RNA was isolated from the homogenate with the RNeasy Mini kit (QIAGEN). Testing by L gene RT-PCR (*9*) showed 16 positive liver and serum samples, which indicated a virus prevalence in the *M. natalensis* population of ≈4% (Table 2). PCR fragments were sequenced (GenBank accession nos. EU914107–EU914122), and Morogoro virus was isolated in cell culture from all 4 PCR-positive serum samples obtained in 2007. Morogoro virus–specific antibodies in serum samples from 2004 and 2007 were measured by IFA assay using Vero cells infected with Morogoro virus. The antibody prevalence was ≈50%, which compares quite well with the 23% prevalence determined in this area 20 years before. In some animals, virus and antibodies were detected (Table 2).

The availability of Morogoro virus L gene sequences from 2004 and 2007, originating from the same host population (trapping sites <1 km apart), provided us with the

Table 2. Prevalence of Morogoro virus and Morogoro virus–specific antibodies in *Mastomys natalensis* mice from Morogoro University campus, Tanzania

Specimen and year of sampling	No. samples	No. (%) virus positive (PCR)	No. (%) antibody positive*	No. (%) antibody plus virus positive
Serum 2004	96	1 (1)†	42 (44)	0
Liver 2004	303	12 (4)†	–	–
Serum 2007	63	4 (6)‡	40 (63)	3 (5)§

*By immunofluorescent antibody (IFA) assay, performed with Morogoro virus-infected cells (cut-off 32).
†Testing was performed with universal Old World arenavirus large (L) gene reverse transcription–PCR (*9*).
‡Testing was performed with Morogoro virus–specific L gene RT-PCR using primers MoroL3359-forward (5′-AGGATTAGTGAGAGAGAGAGTAATTC-3′) and MoroL3753-reverse (5′-ACATCATTGGGCCCCACTTACTATGGTC-3′).
§Titers ranged from 64 to 512.

opportunity to estimate the molecular clock rate for this virus. Phylogenetic reconstruction was performed with the BEAST version 1.4.8 package (http://beast.bio.ed.ac.uk) (*11*) under the assumption of a relaxed lognormal molecular clock and general time reversible (GTR) or Hasegawa-Kishino-Yano (HKY) substitution model with gamma-distributed substitution rate variation among sites (Figure 2 and data not shown). Analysis was run for 2 million Markov chain Monte Carlo steps, which yielded a reliable set of data as verified with the TRACER program (http://tree.bio.ed.ac.uk/software/tracer). Based on GTR and HYK model, 3.2×10^{-3} and 3.4×10^{-3} substitutions per site and year (95% interval of highest posterior density $1.1–6.6 \times 10^{-3}$), respectively, were calculated.

Conclusions

A serologic survey in small mammals from Tanzania identified a hot spot of arenavirus circulation in Morogoro

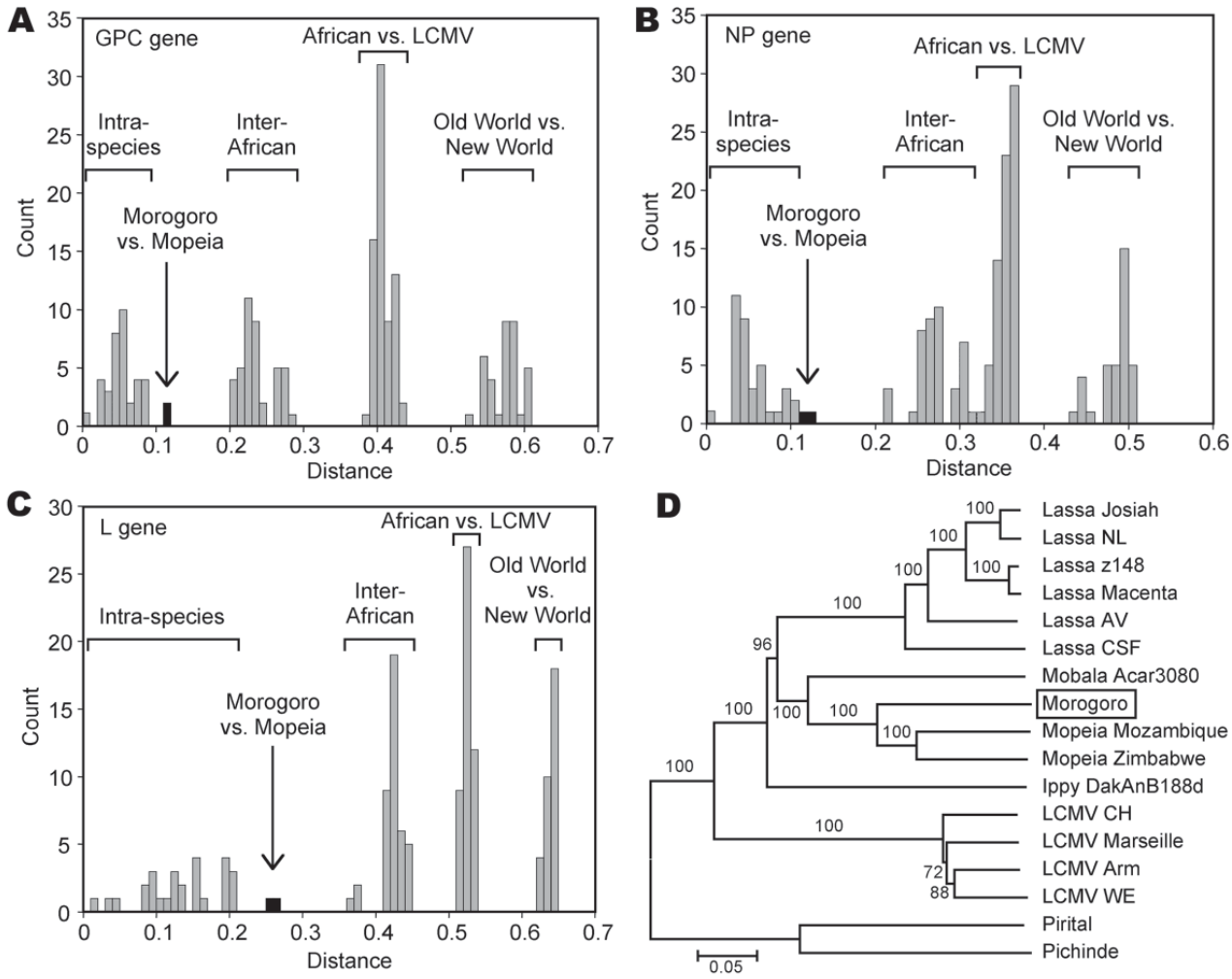

Figure 1. Genetic distances and phylogenetic relationship among arenaviruses, including Morogoro virus. Amino acid sequence diversity was calculated using p distance. Full-length glycoprotein precursor (GPC), nucleoprotein (NP), and large (L) gene amino acid sequences of the following arenaviruses were pairwise compared: Lassa virus (strains Josiah, NL, Z148, Macenta, AV, and CSF), Mobala Acar3080, Morogoro 3017/2004, Mopeia virus (strains Mozambique and Zimbabwe), Ippy DakAnB188d, lymphocytic choriomeningitis virus (LCMV) (strains CH-5692, Marseille, Armstrong, and WE for all genes; Traub and Pasteur for GPC and NP only), Pirital, and Pichinde. Frequency histograms of pairwise distances are shown for A) GPC gene; B) NP gene; and C) L gene. The ranges for intraspecies distances (i.e., pairwise differences between strains of the same virus species); distances between different African arenavirus species; between African arenaviruses and LCMV; and between Old World and New World viruses are marked above the bars. Bars representing the distances between Morogoro virus and the most closely related viruses (Mopeia virus strains) are filled in black. D) Phylogeny of Old World arenaviruses based on full-length L gene amino acid sequences. The tree was inferred by using the neighbor-joining method implemented in the MEGA software package (www.megasoftware.net). The New World arenaviruses Pirital and Pichinde were used as outgroups. Numbers represent bootstrap support (1,000 replications). Identical trees with respect to the phylogenetic position of Morogoro virus (shown in the box) were obtained with full-length GPC and NP amino acid sequences (not shown). Scale bar indicates nucleotide substitutions per site.

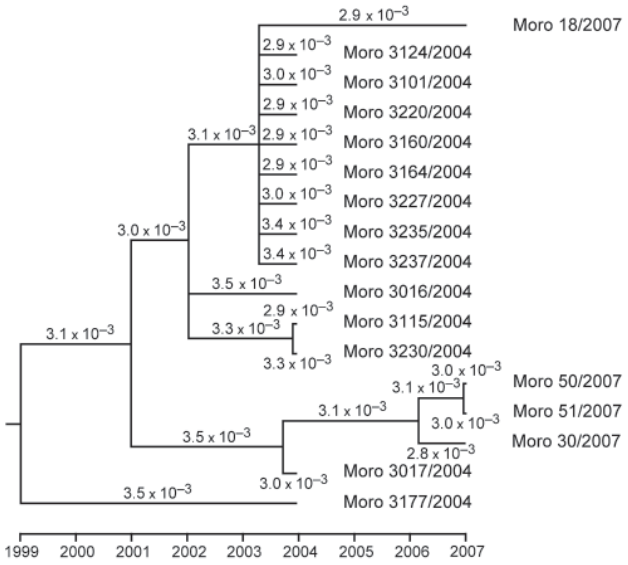

Figure 2. Phylogenetic tree and molecular clock of Morogoro virus based on partial large gene sequences of 17 strains (340 nucleotides; GenBank accession nos. EU914104 and EU914107–EU914122). Phylogeny was inferred with the BEAST v1.4.8 package (*11*) under assumption of a relaxed lognormal molecular clock and general time reversible substitution model with gamma-distributed substitution rate variation among sites. Branches with posterior probability <0.5 were collapsed. The substitution rate per site and year is indicated for each branch. Node ages and rates are median values. Variation in rates among branches is low as calculated with Tracer program (beast.bio.ed.ac.uk/Tracer) indicating a molecular clock in the evolution of Morogoro virus. The same tree topology with similar substitution rates was obtained when assuming the Hasegawa-Kishino-Yano substitution model (not shown).

in the late 1980s. This work is being published now because early attempts to substantiate the existence of the virus failed. The identification of the virus was facilitated by a recently developed pan–Old World arenavirus PCR (*9*) that also led to the discovery of new arenaviruses in rodents from West Africa (*7*). Only a small percentage of *M. natalensis* mice carry Morogoro virus, and a large proportion shows specific antibodies, which indicates that most animals clear the virus during life. Viruses and antibodies, which are presumbably directed to nucleocapsid proteins, also co-circulate, as seen in hantavirus infection in rodents (*12*). Detection of Morogoro virus in the liver is consistent with the organ tropism of Lassa virus in *M. natalensis* mice (*13*). In agreement with studies on Lassa virus strains, the largest genetic distance between Morogoro and Mopeia virus was seen in L gene, which contains several highly variable regions (*14*).

The clock rate estimate of 3×10^{-3} for Morogoro virus L gene is in agreement with that of other RNA viruses (*15*), although it must be interpreted with caution, given that the difference in date between the samples is not large. The tree topology did not correlate with geographic or ecologic sampling data.

The pathogenicity of Morogoro virus for humans is not known, though its phylogenetic clustering with African arenaviruses that are not linked with human disease (*4*–*6*) and the absence of hemorrhagic fever in the area suggest that it does not cause severe disease. Hospital-based investigations are required to estimate the public health relevance of this virus.

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Molecular Model of Prion Transmission to Humans

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To assess interspecies barriers to transmission of transmissible spongiform encephalopathies (TSEs), we investigated the ability of disease-associated prion proteins (PrP^d) to initiate conversion of the human normal cellular form of prion protein of the 3 major *PRNP* polymorphic variants in vitro. Protein misfolding cyclic amplification showed that conformation of PrP^d partly determines host susceptibility.

The agents responsible for the transmissible spongiform encephalopathies (TSEs) are called prions. Although their precise biochemical composition is a matter of debate, they are known to occur in a series of strains, each with a characteristic disease phenotype and host range (*1*). A central event in neuropathogenesis of TSEs is conversion of the normal cellular form of the prion protein (PrP^C) to the pathognomonic disease-associated isoform (PrP^d) (*2*). In the absence of a known nucleic acid genome, it has been proposed that the strain-like properties of different TSE agents are encoded by distinct self-propagating conformational variants (conformers) of PrP^d (*3*). The best developed method available for typing these PrP^d isoforms uses limited proteolysis and classification of the protease-resistant prion protein (PrP^{res}) in terms of the sizes of the nonglycosylated fragment(s) produced and the ratio of the 3 possible glycoforms (*3*). If distinct conformers and glycotypes of PrP^d are responsible for diversity of prion strains, then they would be expected to be able to impose these molecular characteristics onto PrP^C of the same amino acid sequence (when transmitted or replicating within a species) and onto PrP^C of a different primary sequence (when transmitted between species). In support of this theory, the agent responsible for the TSE of cattle, called bovine spongiform encephalopathy (BSE), the accepted cause of variant Creutzfeldt-Jakob disease (vCJD) in humans (*4*), has been shown to be transmissible to at least 7 species (*1*), resulting in propagation of PrP^d that retains the characteristic molecular signature of the original BSE prion strain (*5*–*7*).

Current thinking favors a seeded polymerization model for the conversion of PrP^C into PrP^d, which has led to the development of several cell-free in vitro conversion model systems (*8*). One such system is protein misfolding cyclic amplification (PMCA) (*9*), in which small amounts of PrP^d introduced (seeded) into substrate containing excess PrP^C and other essential conversion cofactors can be amplified to readily detectable levels by sequential cycles of sonication and incubation. We have previously reported that the molecular characteristics, electrophoretic mobility, and glycoform ratio of the PrP^{res} associated with the vCJD PrP^d conformer were faithfully reproduced by PMCA (*10*). However, the efficiency of amplification achieved depended on the substrate's prion protein gene codon 129 (*PRNP*-129) genotype. The most efficient amplification was achieved in a methionine homozygous (*PRNP*-129MM) substrate; the least efficient, in a valine homozygous (*PRNP*-129VV) substrate. To estimate the molecular component of transmission barriers for particular TSE agents between species, we used PMCA reactions to amplify PrP^d associated with vCJD (*10*), bovine BSE (*11*), ovine scrapie (*12*), and experimental ovine BSE (*13*) and substrates prepared from humanized transgenic mouse brain tissue expressing each of the 3 main *PRNP* polymorphic variants found in Caucasian human populations (*PRNP*-129MM, MV, and VV) (*14*).

The Study

We prepared seed and substrate homogenates as 10% (wt/vol) homogenates in PMCA conversion buffer (*10*). Seed homogenates were diluted into substrate homogenates so that all PMCA reactions contained equivalent amounts of PrP^d based on the PrP^{res} levels in each seed homogenate. PrP^{res} levels were determined by Western blot titration that used monoclonal antibody (MAb) 6H4 after limited proteinase K digestion. The reaction mixes were split into 2 aliquots; 1 aliquot was stored immediately at –80°C (−PMCA), and the other was subjected to 48 cycles of PMCA (+PMCA) (*10*). To assess the degree of PrP^d amplification achieved from each seed in each substrate, the samples −/+ PMCA were subjected to limited proteinase K digestion, and PrP^{res} was detected by Western blotting with MAb 6H4 (which recognizes human, bovine, and ovine PrP) and MAb 3F4 (which selectively recognizes only human PrP and would therefore specifically identify PrP^{res} formed from human PrP^C).

Using MAb 6H4 to probe Western blots, we noted amplification of vCJD, bovine BSE, and ovine BSE PrP^{res} in the *PRNP*-129MM substrate (Figure 1, panel A, top) but not in the *PRNP*-129VV substrate (Figure 1, panel A, bottom). Semiquantitative assessment of these Western

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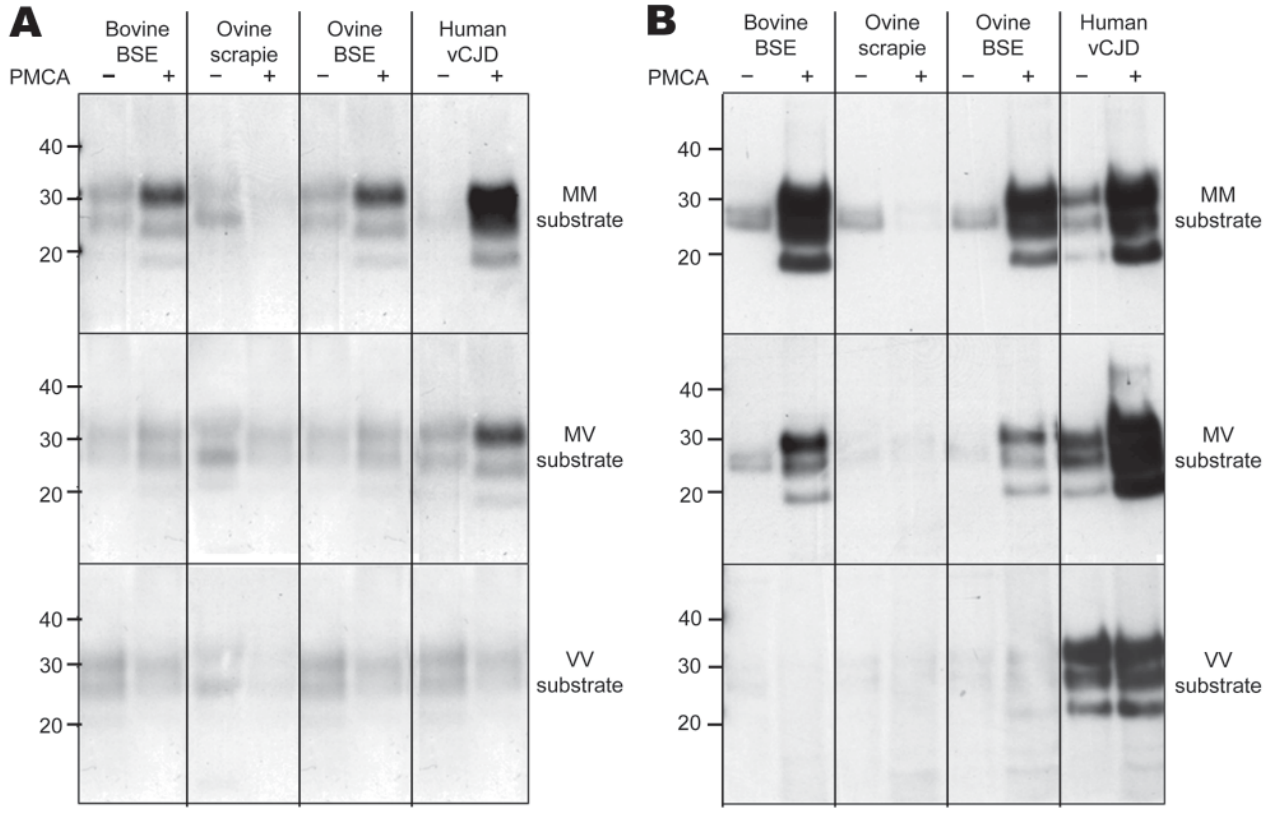

Figure 1. Amplification of PrP^{d} by PMCA from bovine BSE, ovine scrapie, experimental ovine BSE, and human vCJD brain homogenates in substrate homogenates prepared from humanized transgenic mouse brain tissue expressing PrP of each human prion protein gene codon 129 (*PRNP*-129) genotype. A) Amplification of each PrP^{d} type, as determined by Western blotting using MAb 6H4 to detect PrP^{res} after limited proteinase K digestion, in a *PRNP*-129MM substrate (top panel, 3-min exposure), a *PRNP*-129MV substrate (middle panel, 3-min exposure), and a *PRNP*-129VV substrate (bottom panel, 3-min exposure). B) Amplification of each PrP^{d} type, as determined by Western blotting using MAb 3F4 to detect PrP^{res} derived from human PrP after limited proteinase K digestion, in a *PRNP*-129MM substrate (top panel, 30-s exposure), a *PRNP*-129MV substrate (middle panel, 3-min exposure), and a *PRNP*-129VV substrate (bottom panel, 10-min exposure). Limited proteinase K digestion and Western blotting were conducted out as previously described (*11*). MAb 6H4 (Prionics, Schlieren-Zurich, Switzerland) and MAb 3F4 (Dako, Ely, Cambridgeshire, UK) were used at a final concentration of 50 ng/mL. PrP^{d}, disease-associated prion protein; PMCA, protein misfolding cyclic amplification; BSE, bovine spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease; MAb, monoclonal antibody; PrP^{res}, protease-resistant prion protein; MM, methionine homozygous; MV, methionine/valine heterozygous; VV, valine homozygous. Values are in kilodaltons.

blots by densitometry showed that the degree of amplification of vCJD PrP^{res} was considerably greater than that of bovine or ovine BSE in the *PRNP*-129MM substrate (Figure 2, panel A). A more sensitive and discriminatory Western blot conducted by using MAb 3F4 confirmed efficient amplification of vCJD, bovine BSE, and ovine BSE PrP^{res} in the *PRNP*-129MM substrate (Figure 1, panel B, top), weaker amplification in the *PRNP*-129MV substrate (Figure 1, panel B, middle), and little, if any, amplification in the *PRNP*-129VV substrate (Figure 1, panel B, bottom). In all substrates, the amplified PrP^{res} retained the electrophoretic mobility and glycoform ratio associated with BSE-related PrP^{res}. No amplification of ovine scrapie PrP^{res} was evident after PMCA in any of the *PRNP* humanized transgenic mouse brain substrates (Figure 1, panels A, B). The difference between ovine scrapie and ovine BSE in ability to seed amplification in *PRNP*-129MM substrate was a robust phenomenon evident in brain samples from 3 different ARQ/ARQ sheep with each disease (Figure 2, panel B). However, failure of the ovine scrapie seed to amplify was not caused by a general lack of competence to do so or by inappropriate amplification conditions because robust amplification of ovine scrapie PrP^{res} was evident after PMCA in a substrate prepared from normal ARQ/ARQ sheep brain (Figure 2, panel C).

Conclusions

Our results are best appreciated in terms of the molecular interaction between seed PrP^{d} and substrate PrP^{C}, specifically the species-specific amino acid sequence and

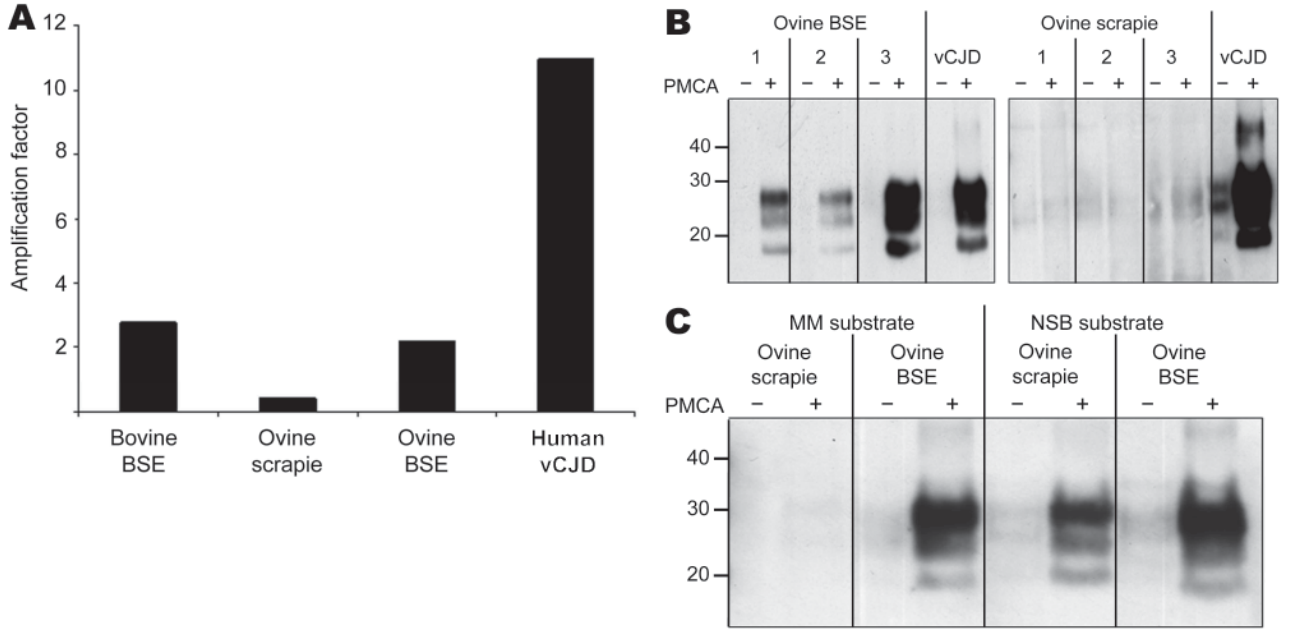

Figure 2. A) Semiquantitative densitometric analysis (optical density × area in mm^2) of Western blot data (Figure 1, panel A, top panel), showing the amplification factors (+PMCA/−PMCA) obtained for all 4 seeds (bovine BSE, ovine scrapie, ovine BSE, and human vCJD in the *PRNP*-129MM substrate. B) Amplification of PrP^d associated with ovine BSE (left) and ovine scrapie (right) from each of 3 different sheep in *PRNP*-129MM substrate as determined by Western blotting using MAb 3F4 to detect PrP^{res} after limited proteinase K digestion. Substrate was seeded with brain homogenates prepared from sheep with confirmed scrapie and BSE such that each PMCA reaction mix contained an equivalent amount of PrP^d according to detection of PrP^{res} by Western blot titration after limited proteinase K digestion. *PRNP*-129MM substrate seeded with vCJD brain homogenate was included as a positive control in each experiment. C) Amplification of PrP^d associated with ovine scrapie and BSE in substrates prepared from *PRNP*-129 methionine homozygous humanized transgenic mouse brain tissue (MM substrate) and NSB substrate. Substrates were prepared as 10% (wt/vol) homogenates in PMCA conversion buffer (*10*). Each substrate was seeded with brain homogenates prepared from sheep with confirmed scrapie and BSE so that each PMCA reaction mix contained an equivalent amount of PrP^d as determined by detection of PrP^{res} by Western blot titration after limited proteinase K digestion. Reaction mixes were divided into 2 lots: 1 was stored immediately at –80°C (−PMCA) and the other was subjected to 48 cycles of PMCA (+PMCA) by using standard conditions (*10*). After limited proteinase K digestion, PrP^{res} in samples −/+PMCA was detected by Western blotting using MAb 6H4. PMCA, protein misfolding cyclic amplification; BSE, bovine spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease; MM, methionine homozygous; PrP^d, disease-associated prion protein; MAb, monoclonal antibody; PrP^{res}, protease-resistant prion protein; NSB, normal ARQ/ARQ sheep brain tissue. Values on the left in panels B and C are in kilodaltons.

PRNP polymorphic status of PrP^C and PrP^d and the PrP^d conformers involved (Table). Regardless of the seed PrP amino acid sequence, the PrP^d conformers associated with bovine BSE, ovine BSE, and human vCJD were amplified in the humanized mouse substrate and displayed similar *PRNP*-129 genotype preferences (*PRNP*-129MM >*PRNP*-129MV >*PRNP*-129VV). In contrast, the PrP^d conformer associated with the ovine scrapie strain, although sharing the same PrP amino acid sequence as the PrP^d in ovine BSE, could not be amplified in any of the *PRNP* humanized mouse substrates but could be amplified in a sheep brain substrate. These observations are consistent with conformation of a TSE agent's PrP^d (rather than solely its amino acid sequence) having a role in determining the susceptibility of a host's PrP^C to conversion. They similarly suggest that these molecular factors could in turn have a powerful influence on disease susceptibility and incubation time.

To date, all clinical cases of vCJD have occurred in persons with the *PRNP*-129MM genotype, as might be predicted from the efficiency of amplification of BSE-related PrP^d shown here. Extrapolating from these results, one would predict that the next genotypic group most likely to show susceptibility to the BSE agent would be heterozygous (MV) at codon 129 of the *PRNP* gene, as previously suggested from the corresponding in vivo transmission studies (*14*).

In the wake of BSE epidemics in the United Kingdom and elsewhere, enhanced surveillance has identified apparently new TSEs (*15*), raising concerns regarding animal and human health. PMCA with suitable substrate sources could provide a rapid way to estimate the molecular component of transmission barriers for particular TSE agents between species, including humans. These estimates could thus indicate whether, like classical scrapie, the agents rep-

Table. Summary of the properties of the sources used in PMCA of vCJD, bovine BSE, ovine scrapie, and experimental ovine BSE PrP^{res}*

Seed homogenate	Species	Bovine†	Human‡	Ovine§	Ovine§
	Disease	BSE	vCJD	BSE	Scrapie
	Tissue	Brain	Brain	Brain	Brain
	PRNP amino acid sequence	Bovine	Human	Ovine	Ovine
	PRNP polymorphism	140MM	129MM	ARQ/ARQ (132MM)	ARQ/ARQ (132MM)
	PrP^d "conformer"	BSE	BSE	BSE	Scrapie
Substrate homogenate¶	Species	Mouse	Mouse	Mouse	Mouse
	Tissue	Brain	Brain	Brain	Brain
	PrP amino acid sequence	Human	Human	Human	Human
	PRNP-129 polymorphism	MM, MV, and VV	MM, MV, and VV	MM, MV, and VV	MM, MV, and VV
	Background genotype	129 Ola $prnp^{-/-}$	129 Ola $prnp^{-/-}$	129 Ola $prnp^{-/-}$	129 Ola $prnp^{-/-}$

*PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease; BSE, bovine spongiform encephalopathy; PrP^{res}, protease-resistant prion protein; PrP^d, disease-associated prion protein; MM, methionine homozygous; MV, methionine/valine heterozygous; VV, valine homozygous.
†Bovine brain tissue was sampled from brain tissue taken from a Friesian cow with terminal BSE (*11*).
‡Human brain tissue (frontal cortex) was sampled from a frozen half brain that had been collected at autopsy with the appropriate consent for tissue retention and research use from a patient methionine homozygous at *PRNP* codon 129, who received a final diagnosis of definite vCJD by established criteria. Ethical approval for its use in this study was covered by LREC 2000/4/157 (J.W.I.).
§Both the ovine scrapie (*12*) and ovine BSE (*13*) brain tissue (hind brain) were sampled from clinically sick sheep. The distinctive disease phenotypes were confirmed by histopathologic, immunohistochemical, and Western blot characteristics.
¶Frozen half brains from inbred transgenic mouse lines expressing human PrP of the 3 major *PRNP* codon-129 genotypes (MM, MV, VV) were used to prepare substrate homogenates. These mice had identical genetic backgrounds, were produced to express human PrP by direct replacement of the murine PrP gene, and all expressed equivalent amounts of human PrP regardless of the *PRNP*-129 genotype (*14*). The transgenic mice were bred under license to the UK Home Office in accordance with the UK Animals (Scientific Procedures) Act of 1986, and the use of brain tissue from these mice was reviewed and approved by the local Ethics Review Committee.

resent little risk for human health or whether, like classical BSE, they represent cause for concern.

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Dr Jones is a postdoctoral research fellow at the National CJD Surveillance Unit, University of Edinburgh. His primary research interests are the application of in vitro PrP^d amplification techniques, such as PMCA, to prion disease research in general and incorporation of these techniques into a confirmatory screening assay to detect vCJD-associated PrP^d in human plasma as a surrogate marker of vCJD infectivity in blood.

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Dobrava-Belgrade Virus Spillover Infections, Germany

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We present the molecular identification of *Apodemus agrarius* (striped field mouse) as reservoir host of the Dobrava-Belgrade virus (DOBV) lineage DOBV-Aa in 3 federal states of Germany. Phylogenetic analyses provided evidence for multiple spillover of DOBV-Aa to *A. flavicollis,* a crucial prerequisite for host switch and genetic reassortment.

European hantaviruses are emerging viruses that can cause hemorrhagic fever with renal syndrome (HFRS) of differing severities. Dobrava-Belgrade virus (DOBV) is a hantavirus that appears in 3 distinct lineages hosted by different *Apodemus* species. The DOBV-Af lineage associated with the yellow-necked mouse (*A. flavicollis*) has caused serious HFRS in southeast Europe with a case-fatality rate ≤12% (*1*,*2*). Human infections with Caucasian wood mouse (*A. ponticus*)–associated DOBV-Ap have resulted in more moderate than severe HFRS in the southern part of European Russia (*3*). Mild-to-moderate human DOBV disease in central and eastern Europe has been connected with infection by DOBV-Aa lineage carried by the striped field mouse (*A. agrarius*) (*3*–*5*). Other *A. agarius*–associated strains, found in Estonia and called Saaremaa virus, have been proposed to form a distinct hantavirus species (*6*). In Germany, human DOBV cases with mild to moderate clinical outcomes have been detected by serologic investigations (*4*,*7*) but only 1 short DOBV-Aa small (S) segment sequence derived from a patient in northern Germany has been identified (*8*). The natural host and the geographic distribution of DOBV in its reservoir host has remained unknown in Germany.

The Study

During 2002 through 2008, a total of 366 *Apodemus* mice were trapped at 7 different sites in Germany (Figure 1). Serologic screening of transudates collected from these rodents by using an in-house DOBV immunoglobulin (Ig) G-ELISA, with a yeast-expressed nucleocapsid protein of DOBV-Af as antigen, identified 16 reactive and 5 equivocal samples of 114 *A. agrarius* trapped at 7 trapping sites in 3 federal states of Germany (Figure 1; online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2017-Techapp.pdf). Additionally, of 237 *A. flavicollis* mice, 1 equivocal sample and 4 DOBV-reactive samples were detected at 4 trapping sites (Figure 1; online Technical Appendix). In contrast, of 15 wood mice (*A. sylvaticus*) originating from 3 trapping sites, none were found to be DOBV-seroreactive. A subsequent focus-reduction neutralization test showed a higher endpoint titer with DOBV-Aa than DOBV-Af (online Technical Appendix) for 6 of the 8 investigated transudates independently, whether originating from *A. agrarius* or *A. flavicollis*.

An initial screening by a large (L) segment–specific nested reverse transcription–PCR (RT-PCR) (*3*) of 67 lung samples, representing all seroreactive (n = 20) and equivocal (n = 6) as well as 36 selected seronegative and 5 serologically not-analyzed animals, showed a 390-nt amplification product for 21 samples representing 16 seroreactive, 4 seronegative, and 1 serologically not-investigated animals (online Technical Appendix). To enable a comparison with the only available DOBV sequence from Germany (H169), an S segment portion of 559 nt was amplified by RT-PCR from 11 lung tissues (online Technical Appendix). In the phylogenetic analyses, all sequences from Germany formed 1 well-supported (PUZZLE [www.tree-puzzle.de]) and bootstrap support values >90%) monophyletic group consisting of 2 clusters. The first cluster contained S segment sequences from district Güstrow (trapping sites Pe1 and Pe3), Lüneburg (trapping site WG), Nordvorpommern (trapping site H), and the previously published DOBV sequence from an HFRS patient from northern Germany (H169; [*8*]; Figure 2, panel A). A second cluster was formed by S segment sequences originating from districts Ostprignitz-Ruppin (trapping sites Ka, To) and Demmin (trapping site K/A1). Notably, the *A. flavicollis*–derived sequences from sites Pe1, H, Ka, and K/A1 clustered together or were identical with *A. agrarius*–derived sequences from the same or neighboring trapping sites, suggesting multiple

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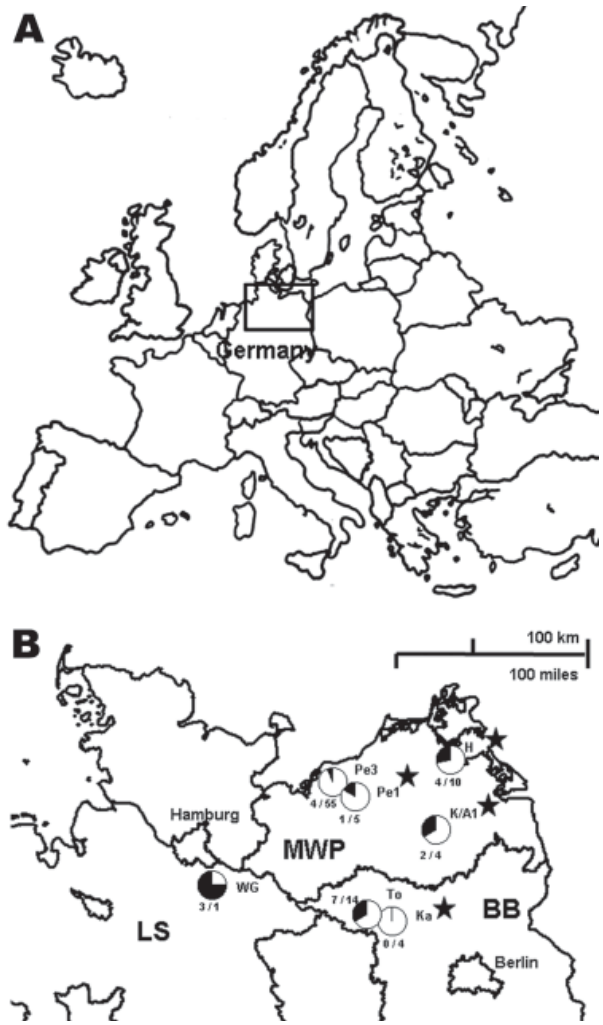

Figure 1. Seroprevalence of Dobrava-Belgrade virus (DOBV) in *Apodemus agrarius* mice within 3 federal states of Germany, central Europe. A) Location of the study area (box). B) Locations of the study sites. WG, Lüneburg district, Lower Saxony (LS); Pe1 and Pe3, Güstrow district; H, Nordvorpommern district, K/A1, Demmin district, all Mecklenburg-Western Pomerania (MWP); To and Ka, Ostprignitz-Ruppin district, Brandenburg (BB). For each trapping site, the rate of seroreactive *A. agrarius* mice is given as a circle (seroreactive and equivocal samples in black, negative samples in white) and with the numbers of seroreactive and equivocal samples/negative samples. At sites Pe1, H, K/A1, and Ka, 1 seroreactive *A. flavicollis* mouse was detected in each location (marked with stars). At site Pe1, 1 equivocal sample was found by the DOBV immunoglobulin (Ig) G ELISA. In addition to *Apodemus* mice, 138 rodents of other species, including 116 bank voles (*Myodes glareolus*), were trapped during the same period at sites Pe1, Pe3, K/A1, Ka, and To, but none of the 136 rodents with available transudates reacted in the DOBV IgG ELISA. Transudates with an optical density (OD) value below the lower cutoff (average of the OD values determined for 2 parallel tests of a negative *Apodemus* spp. serum control; average 0.041) were regarded as negative. Samples with an OD value above the upper cutoff (2-fold of the lower cutoff; average 0.082) were regarded as positive. Samples showing OD values between the lower and upper cutoffs were regarded as equivocal.

spillover infections (Figure 2, panel A; online Technical Appendix).

The sequences from Germany share a common ancestor with the DOBV-Aa sequences originating from Slovakia and Russia. Together, they form a monophyletic group (DOBV-Aa lineage) that is clearly separated from *A. flavicollis*–borne (DOBV-Af) and *A. ponticus*–borne (DOBV-Ap) sequences and from *A. agrarius*–borne Saaremaa virus sequences. Subsequent analysis of nucleotide sequences of the entire nucleocapsid (N) protein– and glycoprotein precursor (GPC)–encoding regions confirmed these findings (Figure 2, panel B; online Technical Appendix). A pairwise comparison between nucleotide and amino acid sequences of the complete N and GPC open reading frames from the novel German DOBV strains showed divergences of 1.5%–8.8% (0.3%–1.4%) and 2.1%–8.3% (0.8%–1.8%), respectively (online Technical Appendix Table 2). The highest identity values on the nucleotide and amino acid sequence level (91.2%–91.7% and 99%–99.7%) were found for an S segment sequence from Denmark (Lolland/1403; GenBank accession no. AJ616854; online Technical Appendix). The nucleotide and amino acid sequence divergence to other DOBV sequences was much higher, reaching 10.1%–14.3% (1%–3.3%) and 12.6%–20.7% (2.9%–9.4%), respectively.

Morphologic species determination for all DOBV-seroreactive and RT-PCR–positive rodents was confirmed by a mitochondrial *cytochrome b* gene-specific PCR (*9*,*10*), sequence determination, and comparison with available GenBank sequences from *A. agrarius* and *A. flavicollis* (online Technical Appendix).

Conclusions

Based on a large panel of the entire N- and GPC-encoding DOBV sequences, we report direct molecular evidence that DOBV in Germany is represented by a genetic lineage associated with *A. agrarius* (DOBV-Aa). In contrast, we found no evidence for the occurrence of DOBV-Af in *A. flavicollis* or other *Apodemus* species from Germany. Consistent with the geographic distribution of *A. agrarius* (*11*) and the report of human DOBV disease exclusively in northern and northeastern Germany, this finding may confirm DOBV-Aa as the sole causative agent of DOBV infections in Germany (*4*; Robert Koch-Institut, SurvStat, www.rki.de).

Previously *A. agrarius*–associated Saaremaa virus was experimentally shown to be able to infect *A. agrarius* and *A. flavicollis* mice (*12*). We report multiple natural spillover infections of *A. flavicollis* by a DOBV strain originally hosted by *A. agrarius*. The observed spillover infections represent a crucial prerequisite for genetic reassortment. This observation is in contrast to other reports from Slovenia and Slovakia where, although *A. agrarius* and *A. flavicollis* are occurring sympatrically, *A. flavicollis*

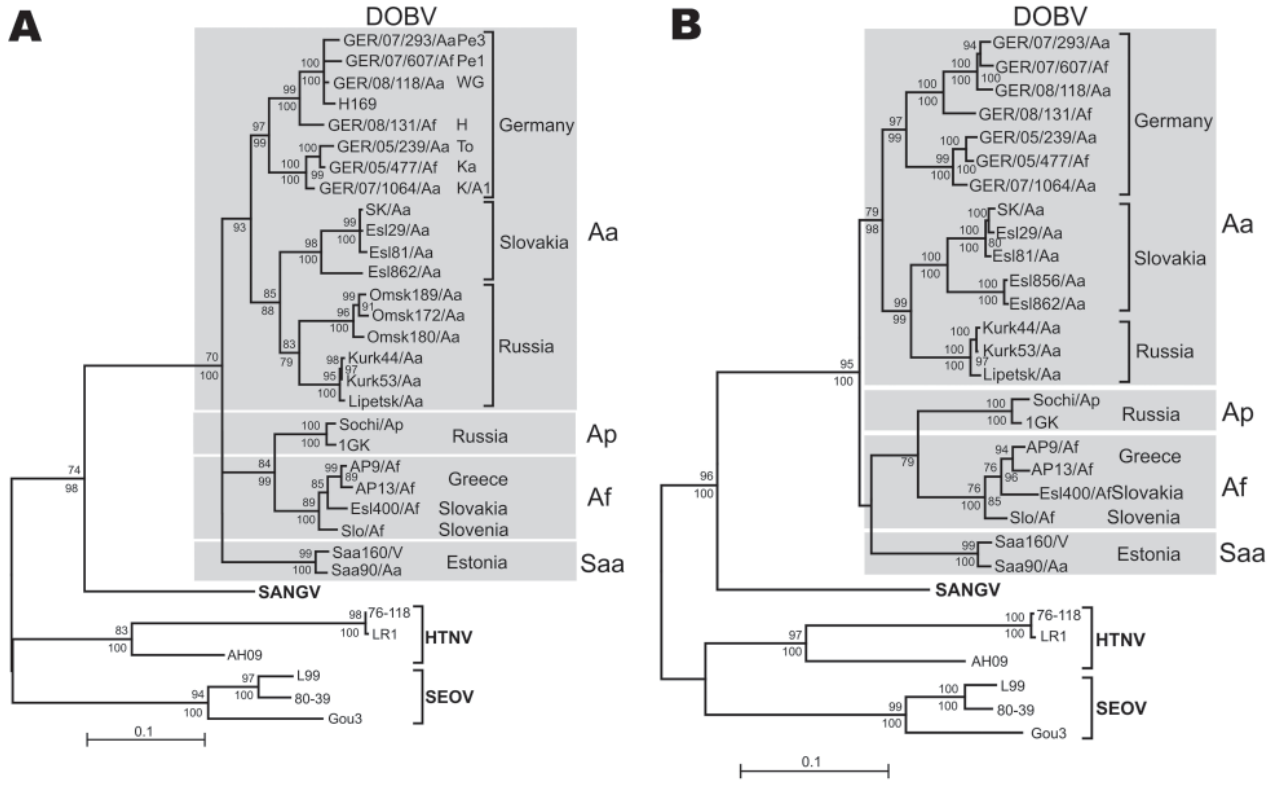

Figure 2. Maximum-likelihood (ML) phylogenetic trees of Dobrava-Belgrade virus (DOBV) based on partial small (S) segment nucleotide sequences of 559 nt (position 377–935) (A) and complete nucleocapsid protein coding nucleotide sequences (S segment open reading frame) (B). The ML trees (Tamura-Nei evolutionary model) were calculated using TREE-PUZZLE package (www.tree-puzzle.de). Scale bars indicate an evolutionary distance of 0.1 substitutions per position in the sequence. Values above the branches represent PUZZLE support values. Values below the branches are bootstrap values of the corresponding neighbor-joining tree (Tamura-Nei evolutionary model) calculated with the PAUP* software (paup.csit.fsu.edu) from 10,000 bootstrap pseudoreplicates. Only values ≥70% (considered significant) are shown. Different DOBV lineages are indicated by gray boxes. SANGV, Sangassou virus; HTNV, Hantaan virus; SEOV, Seoul virus; Saa, Saaremaa virus; Aa, *Apodemus agrarius*; Ap, *A. ponticus*; Af, *A. flavicollis*. WG, district Lüneburg, Lower Saxony (LS); Pe1 and Pe3, district Güstrow; H, district Nordvorpommern, K/A1, district Demmin, all Mecklenburg-Western Pomerania (MWP); To and Ka, district Ostprignitz-Ruppin, Brandenburg (BB). Before tree construction, automated screening for recombination between the S segment sequences was performed using program RDP3 (*15*), which used 6 recombination detection programs: Bootscan, Chimeric, GENECONV, MaxChi, RDP, and SiScan with their default parameters. No putative recombinant regions could be conclusively detected by ≥3 programs and subsequently verified by phylogenetic trees.

is exclusively carrying the DOBV-Af and *A. agrarius* the DOBV-Aa lineage (*4*,*13*). In contrast to our observations, single DOBV-Af spillover infections of *A. sylvaticus* and *Mus musculus* have been reported previously (*14*).

The phylogenetic analyses demonstrated 2 well-separated clusters within the DOBV-Aa lineage. These rodent-derived DOBV sequences in Germany represent a major contribution to the DOBV genomics and phylogenetics. Future investigations should help to identify specific features of these DOBV-Aa strains resulting in its frequent spillover to *A. flavicollis* and to prove a putative adaptation of DOBV-Aa on *A. flavicollis* after spillover, as well as possible reassortment processes.

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Wild Felids as Hosts for Human Plague, Western United States

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Plague seroprevalence was estimated in populations of pumas and bobcats in the western United States. High levels of exposure in plague-endemic regions indicate the need to consider the ecology and pathobiology of plague in nondomestic felid hosts to better understand the role of these species in disease persistence and transmission.

Zoonotic pathogens account for ≈60% of emerging diseases (*1*,*2*). *Yersinia pestis*, a vector-borne bacterium and the causative agent of plague in mammals, is 1 such emergent pathogen (*3*). Plague is maintained among rodent hosts and their fleas; however, spillover into accidental hosts can result in severe illness and death, as well as geographic spread of the disease (*4*).

Domestic cats are a major source of human plague infections in the United States (*5*), putting veterinary workers and pet owners at risk for *Y. pestis* infections. During 1924–2006, a total of 13 human cases of primary pneumonic plague were documented in the United States, and ≥5 were associated with felids (D. Wong, pers. comm.). Twelve cases of plague transmission from nondomestic carnivores to humans have been documented (*5*–*7*), including a fatal case of human pneumonic plague in 2007 that resulted from direct contact with an infected puma (*Puma concolor*) (*8*). Despite the known association of felids with human plague, the prevalence of *Y. pestis* infection in nondomestic cats remains relatively unknown.

Pumas and bobcats (*Lynx rufus*) are 2 of the most widespread felids in North American, with pumas having the greatest range of any wild terrestrial mammal in the Western Hemisphere (*9*). Both species inhabit large territories and travel great distances during dispersal (*9*,*10*). These highly mobile animals may periodically reintroduce *Y. pestis*–positive fleas to distant regions, especially during epizootics (*11*). Consequently, carnivore-aided flea dispersal could play an important role in the spread and persistence of plague during interepizootic periods.

We examined plague exposure in populations of bobcats and pumas in California and Colorado. This gave us an opportunity to evaluate *Y. pestis* seroprevalence in multiple difficult-to-sample, plague-susceptible felid species across a wide geographic area.

The Study

We collected samples from 119 pumas and 212 bobcats (Table 1) in 3 locations in southern California and 2 locations in western and north-central Colorado (Figure) from autumn 2002 through summer 2008. Seventy-seven of these bobcat samples consisted of thoracic fluid collected postmortem from hunter-killed animals. Eight puma samples collected in the 1980s served as historical reference for puma samples from the Colorado Western Slope (i.e., area west of the Continental Divide). Animals were captured, sampled, and released with permission of cooperating agencies after approval by animal care and use committees. Samples were processed according to protocol (*12*).

Thoracic fluid samples were immunoblotted onto nitrocellulose membranes (immuno-blot polyvinylidene fluoride membranes; Bio-Rad, Hercules, CA, USA) and probed with goat-anti-cat-phosphatase labeled antibody to verify the presence of immunoglobulin. Reacted membranes were rinsed 3 times with phosphate-buffered saline, once in Milli-Q (Millipore, Billerica, MA, USA) and were then exposed to a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (alkaline-phosphatase chromogen) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Samples were classified by comparing staining intensity to positive (bobcat/domestic cat serum) and negative controls (water and goat serum).

Serum and thoracic fluid samples were analyzed for *Y. pestis* antibody using a hemagglutination assay according to a standard protocol (*13*). Positive samples were evaluated according to Chu (*13*). If a limited amount of sample was available, serum was diluted 1:4 and considered posi-

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Table 1. Sample sizes for categorical variables, by location, in serosurvey for *Yersinia pestis* in wild felids, western United States, 2002–2008*

Category	Front Range, CO	Orange County, CA	San Diego/Riverside counties, CA	Ventura County, CA	Western Slope, CO	Mean seroprevalence (95% confidence interval)
Species						
Bobcat	0	73	0	61	78	13.77 (4.90–33.07)
Puma	33	5	38	4	36	8.17 (2.97–20.56)
Age						
Young (< 2 y)	5	23	5	29	45	6.70 (2.31–17.87)
Adult (≥2 y)	27	49	27	31	68	16.52 (7.03–34.09)
Sex						
F	20	37	20	32	43	14.01 (5.65–30.70)
M	13	40	18	29	70	8.02 (3.01–19.69)
Season						
Fall	3	18	6	15	21	0
Spring	13	7	18	1	2	23.67 (11.27–43.09)
Summer	6	6	2	3	90	7.49 (0.79–45.06)
Winter	10	47	12	42	112	6.31 (2.74–13.88)

*All samples were serum samples, except for Western Slope bobcats, which were thoracic fluid samples.

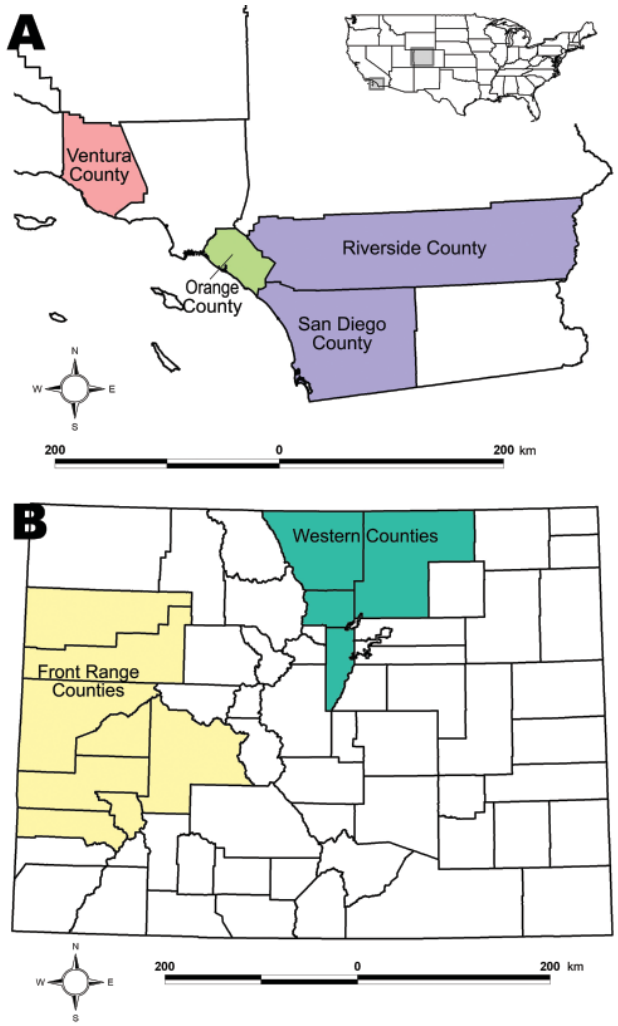

Figure. A) Study locations in California. B) Study locations in Colorado. Inset shows relative locations within the United States.

tive if titers were >32. Larger serum samples were not diluted, and a reading ≥16 was considered positive (*13*).

Data were analyzed by using a logistic link function and binary error, with antibody presence (positive vs. negative) as the outcome variable (SAS version 9.1; SAS, Cary, NC, USA). Estimates used maximum likelihood. Degrees of freedom were calculated by using a Kenward-Roger adjustment. Categorical factors included location, species, age, sex, and capture season. Animals captured in the fall (September–November) and in Ventura County were not plague positive and were omitted. All factors were treated as fixed variables, including location, because of previously reported differences in regional seroprevalence rates.

A total of 76 of 77 thoracic fluid samples had immunoglobulin present, as assessed by visual comparison of immunoblot staining, and were included in *Y. pestis* antibody analysis. Interactions were not significant and were omitted. Mean *Y. pestis* seroprevalence for pumas and bobcats across all locations was 17.7% (95% confidence interval [CI] 13.6%–21.8%). However, considerable variability existed across locations (Front Range, Colorado, mean = 21.1 [95% CI 8.23–44.75]; Orange County, California, mean = 1.23 [95% CI 0.13–10.01]; San Diego/Riverside counties, California, mean = 6.58 [95% CI 1.52–24.33]; Ventura County, California, mean = 0 [NA]; Western Slope, Colorado, mean = 46.03 [95% CI 24.37–69.29]). Species and sex were not significant predictors of plague exposure; however, animal age, geographic location, and capture season were significant (Table 2). Adult animals (≥2 years of age) and animals from the Colorado Western Slope were more likely to be seropositive (Table 1). Sixty-three percent (5/8) of historical puma samples from the Western Slope had detectable plague antibodies, similar to the seroprevalence rate of contemporary puma samples from this region (46.03%). Season also played a

Table 2. Potential fixed-effect predictors of plague exposure in pumas and bobcats, western United States, 2002–2008*

Fixed effect	Num df	Den df	F value	p value
Age	1	287	5.13	**0.024**
Location	4	287	8.36	**<0.0001**
Season	3	287	4.1	**0.0179**
Sex	1	287	2.47	0.117
Species	1	287	1.02	0.314

*Num df, numerator degrees of freedom; den df, denominator degrees of freedom. **Boldface** indicates significance ($p \leq 0.05$).

role, and spring-captured animals were more likely to be seropositive (Tables 1 and 2).

Colorado sample sites showed 51 (38%) positive of 135 animals tested. Seroprevalence rates in the Colorado sample areas were 21% (Front Range) and 46% (Western Slope) respectively, a higher proportion than expected given the severe disease seen in plague infections in some domestic cats (*3*). California sample sites had limited plague seroreactivity, with only 4 (2.2%) of 181 animals positive for plague exposure.

The Colorado Western Slope is near the Four Corners region (i.e., contiguous boundaries of southwestern Colorado, northwestern New Mexico, northeastern Arizona, and southeastern Utah). During 1957–2004, a total of 419 human plague cases were documented in the United States, of which 83% were from this region (*14*). The complex dynamics governing high plague incidence in this region are not fully understood despite extensive research but most likely involve climate, mammalian reservoirs, vector species, and habitat ecotypes (*4*,*7*,*14*).

Conclusions

Plague dynamics often are characterized by epizootics, resulting in interannual variation in infection rates among plague hosts; however, seroprevalence of 8 puma samples collected in the 1980s mirrored contemporary samples collected since 2002 and may indicate high levels of sustained plague activity in the area in this species. Seroprevalence rates were similar across multiple sample years. Vector-borne disease often is highly seasonal because of annual shifts in vector activities and abundance (*4*); however, seasonal patterns based on serologic data must be interpreted with caution because of long-term antibody persistence in some recaptured animals (S.N. Bevins, unpub. data).

Puma and bobcat data from this study suggest exposure followed by recovery. All animals were outwardly healthy. Deaths caused by plague have been documented in wild felids (*8*,*9*,*15*), and the potential for plague exposure remains a concern for field biologists, veterinarians, hunters, and skinners. Field biosafety guidelines have been developed in conjunction with Colorado State University's Biosafety Office as a result of these findings. Recommendations include wearing disposable gloves, long pants, and long-sleeved shirts when handling anesthetized animals and using an N95-rated mask when conducting necropsies or handling deceased animals. Outside of human infections, plague could constitute a problem for felid conservation in areas of high plague activity (*1*,*15*).

Results suggest large numbers of *Y. pestis*–exposed pumas and bobcats. Regular serosurveys that document seroreactivity increases above an original baseline could indicate epizootic activity in felids and other plague hosts. High regional seroprevalence indicate these animals may be involved in the persistence and transmission of *Y. pestis*. This and the documented transmission of plague from nondomestic carnivores to humans (*6*–*8*) emphasize the need to better understand the role of wild felids in plague dynamics.

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Transplacental Transmission of Bluetongue Virus 8 in Cattle, UK

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To determine whether transplacental transmission could explain overwintering of bluetongue virus in the United Kingdom, we studied calves born to dams naturally infected during pregnancy in 2007–08. Approximately 33% were infected transplacentally; some had compromised health. In all infected calves, viral load decreased after birth; no evidence of persistent infection was found.

Bluetongue virus (BTV) is generally transmitted between ruminant hosts by *Culicoides* biting midges, and infection may result in the disease called bluetongue. In 2006, a strain of BTV-8 caused the first outbreak of bluetongue in northern Europe (*1*). Although adult *Culicoides* midges are absent from this region during winter for long enough to interrupt normal transmission, BTV-8 survived the winters of 2006–07 and 2007–08.

Several mechanisms have been suggested to explain the overwintering of BTV, one of which is transplacental transmission (*2*). Tissue-attenuated strains of BTV are sometimes capable of crossing the placenta and infecting fetuses in utero (*3*), and transplacental infection has been reported from the field after use of live attenuated vaccines (*4*). However, many wild-type strains of BTV failed to cross the placental barrier when cows were infected during pregnancy (*5*). Additionally, although a few studies have reported experimental transplacental infection with wild-type strains, these studies did not recover infectious virus from live offspring (although many field strains do not grow in tissue culture) and suggested that fetal infection often resulted in deformation, stillbirth, or abortion (*6*,*7*). Collectively, this information led to the assumption that only viruses passaged in tissue culture had the potential to overwinter by transplacental transmission (*8*). However, in 2008, nonlethal transplacental transmission of BTV-8 was detected in Northern Ireland (*9*). To examine the occurrence, rate, and consequences of transplacental BTV-8 transmission in the United Kingdom, we studied calves born to dams naturally infected with BTV-8 during pregnancy.

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The Study

After obtaining owners' permission, we sampled calves born to previously infected dams during the vector-free period of December 20, 2007 to March 15, 2008. Farmers were also asked to report any births, abortions, or stillbirths from BTV-infected dams outside the vector-free period. Blood samples from live calves were taken as soon as possible after birth (usually within 4 days) and tested by using a real-time reverse transcription–PCR (rRT-PCR) (*10*) and the Pourquier c-ELISA kit (IDEXX, Chalfont St. Peter, UK). When possible, information about the health of the calf was obtained, dams were sampled alongside their calves, and placenta samples were collected. Calves with positive BTV RNA results were resampled at 2–3 week intervals. In total, 61 calves were tested and 21 (including 1 set of twins) had detectable levels of BTV RNA in their blood or organs (online Appendix Table, available from www.cdc.gov/EID/content/15/12/2025-appT.htm). The transplacental transmission rate was 33% (95% confidence interval 22%–47%).

All calves except calf 21 and calf X, each of which had not consumed colostrum before sampling, had antibodies against BTV. Calf 21 was also negative for BTV RNA, but calf X showed the highest viral load in the blood (online Appendix Table). Virus isolation in KC cells (*11*) was attempted for all calf blood samples with a cycle threshold (Ct) <29, but virus was isolated from calf X only. Viral RNA load in all calves tested declined over time, and almost all calves were rRT-PCR negative by the end of the study (Table).

When the calves were first sampled, 52 dams were also tested. The RNA load in the calves always exceeded that of their dams, and 7 of the 20 dams giving birth to BTV-positive calves had no detectable viremia.

Of the 21 BTV RNA–positive calves, 5 had compromised health. Calves Y, X, and 33 were born weak and died within hours, days, and weeks after birth, respectively, and calves 13 and 29 exhibited dummy calf syndrome (*12*). All calves except calf 33 were examined postmortem and had

Table. Bluetongue virus real-time reverse transcription–PCR results from follow-up sampling of calves with initial positive results, United Kingdom, December 20, 2007, to March 15, 2008*

Calf no.	First BTV result, age, d (Ct)	Retest results, age, d (Ct)					Age, d, when PCR negative	Estimated gestation, d†
		Retest 1	Retest 2	Retest 3	Retest 4	Retest 5		
1	15 (25)	28 (26)	44 (26)	58 (28.5)	72 (32.5)	91 (neg)	91	82–219
3	38 (31)	47 (32)	61 (35.5)	81 (neg)	NT	NT	81	106–243
10	79 (32)	106 (33.5)	120 (34)	137 (neg)	158 (neg)	NT	137	140–197
12	81 (28)	108 (30)	122 (31)	139 (34)	160 (neg)	NT	160	142–199
13	4 (33)	31 (36.5)	45 (neg)	62 (neg)	83 (neg)	NT	45	65–122
14	28 (26)	48 (29)	55 (32)	69 (neg)	86 (neg)	107 (neg)	69	154–209
15	70 (32)	97 (neg)	111 (neg)	128 neg)	149 (neg)	NT	97	196–251
20	17 (31)	44 (32.5)	58 (33.5)	75 (neg)	96 (neg	NT	75	78–128
25	27 (29.5)	41 (29)	55 (30.5)	69 (36)	NT	NT	>69‡	145–202
28	1 (23)	26 (25)	35 (26)		NT	NT	>35‡	101–181
29	1 (27)	12 (27.5)			Calf died			45–182
41	47 (28)	61 (29.5)	NT	NT	NT	NT	>61‡	79–126
45	22 (27)	40 (30.5)	61 (34)	NT	NT	NT	>61‡	52–130
47	25 (26.5)	39 (29)	66 (38)	NT	NT	NT	>66‡	52–189
49 (twin with 50)	46 (29)	60 (36)	87 (neg)	NT	NT	NT	87	73–136
50 (twin with 49)	46 (29)	60 (36.5)	87 (neg)	NT	NT	NT	87	73–136
55	21 (25.5)	48 (31.5)		NT	NT	NT	>48‡	34–172

*BTV, bluetongue virus; Ct, cycle threshold; neg, negative: NT, not tested.
†Estimated stage of gestation at which transplacental infection may have occurred
‡These calves could not be followed up for farm management reasons or because the project had ended.

negative PCRs for bovine viral diarrhea virus (S.W., pers. comm.). Although calf X died of colisepticemia, this illness probably resulted from the calf's weakness and inability to consume colostrum. No infectious cause for the early postnatal death of calf Y, other than bluetongue, was identified; pathologic findings for calves 13 and 29 are described elsewhere (S.W. et al., unpub. data). Calf 27, which had negative BTV test results, was born with hypermobility of the fetlock joints, unilateral carpal valgus, and arthrogryposis. All other calves were reported to be healthy.

Time windows for possible in utero infection of each calf were calculated according to the BTV testing history of the dam and the birth date of the calf (Figure). These windows were used to investigate effect of stage of gestation on the probability of transplacental transmission. To account for uncertainty in the date of infection, we used Bayesian methods (online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2025-Techapp.pdf). The probability of transplacental transmission increased with the time of gestation during which the dam became infected (β_1 0.033; 95% credibility interval 0.014–0.063).

Conclusions

This detailed field study, which combines data on BTV infection in cows with data on transplacentally acquired infection in their offspring, demonstrates that the BTV-8 strain circulating in northern Europe can cross the bovine placenta in a high proportion (33%) of cases and infect calves when dams are infected during pregnancy. A similar study in continental Europe suggested a rate of ≈10% (*13*). However, because the transmission season was longer in some of these countries, many seropositive dams could have been infected before pregnancy, leading to underestimation of the probability of transplacental infection. In our study, we tested only calves from dams infected between August and December 2007 and known to be pregnant at the time of infection. Furthermore, analysis of our data suggests that transplacental transmission is more likely when infection occurs later in gestation; indeed, most of the dams in this study would have been in the second or third gestation trimester when infected (Figure), which may have increased our estimated rate over that found in continental Europe.

Transplacental transmission is of particular concern for policy makers because it may result in the birth of immune-tolerant, persistent carriers, as has happened with bovine viral diarrhea virus (*14*). In our study, all BTV-positive calves other than X and Y were tested after they had received colostrum and, hence, maternal antibodies. The presence of BTV antibodies in calf Y suggests that fetal antibody formed in response to in utero infection, yet calf X had no detectable antibodies against BTV despite strongly positive rRT-PCR results. Calf X was infected late in gestation (Figure), when it should have been capable of mounting its own antibody response (*15*). Antibody-negative PCR-positive calves have been reported elsewhere (*13*). Follow-up testing is needed to assess whether such calves remain persistently infected; however, because calf X died a few days after birth, follow-up testing was not possible.

RNA declined in all retested calves (Table); most were PCR-negative by the end of the study, including dummy calf 13. Therefore, our results do not suggest that transpla-

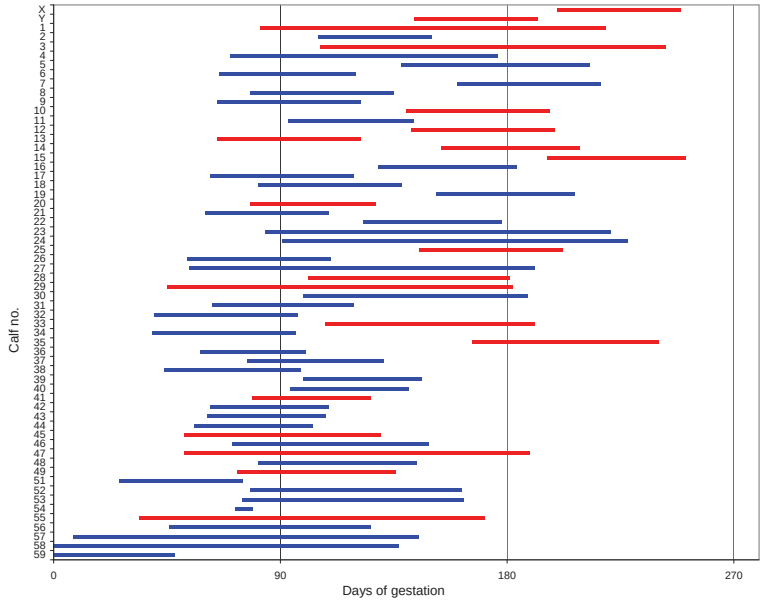

Figure. Estimated gestation period at infection of the dam in relation to occurrence of transplacental transmission. Bluetongue virus (BTV) test data for the dams and birth dates of the calves were used to calculate the window of gestation when the dam could have become infected (see online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2025-Techapp.pdf, for details). The calculated infection windows are shown in red for BTV-positive calves (transplacental infection did occur) and in blue for BTV-negative calves (transplacental infection did not occur). Because calves were conceived naturally, the exact date of conception is not known, although all were considered to have been born at full term.

cental infection with BTV-8 results in subclinical, persistent carriers. Nonetheless, the finding that some calves may be born with deformaties after the virus has cleared may lead to underestimation of the economic effects of BTV; calf 27, which was born with limb deformities to a BTV positive dam, could be such a case.

Live virus has been successfully isolated from only 4 transpacentally infected calves (including calf X described in this study), all of which received either no maternal colostrum or only pooled colostrum (*9*,*13*). Further work is needed to assess whether infectious virus can be isolated from healthy transplacentally infected calves that have colostrum-derived maternal antibodies, because infectious virus needs to be present if transplacental infection is to play a major role in overwintering. In conclusion, future emerging BTV strains should be considered to have the potential for transplacental transmission until investigations show otherwise.

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Echinococcus vogeli Infection in a Hunter, French Guiana

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Echinococcus vogeli infection in a hunter from the rain forest of French Guiana was confirmed by imaging and mitochondrial DNA sequence analysis. Serologic examination showed typical patterns for both alveolar and cystic echinococcosis. Polycystic echinococcis caused by *E. vogeli* may be an emerging parasitic disease in Central and South America.

Echinococcosis is one of the most lethal helminthic zoonoses worldwide. The 4 species of the genus *Echinococcus* are *E. granulosus* sensu lato, now including 5 independent species (*1*,*2*), which causes cystic echinococcosis (CE); *E. multilocularis,* which causes alveolar echinococcosis; *E. vogeli,* which causes polycystic echinococcosis (PE); and *E. oligarthrus,* which causes the recently described unicystic echinococcosis (*3*–*6*). Among these species, *E. oligarthrus* and *E. vogeli* are neotropical species localized exclusively in Central and South America (*5*,*6*). Only 3 cases of *E. oligarthrus* infection have been reported in the literature (1 from Brazil, 1 from Venezuela, and 1 from Surinam); 168 *E. vogeli* cases have been reported in 12 countries in Central and South America. To date, there have been no reports of neotropical echinococcosis in Bolivia, Paraguay, Guyana, or French Guiana (*5*,*6*). *E. granulosus* occurs sympatrically in South America, whereas *E. multilocularis* does not occur there at all (*3*,*5*). As both *E. vogeli* and *E. oligarthrus* have primarily sylvatic life cycles and the diagnosis is usually based on histopathologic examination of resected lesions, the number of human cases might be underestimated because of the small number of patients who receive surgical treatment (*5*,*6*). We report a case of human infection from French Guiana caused by *E. vogeli*.

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The Study

In April 2006, a 72-year-old man was admitted to a local hospital in Cayenne, French Guiana, for abdominal pain and a palpable epigastric mass. The patient hunted jaguars in the rain forest of French Guiana and owned dogs. He had no history of travel outside French Guiana. An exploratory laparotomy performed in June 2006 showed a hard, whitish liver tumor, deemed unresectable. Histopathologic examination of a biopsied sample of the tumor showed multilocular cysts. Albendazole treatment was started immediately after surgery. In January 2007, the patient was referred to the Department of General, Endocrine, and Digestive Surgery at the Saint-Louis Hospital, Paris, France. Computed tomography showed a multilocular hypoattenuating cystic mass in the left side of the liver, infiltrating the left glissonian pedicle up to the hepatic hilum (Figure 1, Panel A). Magnetic resonance imaging showed a well-defined, thin-walled, multilocular cystic mass (11 × 10 × 12 cm) involving segments II, III, and IV of the liver (Figure 1, Panel B) and multiple intraperitoneal cysts. The cysts appeared markedly hyperintense on T2-weighted images, hypo-intense on T1-weighted images, and showed slight enhancement of the septa after gadolinium injection. The patient underwent a left hepatectomy and resection of intraperitoneal cysts. Analysis of the operative specimen showed multiple large and small parasite hooks, with mean lengths of 32.7 μm ± 1.6 μm and 42.7 μm ± 0.7 μm, respectively (Figure 2).

Two serum samples from the patient (one obtained in May 2006, the other in December 2006) were analyzed by several immunologic techniques; all indicated infection with *Echinococcus* spp. Commercial ELISAs using *E. granulosus* antigens (Bordier Affinity Products, Lausanne, Switzerland, and Biotrin International, Antony, France) and the *E. multilocularis*-specific Em2^{plus}-ELISA (Bordier Affinity Products, Switzerland) were both strongly positive. Confirmative Western blots (LD Bio Products, Lyon, France) showed a shadow at 16–18 kDa, which is characteristic for *E. granulosus* infection, on both samples. Additional Western blots carried out at Asahikawa Medical College (Asahikawa, Japan) using recombinant antigens (RecAgB8/1, more specific for *E. granulosus*, and RecEm18, more specific for *E. multilocularis*; *7*,*8*) also showed strong responses. On the basis of serologic patterns obtained with recombinant antigens, with no knowledge of the patient's clinical background, travel history, or images of the lesion, *E. granulosus* infection with many multiple cysts, advanced *E. multilocularis*, or advanced *E. vogeli* infection was suspected (*7*,*9*).

[1]These authors contributed equally to this article.

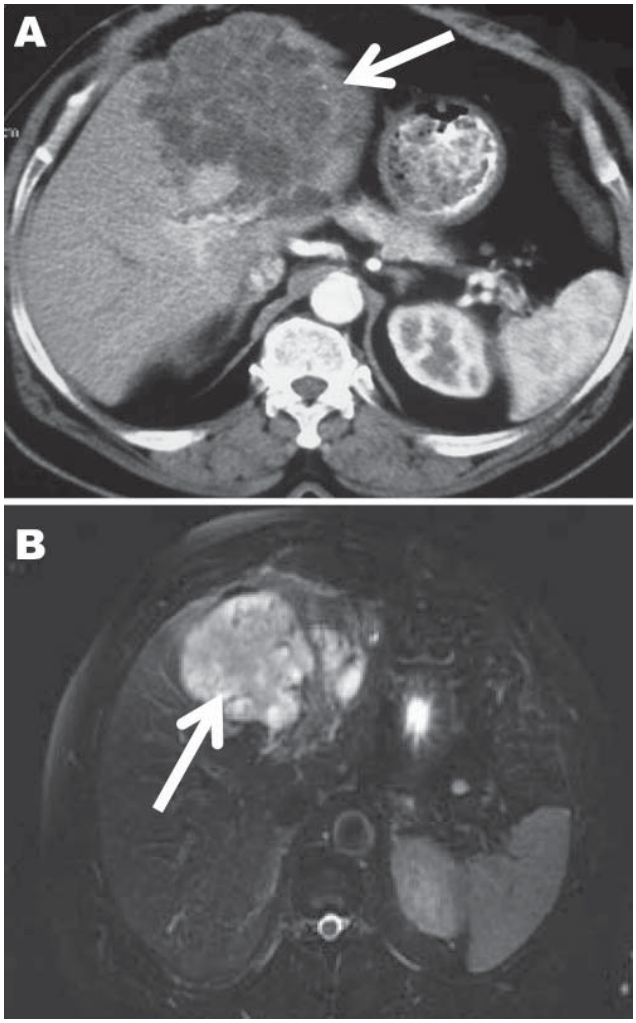

Figure 1. Computed tomography (A) and magnetic resonance (B) images of the liver of a 72-year-old man from French Guiana with polycystic echinococcosis affecting the left side of the liver. White arrows indicate the multicystic liver lesion.

Molecular identification was carried out with reference to the GenBank database by using a highly polymorphic DNA target (*10*). DNA from the liver lesion was extracted by using the High Pure PCR Preparation Kit (Roche, Mannheim, Germany). A part of the *cox1* mitochondrial gene was sequenced by using the *E. vogeli*–specific primer set (*cox1*_F: 5′-TTAATTTTGCCTGGGTTTGG-3′ and *cox1*_R: 5′-ACGACCCATATGATCCCAAA-3′). A sequence of 492 bp was obtained with the ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA) and was compared with *Echinococcus* spp. sequences published in the GenBank database (online Appendix Table, available from www.cdc.gov/EID/content/15/12/2029-appT.htm). The sequences were aligned by using BioEdit 7.0.9.0 (*11*), and sequence identity matrix was generated based on the percentage of base pairs in common between species. The *cox1* sequence was found to be 100% identical to *E. vogeli* species originating from Colombia (GenBank accession no. AB208546; *2*) and was clearly distinguishable from all other *Echinococcus* species (online Appendix Table).

On the basis of imaging showing numerous multiple cysts, serologic examination showing typical patterns for both alveolar and cystic echinococcosis, and the life history of the patient, the diagnosis of polycystic echinococcosis caused by *E. vogeli* could have been made before surgical intervention (*5*,*7*,*9*). The immunoblot showing a strong antibody response to recombinant AgB suggested a large volume of cyst fluid. Therefore, the immunoblot showing strong responses to both recombinant Em18 and AgB may be a typical pattern for advanced *E. vogeli* infection (data not shown). Because few studies using serologic analysis on human *E. vogeli* cases have been published, it would be useful to study antibody responses using recombinant antigens with large numbers of such patients and to compare the results with patterns observed with alveolar and cystic echinococcosis (*7*,*9*).

After surgery, identification of parasite hooks was carried out. The hooks showed the characteristic shape of *E.*

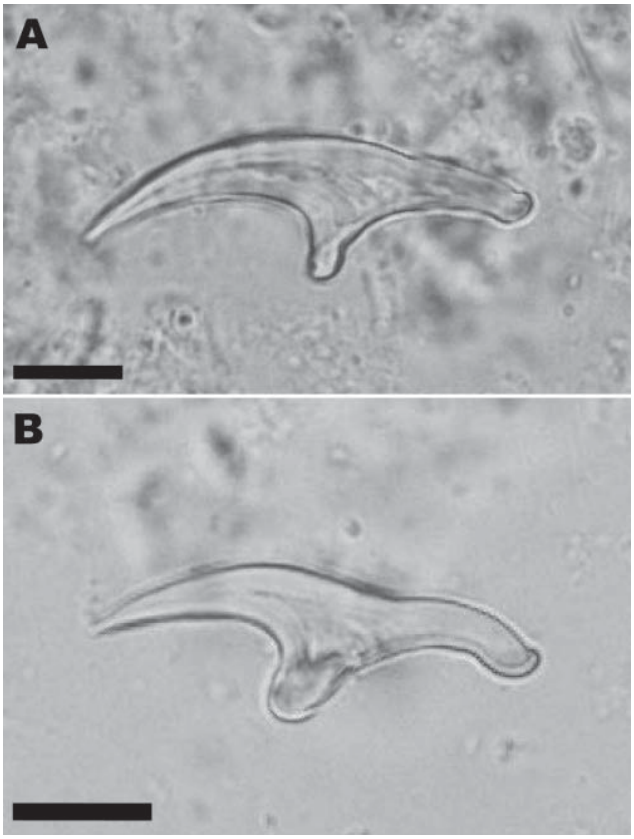

Figure 2. Large (A) and small (B) hooks from *Echinococcus vogeli* protoscoleces in the liver lesion of a 72-year-old man from French Guiana. Scale bars = 10μm.

vogeli and thus differed from *E. granulosus* (mean lengths of large hooks 25.9–35 μm, and small hooks 22.6–27.8 μm) and *E. oligarthrus* (30.5–33.4 μm and 25.4–27.3 μm, respectively) (*12*,*13*). *E. granulosus* and *E. oligarthrus* also occur in South America. The presence of hooks indicated that the parasite lesion was fertile in our patient, as shown in ≈50% of cases (*5*). Based on mitochondrial DNA analysis, the parasite identification was confirmed as *E. vogeli*. Further molecular studies on the haplotypes of this species may give information concerning the genetic diversity and circulation of the parasite in South America (*14*).

Albendazole has been used for medical management of alveolar and advanced cystic echinococcosis (*3*). Several instances of its efficacy on polycystic echinococcosis have been reported, but given the primacy of surgical management of these infections, albendazole will probably remain an additional treatment (*5*).

Neotropical echinococcosis cases are rare compared with alveolar and cystic echinococcosis (*5*). This rarity is probably because of the sylvatic life cycle of these species. However, because domestic dogs have been introduced to areas where *E. vogeli* is present in its natural cycle, the potential for transmission of the parasites from dogs to humans by close contact exists. The at-risk population mainly lives in rural areas and has limited access to medical services, which strongly suggests that many infected persons cannot receive adequate treatment for this underestimated disease.

Conclusions

We report an autochthonous case of *E. vogeli* infection documented in French Guiana. Further investigations are needed to improve the serologic diagnosis of this infection and to define its typical serologic pattern compared with echinococcosis. Healthcare providers need to be alert to the existence of neotropical echinococcosis and should consider the possibility of its emergence in Central and South America. Although rare, this disease is still lethal in untreated cases.

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Recombination in Vaccine and Circulating Strains of Porcine Reproductive and Respiratory Syndrome Viruses

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Em2007, a porcine reproductive and respiratory syndrome virus (PRRSV) variant with a unique 68 aa deletion in Nsp2, was recently isolated in China. Phylogenetic and molecular evolutionary analyses indicated that Em2007 is a natural recombinant between a vaccine strain of PRRSV and circulating virus. We also tested its pathogenicity in piglets.

Porcine reproductive and respiratory syndrome (PRRS) is now considered one of the most economically important diseases in countries with intensive swine industries. The causative agent, PRRS virus (PRRSV), is a member of the family *Arteriviridae* (*1*). The genome of PRRSV is ≈15 kb and encodes 9 open reading frames (ORFs). Two distinct genotypes of PRRSV share only ≈60% nucleotide identity and are represented by the North American prototype VR-2332 and the European prototype Lelystad virus (LV) (*2*). Sequence differences have also been found among isolates of the same genotype, particularly in the Nsp2 regions within ORF1a, and ORF5 (*3*). Mutation and genetic recombination play an important role in the evolution of PRRSV (*4*–*6*).

Since May 2006, porcine high fever syndrome, caused by highly pathogenic PRRSV and characterized by high fever and high death rates in pigs of all ages, has emerged in China and affected >20 million pigs (*7*–*9*). Genomic analysis showed that nearly all of the emerging highly pathogenic PRRSVs isolated from this outbreak share a unique discontinuous deletion of 30 aa in Nsp2 (*7*–*10*). However, a novel PRRSV variant, with a 68 aa deletion in Nsp2, emerged in central China in 2007. We report the unique genetic characteristics of this novel variant and its pathogenicity in piglets.

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The Study

At the end of 2007, a smaller cDNA fragment than the expected size was observed from a fetal piglet when a diagnostic reverse transcription–PCR (RT-PCR) was performed to amplify the unique genetic marker of the highly pathogenic PRRSV, indicating that a novel PRRSV variant was found. This strain, designated Em2007, was subsequently isolated and the full-length genomic sequence was determined. The genome of Em2007 was 15,272 bp, including the poly(A) tail (GenBank accession no. EU262603), and shared 87.6% and 57.9% sequence identity with VR-2332 and LV, respectively, indicating that Em2007 belongs to the North American genotype. The Nsp2 gene of Em2007 was 2,736 bp and encoded 912 aa, with a unique continuous deletion of 68 aa at positions 499–566, relative to strain VR-2332 (online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2032-Techapp.pdf). This unique deletion is substantially different from previous PRRSV isolates with deletions in Nsp2 (*3*,*7*–*11*).

To establish the genetic relationships of Em2007, we constructed phylogenetic trees using the neighbor-joining method based on the full-length genome. Results showed that Em2007 formed a minor branch, which was located in the middle of 2 clusters represented by CH-1a (the first PRRSV isolated in China in 1996) and JXA1 (the highly pathogenic PRRSV isolated in China in 2006), respectively (data not shown).

We also compared the sequence identity of individual Em2007 ORFs with representative PRRSV isolates and found that all ORFs have highest identity (>92%) with CH-1R (an attenuated vaccine strain used in China), except for Nsp2 (80.2%). Because recombinations have been reported in PRRSV in previous studies (*6*), we speculated that Em2007 is a mosaic. To test our hypothesis, we used 3 approaches to detect possible recombination events within Em2007.

First, SimPlot, which calculates and plots the percent identity of a query sequence against a panel of reference sequences in sliding windows (*12*), was performed using Em2007 as a query. Based on a set of complete genome sequences, including 56 Chinese PRRSVs isolated during 1996-2008, three representative North American strains (VR-2332, MN184B, and P129), and 2 attenuated vaccine strains (RespPRRS, CH-1R) (the origin of all strains is listed in the Table), the SimPlot graph clearly showed that Em2007 was generally closer to CH-1R than to any other strain. However, there were 3 narrow zones showing disproportionately low levels of similarity between the 2 strains compared to other regions (Figure 1, panel A). Notably, the 3 narrow zones of Em2007 had high levels of similarity with WUH1 (a highly pathogenic PRRSV, isolated in Wuhan, China in 2006). These results indicated that Em2007 is a possible recombinant and CH-1R and WUH1 are 2 putative parental-like strains. Recombination

Table. Origin and GenBank accession numbers of 61 PRRSV isolates from China and representative strains from North America used in this study*

Isolate no.	Strain	Country of origin	GenBank accession no.	Isolate no.	Strain	Country of origin	GenBank accession no.
1	CH-1a	China	AY032626	32	HUN4	China	EF635006
2	BJ-4	China	AF331831	33	HuN	China	EF517962
3	PRRSV01	China	FJ175687	34	JXwn06	China	EF641008
4	PRRSV02	China	FJ175688	35	07QN	China	FJ394029
5	PRRSV03	China	FJ175689	36	GD	China	EU825724
6	HB-1(sh)/2002	China	AY150312	37	CG	China	EU864231
7	HB-1-3.9	China	EU360130	38	NM1	China	EU860249
8	HB-2(sh)/2002	China	AY262352	39	07NM	China	FJ393456
9	GS2002	China	EU880443	40	XH-GD	China	EU624117
10	GS2003	China	EU880442	41	Em2007	China	EU262603
11	GS2004	China	EU880441	42	Henan-1	China	EU200962
12	CH2002	China	EU880438	43	Jiangxi-3	China	EU200961
13	CH2003	China	EU880440	44	SX2007	China	EU880434
14	CH2004	China	EU880439	45	WUH1	China	EU187484
15	HN1	China	AY457635	46	LN	China	EU109502
16	NB/04	China	FJ536165	47	SHH	China	EU106888
17	SHB	China	EU864232	48	GD2007	China	EU880433
18	CC-1	China	EF152486	49	BJ	China	EU825723
19	HUB1	China	EF075945	50	07BJ	China	FJ393459
20	HUB2	China	EF112446	51	HN2007	China	EU880437
21	HEB1	China	EF112447	52	07HEBTJ	China	FJ393458
22	JSyx	China	EU939312	53	07HEN	China	FJ393457
23	JX143	China	EU708723	54	CH-1R	China	EU807840
24	JXA1	China	EF112445	55	GS2008	China	EU880431
25	SY0608	China	EU144079	56	XL2008	China	EU880436
26	TP	China	EU864233	57	HPBEDV	China	EU236259
27	JX2006	China	EU880432	58	VR-2332	USA	AY150564
28	S1	China	DQ459471	59	P129	USA	AF494042
29	BJsy06	China	EU097707	60	RespPRRSMLV	USA	AF066183
30	TJ	China	EU860248	61	MN184B	USA	DQ176020
31	NX06	China	EU097706				

*PRRSV, porcine reproductive and respiratory syndrome virus.

was further analyzed by Bootscan, a program for the detection of recombination events, and the Genetic Algorithm for Recombination Detection (GARD) (*13*). Six potential recombination breakpoints, with maximal χ^2, were found (Figure 1, panel B), indicating that 3 recombination events have taken place within Em2007. Two recombination fragments (1,457–2,312 and 3,245–4,584) are located in Nsp2; the third (8,195–9,168) is located in Nsp9.

Phylogenetic trees of nucleotide sequences of each recombination region defined by GARD, including flanking regions, were further reconstructed by the neighbor-joining method. A large discrepancy ($p<0.001$, by Shimodaira-Hasegawa test) between phylogenetic trees inferred for each recombination region constitutes powerful evidence for recombination (online Technical Appendix). In addition, a retrospective survey found that the fetal piglet from which Em2007 was isolated was from a farm in Wuhan, China, and CH-1R was used on this farm to control PRRS, indicating the potential for recombination between CH-1R and WUH1. This evidence further supported the possibility that Em2007 is a natural recombinant between CH-1R and WUH1.

To test the virulence of Em2007, 40-day-old PRRSV-free piglets (9 piglets in each group) were inoculated intramuscularly with $10^{5.0}$ mean tissue cultures infectious doses/2 mL of Em2007, CH-1a, and WUH1, respectively. Control piglets were inoculated with Dulbecco minimal essential medium. Clinical signs and rectal temperature were recorded daily. Two piglets from each group were euthanized and necropsied at 7 and 10 days postinoculation, and organs including lung, brain, spleen, kidney, liver, intestines, and lymph nodes were collected for viral load analyses and histopathologic examinations. The remaining 5 piglets in each group were observed for 21 days to evaluate death rates.

Results showed that piglets inoculated with CH-1a experienced only temporary fever and mild respiratory symptoms. Obvious clinical signs, including inappetence, lethargy, high and continuous fever, red discolorations in the body, and blue ears were observed in piglets inoculated

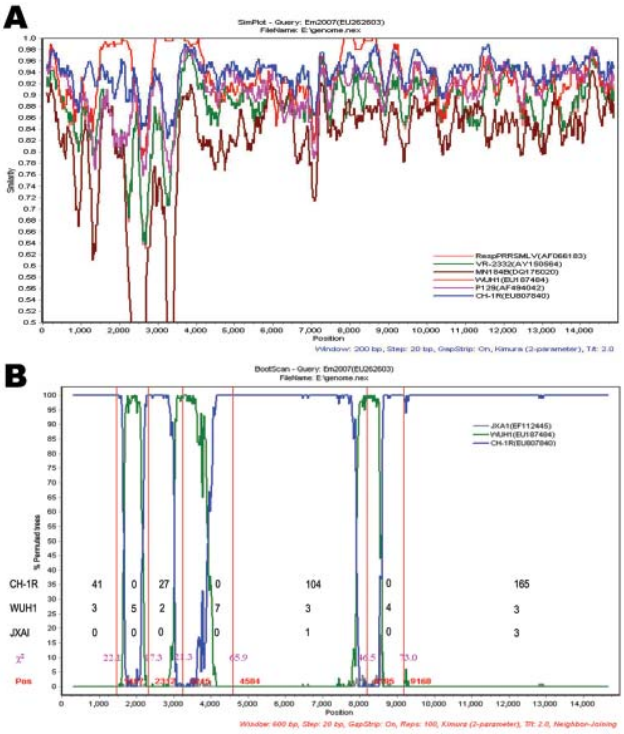

Figure 1. Recombination event analyses of the Em2007 strain of porcine reproductive and respiratory syndrome virus (PRRSV). A) Similarity plot analysis using Em2007 as query sequence. Analysis made use of a sliding window of 200 bases and a step size of 20 bases. The y-axis shows the percentage similarity between the selected PRRSV sequences and the query sequence. The other comparisons are not shown for clarity. B) Bootscan analysis using Em2007 as the query sequence. JXA1 is used as the outgroup to determine the breakpoints. The y-axis shows the percentage of permutated trees using a sliding window of 600 bases and a step size of 20 bases. Red vertical lines and numbers indicate the recombination breakpoints identified by the Genetic Algorithm for Recombination Detection. Pink numbers indicate the maximal χ^2 value of each breakpoint. Numbers corresponding to CH-1R, WUH1, and JXA1 indicate the quantity of informative sites in 7 zones defined by 6 recombination breakpoints, respectively.

with WUH1 (Figure 2, panel A). Furthermore, severe interstitial pneumonia (online Technical Appendix) and nonsuppurative encephalitis cases were also observed 7 and 10 days postinoculation. Four of 5 piglets died within 21 days after inoculation (Figure 2, panel B). Piglets inoculated with Em2007 also showed similar clinical signs to those seen in the WUH1 group. However, the interstitial pneumonia and nonsuppurative encephalitis were mild and no deaths occurred throughout the experimental period in Em2007 group. The results of viremia and viral load also indicated that Em2007 was more mild than WUH1, but of substantially higher virulence than CH-1a (data not shown).

Conclusions

Em2007, a PRRSV variant with a unique continuous deletion of 68 aa in Nsp2, was isolated in China. This variant is a natural recombinant between an attenuated PRRSV vaccine strain CH-1R and a highly pathogenic PRRSV strain, WUH1. Animal experiments demonstrated that while Em2007 has higher virulence than CH-1a, the parental strain of CH-1R, it is attenuated relative to WUH1.

Previous studies have shown that genetic recombination occurs between attenuated vaccine strains of PRRSV grown together in culture (*14*). This study demonstrates for the first time that natural recombination can occur between vaccine and field strains, suggesting that live vaccines have the capacity to shape PRRSV evolution by homologous recombination with circulating virus.

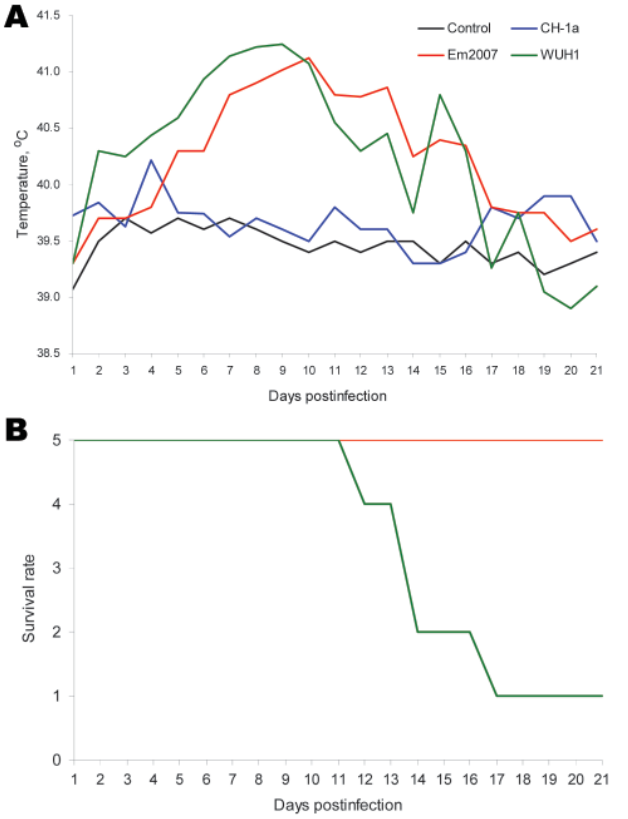

Figure 2. Pathogenicity comparison among the Em2007, CH-1a, and WUH1 strains of porcine reproductive and respiratory syndrome virus (PRRSV). Forty-day-old piglets (9 piglets in each group) free of PRRSV were inoculated intramuscularly with $10^{5.0}$ mean tissue culture infectious doses/2 mL of Em2007, CH-1a, WUH1, respectively. Two piglets from each group were euthanized and necropsied at 7 and 10 days postinoculation (dpi) for viral load analyses and histopathologic examinations. The remaining 5 piglets in each group were used to evaluate rate of death. Mean rectal temperature (A) and survival rate (B) of each group were recorded for 21 dpi.

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Molecular Epidemiology of Glanders, Pakistan

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We collected epidemiologic and molecular data from *Burkholderia mallei* isolates from equines in Punjab, Pakistan from 1999 through 2007. We show that recent outbreaks are genetically distinct from available whole genome sequences and that these genotypes are persistent and ubiquitous in Punjab, probably because of human-mediated movement of equines.

Glanders is an equine disease that was recognized by Hippocrates and Aristotle (*1*). It is caused by the bacterium *Burkholderia mallei*, an obligate pathogen of horses, donkeys, and mules (Equidae), with occasional infections in felines, canines, and humans (*2*,*3*). Strict regulation of equines has reduced the range of this once globally distributed disease to a few endemic foci in South and Central America, the Middle East, and parts of Africa and Asia (*2*,*4*). This emerging disease has only recently regained attention following the listing of *B. mallei* as a Category B agent by the US Centers for Disease Control and Prevention (*2*). Although outbreaks are common in regions of disease endemicity, much of what is known about the ecology and natural population dynamics of *B. mallei* relies on indirect evidence and expert opinion, with little to no knowledge concerning its genetic diversity (*2*,*3*). We genetically characterized 15 samples of *B. mallei* from recent outbreaks in Pakistan to provide additional knowledge of how this disease of antiquity is transmitted throughout endemic regions today.

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The Study

We obtained clinical samples and background information from 15 glanderous equids in Punjab, Pakistan from 1999 through 2007 (Table; online Appendix Table, available from www.cdc.gov/EID/content/15/12/2036-appT.htm). Research on equine subjects was approved by the Synopsis Scrutiny Committee and Animal Ethics Committee, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. Samples were plated on brain-heart infusion (BHI) agar with 4% glycerol and incubated for 24–30 hours at 37°C. Individual colonies were inoculated into BHI broth containing 4% glycerol and were incubated with shaking for 36 h at 37°C. An aliquot of broth (1.5 mL) was centrifuged at 13,000 rpm for 15 min. Genomic DNA was extracted from the resulting pellets using standard digestion buffer and phenol-chloroform extraction protocols (*6*).

For genotyping, we screened 23 loci (online Appendix Table) from a previously established 32-marker multiple locus variable number of tandem repeats (VNTR) analysis system designed for *B. pseudomallei* and *B. mallei* (*7*). *In silico* genotyping of the same loci was also performed for 10 whole genome sequences (WGS) of *B. mallei* (*8*; online Appendix Table). VNTR markers have higher mutation rates than other genetic markers, which make them inappropriate for determining deep levels of evolutionary relatedness. VNTRs, however, are appropriate for 1) discrimination between closely related isolates, 2) determination of the degree of relatedness among isolates, and 3) discernment of population structure on a spatial scale (*7*,*9*,*10*). This utility is especially important for *B. mallei* because it is a recently emerged clone of *B. pseudomallei* and has been shown to be genetically monomorphic with typing methods such as multilocus sequence typing (*11*). To compare the genetic diversity of our Punjab isolates to that of sequenced strains, we performed a phylogenetic analysis on the 23 loci using the neighbor-joining algorithm in PAUP* 4.0b (*9*). To determine the genetic relationships among the Punjab population itself, we performed the same analysis using only the Punjab isolates and polymorphic loci (n = 15 loci).

Combined analysis of the Punjab isolates and WGS showed that the Punjab isolates are phylogenetically distinct from WGS (Figure 1). This finding was also demonstrated in the values for average pairwise distance (APD), where the APD among Punjab isolates is 2× lower than the APD calculated for either the entire phylogeny or the WGS alone (Figure 1). Therefore, the Punjab isolates represent only a small amount of the genetic diversity demonstrated in this pathogen. Phylogenetic analysis of the Punjab isolates alone placed 14 of the 15 samples into 3 distinct clades with 1 sample standing alone (Figure 2). Most samples (9/15) belong to clade A, whose isolates are both

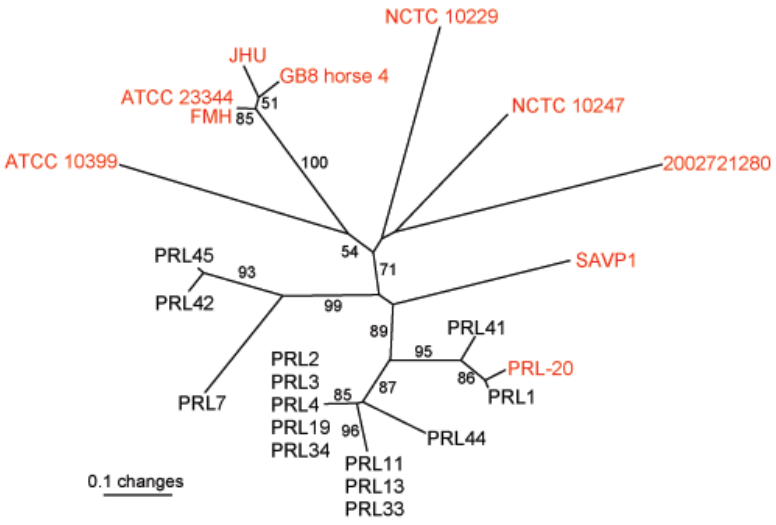

Figure 1. Unrooted neighbor-joining tree based on 23 variable number tandem repeat loci demonstrating that the Punjab isolates (black text and PRL-20) are genetically distinct from and less diverse than available whole genome sequences (WGS, red text) (*7*). Statistical supports for branches based on 1,000 bootstrap iterations are shown. Sample PRL-20 is shown in red text because it is also available as a whole genome sequence; therefore, it was used in all 3 situations where an average pairwise distance (APD) was calculated. Among 10 WGS, the average pairwise distance was 0.607; between 10 WGS and Punjab isolates, average pairwise distance was 0.627; and among 15 Punjab isolates, average pairwise distance was 0.312. These results indicate that the Punjab isolates are more closely related to each other than to the sequenced strains because the APD among Punjab isolates is 2× lower than the APD calculated in the other 2 situations.

temporally and geographically diverse, suggesting that this lineage is ecologically established in Punjab.

Because of the limited sample size, many of the patterns observed from these data may result from sampling bias. However, even a limited amount of genotypic data can be useful in formulating hypotheses regarding the dispersal of *B. mallei*. For example, the presence of samples from Faisalabad in each clade suggests that this district may be a center of diversity in the province (Figure 2) but this does not indicate a lesser degree of diversity in other districts where fewer samples were collected.

The diversity seen in the district of Faisalabad may result from either 1) the industrial nature of Faisalabad or 2) from high endemism of *B. mallei* in the region. Currently, the district has ≈10,000 horses and mules and >44,000 donkeys, plus other transient equines (*12*). Many equines move through and work in the city, potentially introducing strains from surrounding regions. Because horses and mules can be positive but asymptomatic for glanders (*13*), many hosts are available to maintain strains throughout the region. Communal stables and water troughs are common throughout the district and *B. mallei* has been isolated from these water troughs (A. Naureen, unpub. data). Furthermore, *B. mallei* can remain viable in contaminated stables for up to 6 weeks (*14*) and in sterile tap water for up to 4 weeks (*15*), which provides an environment for establishment and retention of *B. mallei* populations in Faisalabad.

Combining phylogenetic with epidemiologic data reveals how *B. mallei* disseminates throughout a region. For example, epidemiologic data suggests that 2 horses from a farm in the district of Sargodha (PRL3 and PRL4) contracted glanders while at a polo club in the Lahore district. This is supported by VNTR data, as these 2 isolates clustered phylogenetically with one of the samples obtained from an outbreak that occurred at the same polo club 3 months prior (groups 5 and 6, Table). Furthermore, at the time of the PRL3 and PRL4 infections, a co-resident mule with no previous travel history (PRL44) was negative for glanders, making it unlikely that these horses acquired glanders from their farm. This mule was positive for glanders ≈1.5 years later, and the isolate obtained from its infection clustered phylogenetically with the samples from the polo club and

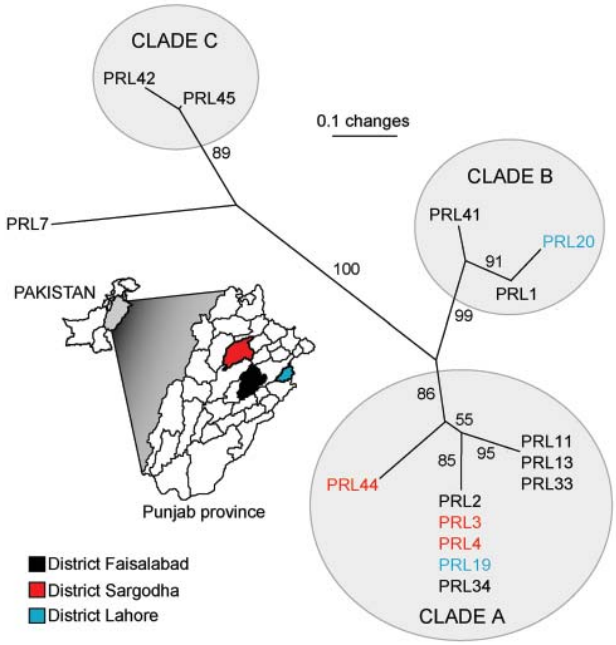

Figure 2. Unrooted neighbor-joining tree showing phylogenetic relationships among 15 samples of *Burkholderia mallei* from the Punjab Province, Pakistan. Statistical support for each branch derived from 1,000 bootstrap iterations are shown. Sample names are color-coded to match their district of origin in reference to the inset map of the Punjab Province. Approximate linear distances between districts are Faisalabad to Lahore ≈120 km, Faisalabad to Sargodha ≈84 km, Lahore to Sargodha ≈168 km.

Table. Spatial, temporal, and phylogenetic relationships among *Burkholderia mallei* infections in equids, Punjab Province, Pakistan*

Epidemiologic group and subgroup designation (isolate names)†	Description	Clade(s)‡	Total VNTR differences between subgroups
Group 1 Group 1a (PRL2) Group 1b (PRL11, PRL13)	Strains were collected from an outbreak among Faisalabad Mounted Police Horses (n = 18) in June 1999. Biochemical test results (based on Analytical Profile Index 20E strips; BioMériux, Marcy l'Etoile, France) differed nonsubstantially (data not shown). Therefore, only 3 isolates were evaluated by using VNTR. Strains PRL11 and PRL13 were isolated from horses that were kept at 2 stables ≈8 km from each other, but the 2 stables had a history of mixing.	A	3/15 loci
Group 2 Group 2a (PRL42) Group 2b (PRL45)	Samples came from 2 sporadic cases of glanders in draught mules from the Faisalabad district in 2007. Reports indicate that these animals drank from communal water troughs available in different zones of Faisalabad.	C	1/15 loci
Group 3 Group 3a (PRL1) Group 3b (PRL41) Group 3c (PRL7)	Samples came from 3 sporadic cases of glanders in draught equids from the Faisalabad district during different years (2002, 2006, and 2000, respective to subgroup listing). Reports indicate that these animals drank from communal water troughs available in different zones of Faisalabad.	B§ B, no clade¶	2/15 loci§ 14/15 loci¶
Group 4 Group 4a (PRL33) Group 4b (PRL34)	Samples were obtained from 2 donkeys that worked and were housed together in a brick factory in the district of Faisalabad. Samples were collected 3 weeks apart in 2007, and the strains were passaged 3× in guinea pigs before DNA was extracted for VNTR evaluation.	A	3/15 loci
Group 5 Group 5a (PRL19) Group 5b (PRL20)	In September 2005, an outbreak of glanders occurred at the Lahore Polo Club. Two isolates were obtained from separate horses in this group, and each isolate had a different biochemical profile (data not shown).	A, B	8/15 loci
Group 6 Group 6a (PRL3, PRL4) Group 6b (PRL44)	From November 2004 to March 2005, two horses from a farm in Sargodha (PRL3, PRL4) participated in matches at the Lahore Polo Club. Horses were returned to their farm in late spring 2005. In the fall of 2005, there was a glanders outbreak at the Lahore Polo Club (see Group 5 above). In December 2005, the 2 horses on the Sargodha farm tested positive for glanders after being housed together during the winter. A mule (PRL44) that was also present at the Sargodha farm tested negative for glanders at this time. Approximately 2 years later, the same mule tested positive for glanders after reports of 6 months' standing nasal discharge. Records indicate the mule was brought to the farm at a young age from the city of Multan and never left the farm before onset of symptoms.	A	4/15 loci

*VNTR, variable number tandem repeats.
†Samples from equines with similar histories were assigned to the same epidemiologic group (e.g., Group 1, Group 2). Subgroups were defined based on VNTR data; samples with identical VNTR genotypes were assigned to the same subgroup (e.g., PRL11 and PRL13 in Group 1b).
‡See Figure 2.
§Data are comparing subgroups 3a and 3b to each other (PRL1 and PRL41) and excluding subgroup 3c (PRL7).
¶Data are comparing subgroups 3a and 3b combined (PRL1 with PRL41) to subgroup 3c (PRL7).

Sargodha horses. Therefore, we hypothesize that the infected horses either directly transferred the disease to the mule or they contaminated a source on the farm which subsequently led to the mule's infection. Environmental sampling would be required to identify the original infection source for the horses and subsequent transmission route to the mule. Nevertheless, this case shows a strain that was transferred a distance of 168 km, demonstrating that human-mediated movement of equines can influence the distribution of *B. mallei* genotypes. This case also suggests that a strain can persist for ≈1.5 years.

Other cases in the province demonstrate that infections either stem from similar strains or are caused by multiple strains. For example, samples that were placed in the same epidemiologic group cluster together phylogenetically (groups 1, 2, and 4; Table), indicating communal infections similar to the cases described above. In contrast, epidemiologic group 5 (PRL19 and PRL20) was separated into 2 distinct clades (Figure 2), indicating that this outbreak was caused by multiple strains. Therefore, it should not be assumed that an outbreak of glanders is always caused by a single strain.

Conclusions

Our study suggests that numerous lineages of *Burkholderia mallei* are present in Punjab, Pakistan, and that these lineages persist across geographic space and time. Despite this, these isolates appear to be genetically distinct from other studied strains. The economics and use of equines likely contribute to the persistence of glanders in this region because modern methods for control of this disease (monitoring and euthanasia) are not viable options.

Therefore, other solutions to curbing the spread of glanders need to be identified. We suggest that a focus on finding methods to improve the sanitary conditions of communal water troughs and stables may lead to a practical solution for disease reduction and containment. Finally, our study demonstrates the utility of VNTRs paired with extensive epidemiologic data for analyzing the distribution of *B. mallei* genotypes throughout endemic regions.

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Aleutian Mink Disease Virus and Humans

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Reports of a possible relationship between Aleutian mink disease parvovirus (AMDV) and human infection are rare. However, 2 mink farmers with vascular disease and microangiopathy similar to that in mink with Aleutian disease were found to have AMDV-specific antibodies and AMDV DNA. These findings raise the suspicion that AMDV may play a role in human disease.

Autonomous parvoviruses, such as Aleutian mink disease virus (AMDV), cause a broad spectrum of diseases in animals and man. Acute disease manifests itself as a lytic infection of rapidly dividing cells; chronic disease reflects a restricted or abortive infection of specific cell types (*1*). Aleutian disease (AD) is known to produce clinical signs in mink and ferrets only (*2*,*3*), although other mammals have reportedly been antibody positive.

In adult mink, AD is a persistent, slowly progressive AMDV infection in which a dysregulated immune system and a postinfectious antibody response cause an immune complex–mediated vasculitis (*2*). Perivascular and glomerular immune complexes (*2*,*4*,*5*) can cause membranoproliferative glomerulonephritis (*6*) and segmental or circumferential arteritis (*4*) with mononuclear infiltration, fibrinoid necrosis and deposits, and increased intimal cellularity. Mononuclear cells may surround the vessel, and connective tissue proliferation and necrosis in the tunica elastica media narrow the lumen (*7*). In mink kits, AD causes an acute cytopathic infection of alveolar cells, which leads to respiratory distress and death (*8*).

Reports of a possible relationship between AMDV and human infection are rare (*9*). Histopathologic features like those in AMDV-infected mink have been described for 2 patients in the early 1960s (*10*). Exposed laboratory workers have had persistent anti-AMDV antibodies for up to 18 months; however, injection of their antibody-positive blood into Aleutian mink caused neither lesions nor AMDV-antibody production (*11*). In vitro studies have demonstrated a permissive infection (production of infectious progeny) of human macrophages with the Utah I strain of AMDV (*12*). We report finding anti-AMDV antibodies and AMDV genome in tissue from 2 mink farmers with relevant virus exposure and clinical disease similar to that in mink with AD.

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The Study

We examined AMDV antibody from each of the 2 patients by countercurrent and line electrophoresis (*13*). AMDV DNA was identified by standard and nested PCR. DNA was extracted from lymph nodes (patient 1) and from peripheral blood and bone marrow (patient 2) before amplification with AMDV-specific primers. AMDV DNA was identified by 2 different sets of primers in the standard PCR (5–600 bp) and with 2 complete different internal primers in the nested PCR (200 bp). PCR products were cloned, and some clones were sequenced to confirm the presence of AMDV DNA. All PCR reactions were done with appropriate controls.

Patient 1 was a mink farmer who had been exposed to AMDV-infected mink for 10 years. When he was 22 years of age, toe ulceration and claudication developed. Arteriography showed bilateral occlusions of several lower limb arteries and associated development of a collateral network of vessels. At the age of 25, he underwent embolectomy, and the removed tissue showed vessel wall inflammation with a granulomatous appearance but no necrotizing lesions or epitheloid or eosinophilic infiltration. Over the next 10 years, despite surgical attempts to revascularize and treatment with anticoagulant drugs, his condition deteriorated: his renal, mesenteric, and axillary arteries became stenosed, and his right leg was amputated. Antibodies to AMDV were found in his serum at the end of these 10 years and at 2 subsequent measurements after 1 additional year. An abdominal aortic biopsy showed adventitial lymphoplasmacytic cell infiltration and minimal atherosclerosis (Figure 1). A lymph node biopsy sample showed modest reactive changes and T-zone hyperplasia, and AMDV DNA was identified in the sample. At 35 years of age, the patient had a positive serologic result for anti-AMDV antibodies and severe claudication. Subsequent testing 1 and 2 years later showed negative results for AMDV antibodies and AMDV genome. The patient died in 1999, at 40 years of age, at which time his clinical condition resembled that of bilateral pneumonia. No specific infectious agent was identified. Postmortem examination showed periarterial collagen deposits, adventitial focal mononuclear accumulations, neutrophil infiltration in the media, fibrosis-related hyperplasia, lipid deposition and calcifications of the intima, and microabscesses within intraluminal thrombotic material.

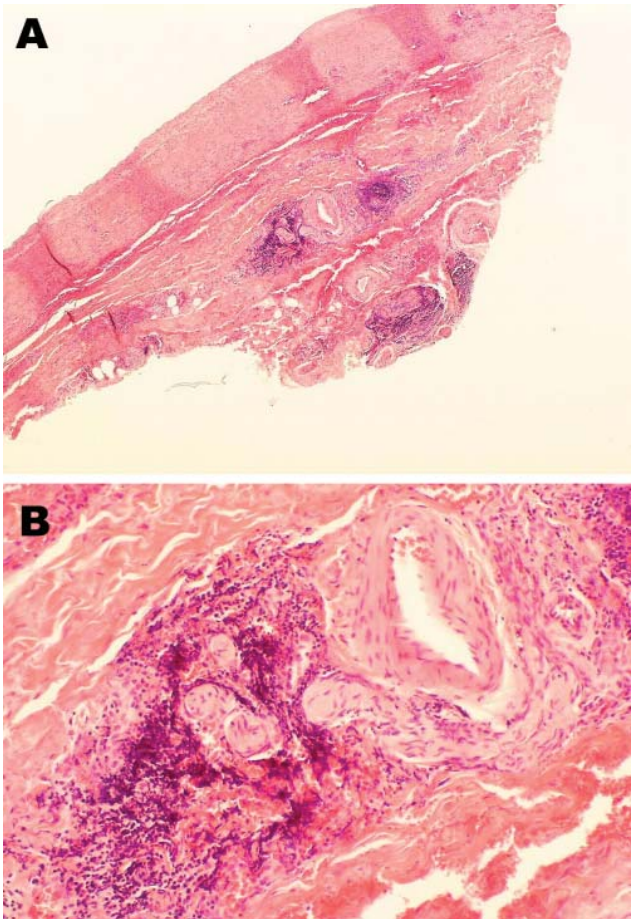

Figure 1. Histopathologic appearance of abdominal aortic biopsy sample from 35-year-old mink farmer in Denmark who had been exposed to Aleutian mink disease parvovirus–infected mink for 10 years (patient 1). A) Perivascular, adventitial lymphoplasmacytoid infiltration. Original magnification ×4. B) Minimal atherosclerotic changes. Original magnification ×20.

Patient 2 was also a mink farmer. He had been exposed to AMDV since the age of 20. At 54 years of age, 2 years after an extensive outbreak of AMDV among his mink, he was hospitalized for chronic glomerulonephritis. A renal biopsy sample showed endocapillary and mesangial proliferative glomerulonephritis with abundant focal semilunes (Figure 2, panel A). Immunofluorescence showed anti-immunoglobulin M and anti-C3 antibodies localized to the renal capillaries. Electron microscopy showed organized fibrillar deposits of stacked microtubules of ≈20 nm (Figure 2, panel B), consistent with fibrillary glomerulonephritis (*14*), an idiopathic condition characterized by polyclonal immune deposits with restricted gamma isotypes. No seroimmunologic information was available for the patient at this time. Immunosuppression improved his renal function, and he remained stable while receiving continuous immunosuppressive medication.

Eight years later, patient 2 was readmitted to the hospital for diarrhea, vomiting, pyrexia, asthenia, and confusion. Cerebrospinal fluid contained high levels of protein and pleocytosis, but no specific infectious agent could be isolated from the cerebrospinal fluid or blood. Magnetic resonance imaging showed an increased meningeal signal over both cerebral hemispheres. Subsequent investigations repeatedly demonstrated anti-AMDV antibodies and AMDV DNA in peripheral blood and bone marrow. Serum was still positive for AMDV antibodies 2 years later. Despite treatment with antimicrobial drugs, the patient further deteriorated and died in 2004, at age 63, after an additional year of hospitalization. At postmortem examination, the kidneys were reduced in size with evidence of cortical attenuation. Increased mesangial hypercellularity was observed. An adenocarcinoma of the right lung had metastasized to the su-

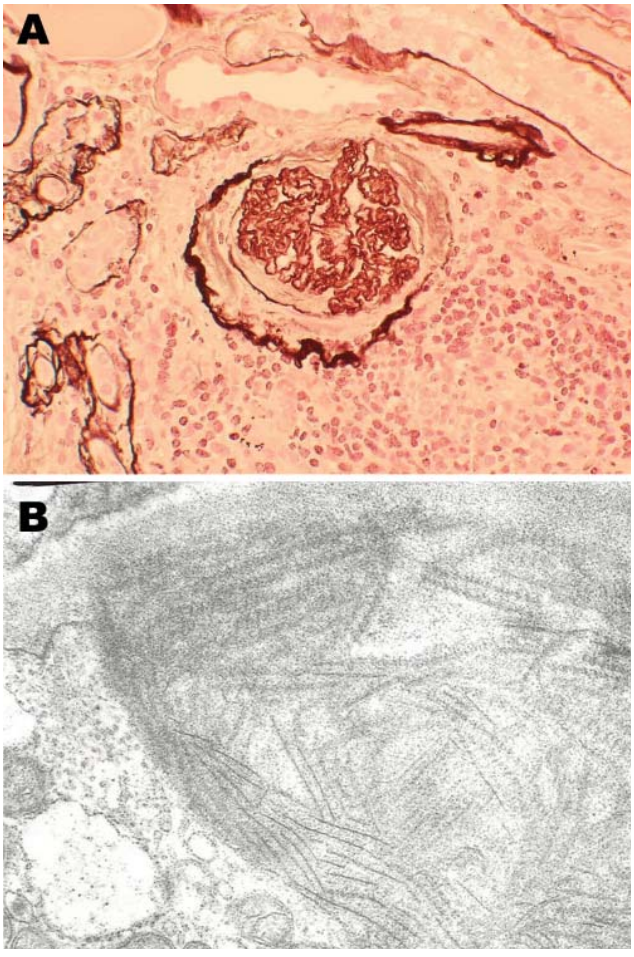

Figure 2. Histopathologic appearance of renal biopsy sample from 54-year-old mink farmer in Denmark who had been exposed to Aleutian mink disease parvovirus–infected mink for 34 years (patient 2). A) Glomeruli with hypercellularity and crescents. Original magnification ×20. B) Electron microscopy showing distinct extracellular deposits of coarse 20-nm fibrils (microtubular structures) characterized as immunotactoid glomerulopathy. Original magnification ×100,000.

prarenal glands, liver, and mesenterium. Coronary arteries and the aorta were moderately atherosclerotic.

Conclusions

The clinical history, histopathologic features, and molecular findings for the 2 mink farmers exposed to AMDV were similar to those described for AD in mink. The combination of clinical and laboratory findings is unique for these patients compared with previous reports. These 2 patients had micro- and macroangiopathic lesions and prolonged persistence of serum antibodies to AMDV and AMDV DNA.

On the basis of its early onset, dissemination, and severity, the slight atherosclerosis found in some histopatologic specimens from patient 1 represent a consequence of the pre-existing arteritis rather than a primary condition. Buerger disease is unlikely on the basis of cytopathologic and histopathologic findings, and other vasculitic disorders were excluded on the basis of serologic findings. The arteritis was similar to the autoimmune vascular lesions accompanying AD in mink with adventitial lymphocytic infiltration (*4*). The histopathologic findings for patient 2, in whom the autoimmune glomerulonephritis was diagnosed 8 years before the first measurement of anti-AMDV antibodies, resembled the immune complex–mediated glomerulonephritis in mink with AD (*6*).

For patient 1, anti-AMDV antibodies persisted 4 years from his last exposure to mink, exceeding the longest reported duration of positive AMDV response in a human in the absence of virus exposure (*11*). Similarly, patient 2 had a long-lasting antibody response, although under potentially persisting exposure. The persistence of anti-AMDV antibodies in patient 2 may reflect host-related factors in the modulation of immune response to chronic antigen stimulation. In mink, host-related factors influence their susceptibility to AMDV infection and correlate with clinical manifestations from an asymptomatic condition to overt disease. One may speculate whether the overrepresentation of malignant lymphoma in mink farmers could reflect a part of a disease spectrum paralleling monoclonal plasma cell proliferation in mink (*15*).

That AMDV DNA was found only in the first biopsy sample from patient 1 may weaken the hypothesis of virus replication. However, it may reflect technical difficulties with DNA amplification after paraffin embedding of the specimen or a possible later clearance of the virus from infected tissues. Regardless, we have described our clinicopathologic and molecular findings with the goal of raising awareness about the possible role of AMDV replication in human disease.

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Spatiotemporal Dynamics of Hemorrhagic Fever with Renal Syndrome, Beijing, People's Republic of China

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We used geographic information systems to characterize the dynamic change in spatial distribution of hemorrhagic fever with renal syndrome (HFRS) in Beijing, People's Republic of China. The seasonal variation in its incidence was observed by creating an epidemic curve. HFRS was associated with developed land, orchards, and rice paddies.

Hemorrhagic fever with renal syndrome (HFRS), a rodent-borne disease caused by hantaviruses (family *Bunyaviridae*), is characterized by fever, acute renal dysfunction, and hemorrhage manifestations. Various rodent species are natural hosts and serve as sources of infection (*1*). Humans usually acquire hantavirus infection by contact with or inhalation of aerosols or excreta from infected rodents (*2*,*3*). In the People's Republic of China, HFRS is caused mainly by 2 types of hantaviruses, Hantaan virus (HTNV) and Seoul virus (SEOV), each of which has coevolved with a distinct rodent host. HTNV is associated with *Apodemus agrarius*, whereas SEOV, which causes a less severe form of HFRS, is associated with *Rattus norvegicus*. Although HFRS infection has long been recognized in many places throughout mainland China, HFRS was first reported in metropolitan Beijing in 1997. Since then, the natural foci have been established, and human cases were continuously diagnosed in the new disease-endemic region (*4*).

The presence and transmission of hantavirus depend on the distribution and infection of its animal hosts, which largely determine the incidence and extent of HFRS; such distribution and infection are usually determined by environmental elements (*5*,*6*). Ecologic studies in China demonstrated that elevation, precipitation, temperature, vegetation type, and soil type influenced transmission of HTNV (*7*,*8*). However, these studies were conducted on a relatively large scale, usually at the county or even province level. Environmental factors driving variability in HFRS incidence at a finer scale (e.g., township) remain poorly understood. The availability of detailed records of HFRS cases and environmental information in the newly established disease-endemic region provides an opportunity to explore possible factors underlying the emergence of the rodent-borne disease. In this study, we aimed to learn the current situation of endemic HFRS in Beijing, characterize its spatiotemporal distribution, and identify environmental factors possibly contributing to the incidence of the disease.

The Study

The study area covered metropolitan Beijing (between 115°20′ and 117°30′ E, and 39°28′ and 41°05′N), including 220 townships of 18 districts with an area of 16,800 km^2. The data on reported HFRS cases were obtained from the National Notifiable Disease Surveillance System, which included information about sex, age, residential address, and onset date of symptoms for each case.

A total of 852 HFRS cases were reported in Beijing metropolis during 1997–2006. The annual incidence of each district was calculated by using the fifth national census data in 2000 and mapped by using a geographic information system (GIS) technique by digitalizing village, street, and boundaries on the 1:100,000 topographic map of Beijing in ArcGIS 9.0 software (ESRI Inc., Redlands, CA, USA). Each HFRS case was geocoded according to residential address, and a layer including information about HFRS cases was created and overlayed on the digital map. By 2000, the disease had affected all the area of the city. However, the incidence in each district varied during the 10-year study period (Figure 1).

An epidemic curve was created to show the temporal distribution of HFRS in Beijing. The annual incidence had sharply increased from 1997 to 1999; thereafter it fluctuated around 0.8 ± 0.2/100,000 persons (Figure 2). Within each year, the incidence varied markedly; most cases occurred in winter and spring, usually peaking in April, although the disease was reported in almost every month (Figure 2).

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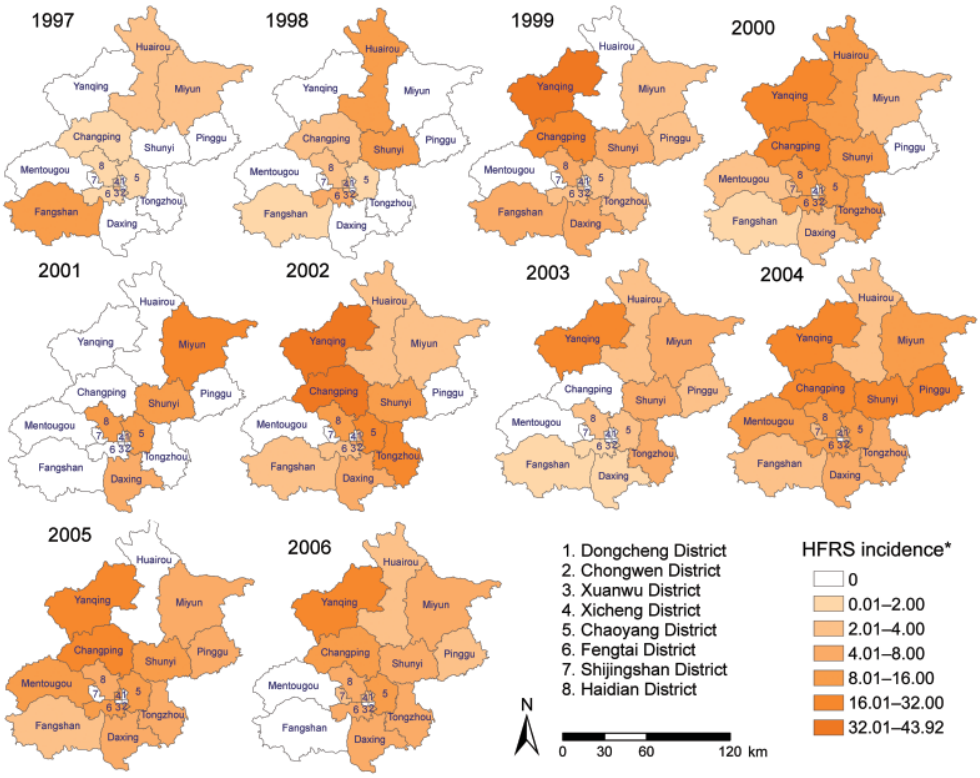

Figure 1. Yearly distribution of hemorrhagic fever with renal syndrome, Beijing, People's Republic of China, 1997–2006. *Per 100,000 population.

To study factors related to HFRS spread, we compared the incidence with various environmental indicators. Data on elevation were collected from a digital elevation model. Land cover data were derived from 2005 Landsat 7 enhanced thematic mapper image by using ENVI 4.0 software (Research Systems Inc., Boulder, CO, USA). Land use types were classified as follows: built-up land, water body, dry land, scrub, orchard, irrigable land, rice paddies, and forest. Average elevation and area of different land use type of each township were calculated by ArcTools and Spatial Analyst module in ArcGIS 9.0 software (ESRI Inc.).

To determine the associations between the number of HFRS cases per township during the study period and elevation, as well as land use type, we performed standard Poisson regression analysis by using STATA 9.1 software (StataCorp LP, College Station TX, USA) (*9*). The percentage change in incidence in response to the value change of the variable by a given amount and its 95% confidence interval and corresponding p value were estimated after correction for overdispersion. The Poisson regression analysis indicated that built-up land, orchard, and rice paddies were significantly associated with HFRS at $p<0.05$ (Table). The incidence rose with increasing coverage of orchard and rice paddies, but dropped with decreasing coverage of built-up land.

Conclusions

Since the first local infection was reported in 1997, HFRS cases have occurred in all 18 districts of Beijing, with a fairly stable annual incidence since 1999. The dynamic change in spatial distribution confirmed the focal nature of the rodent-borne disease. The seasonality is one of the epidemiologic characteristics of HFRS, and further indicates that HFRS in the region is caused by SEOV with domestic rats, with mainly *R. norvegicus* as the source of infection (*10*,*11*) The rat population apparently peaks in winter, resulting in a lagged impact on transmission and

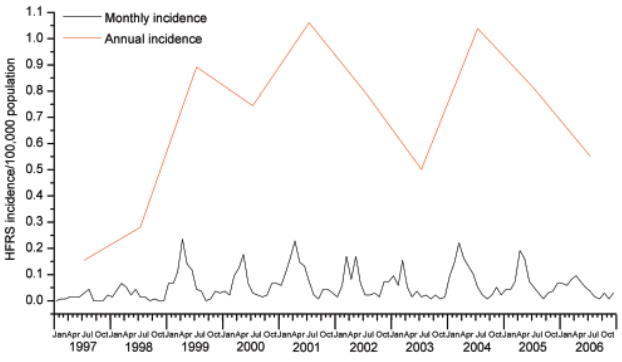

Figure 2. Temporal distribution of hemorrhagic fever with renal syndrome (HFRS), Beijing, People's Republic of China, 1997–2006.

Table. Poisson regression analysis of environmental factors in relation to HFRS incidence in Beijing, People's Republic of China*

Variable (unit)†	% Change‡	95% CI	p value
Elevation (10 m)	−0.47	−15.9 to 17.8	0.96
Built-up land (1%)	−0.82	−1.50 to 0.14	0.02
Water body (1%)	−0.29	−5.88 to 5.63	0.92
Dry land (1%)	1.44	−0.21 to 3.12	0.09
Scrub (1%)	−1.26	−4.66 to 2.52	0.48
Orchard (1%)	4.33	1.71 to 7.00	<0.01
Irrigable land (1%)	1.22	−0.10 to 2.56	0.07
Rice paddies (1%)	27.8	4.4 to 56.3	0.02
Forest (1%)	0.60	−0.52 to 1.73	0.30

*HFRS, hemorrhagic fever with renal syndrome; CI, confidence interval.
†Built-up land comprised human residences, industrial land, and land occupied by all kinds of roads. Water body comprised lakes, reservoirs, ponds, and watercourses of all kinds. Dry land comprised nonirrigated fields for planting crops. Scrub comprised bushes and shrubs. Orchards were areas producing fruits and raw materials for industry or for beverages. Irrigable land comprised fields under irrigation for planting crops. Rice paddies were fields for planting rice. Forest included areas with dense trees.
‡Percentage change in incidence rate if the value of the variable changed by the given amount.

seasonal variation of HFRS. In addition, rats are more active in homes in the winter because of cold temperature outside; this increases the chance for humans to acquire the infection by contact or inhalation of aerosols and secretions from infected rodents.

Our findings suggest that residents of townships with not too much built-up land but with orchard or rice paddies are at highest risk for infection. The land-use variables (acting as economic development indicators) are likely to be suitable for predicting the presence and distribution of HFRS in Beijing, where the disease recently emerged and economic development has greatly increased. Suitable control measures, such as removing rodents and preventing them from entering houses and human food storage buildings, should be taken to reduce incidence in this new disease-endemic region. However, the emergence and endemicity of HFRS are not determined only by the economic development activities, although they are important environmental contributors to the transmission of the disease. Biologic, ecologic, and social factors such as population immunity level, abundance and infection rate of host rodents, and human behavior, also may affect transmission of HFRS. Further epidemiologic and ecologic studies are required to understand the exact variables contributing to the emergence and extension of the disease during urbanization.

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.

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Age Patterns of Persons with Campylobacteriosis, England and Wales, 1990–2007

Iain A. Gillespie, Sarah J. O'Brien, and Frederick J. Bolton

To explore hypotheses for age-related changes in the incidence of *Campylobacter* infections in England and Wales during 1990–2007, we analyzed electronic laboratory data. Disease incidence reduced among children, and the greatest increase in risk was among those >60 years of age. Risk factors for campylobacteriosis in the elderly population should be identified.

Campylobacter infection remains a major public health problem worldwide. The infection is unpleasant, although generally self-limiting, and most patients experience acute enteritis for 7 to 10 days (*1*). Approximately one tenth of patients with laboratory-confirmed cases require hospital treatment as a result of their illness (*2*), and a range of gastrointestinal, arthritic, and neurologic sequelae add to disease effects (*3*). Although food is likely the main source of transmission to humans, most human infections cannot be explained by recognized risk factors.

In the late 1970s, when the role of *Campylobacter* spp. in human gastrointestinal disease had been newly appreciated, the number of laboratory-confirmed infections in England and Wales began to rise; 8,956 cases were reported in 1980 and 33,234 in 1989 (Health Protection Agency, unpub. data). This increase was largely artifactual, reflecting increased scientific interest in, and testing for, *Campylobacter* spp. and improvements in media and methods for isolating them (*4*,*5*). The incidence continued to rise throughout the 1990s and peaked in 2000 at 58,236 cases. The reasons for this increase are unknown; further methodologic improvements or increased surveillance activity in that decade cannot fully explain it. Incidence rapidly decreased between 2000 and 2004 (from 57,674 to 44,294 cases; 24% decrease; Health Protection Agency, unpub. data), after which incidence increased for 3 consecutive years; provisional total was 51,758 cases in 2007 (Health Protection Agency, unpub. data). The reasons for these recent changes in incidence are again unknown. To explore hypotheses for changes in incidence related to age, we analyzed electronic laboratory data for *Campylobacter* infections reported in England and Wales from 1990 through 2007.

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The Study

Data on all *Campylobacter* isolates obtained from fecal or lower gastrointestinal tract samples, reported in England and Wales from 1990 through 2007, were extracted from the national laboratory database (LabBase) and stored in a Microsoft Access (Microsoft Corp., Redmond, WA, USA) database. Cases were assigned to 10-year age groups and to geographic areas on the basis of laboratory location (northern, mid-country, southern). The season was assigned on the basis of the earliest available specimen date (spring, March–May; summer, June–August; autumn, September–November; winter, December–February). Data on cases of cryptosporidiosis and nontyphoidal salmonellosis for the same period were extracted and manipulated as above for comparative purposes. Denominator data for England and Wales for the same period were obtained from the Office for National Statistics. Data were analyzed in Microsoft Excel 2003 and Stata version 10 (StataCorp, College Station, TX, USA). Estimates of incidence per 100,000 population were calculated throughout; relative risks (RR) and 95% confidence intervals (CIs) were calculated as required.

From 1990 through 2007 in England and Wales, 838,436 cases of *Campylobacter* infection were reported; patient age was available for 810,632 case-patients (96.7%). From 1990 through 1999, incidence increased in all age groups (Figure 1), but the increase was proportionate to increasing age (0–9 years of age RR 1.07 [95% CI 1.03–1.10]; 10–19 years RR 1.47 [95% CI 1.41–1.55]; 20–59 years RR 1.78 [95% CI 1.75–1.81]; ≥60 years RR 2.51 [95% CI 2.41–2.61]). From 2000 through 2004, incidence declined in all age groups. However, although the degree of decline was similar for those 0–9 years of age (RR 0.77 [95% CI 0.74–0.8]), 10–19 years (RR 0.73 [95% CI 0.70–0.76]), and 20–59 years (RR 0.75 [95% CI 0.74–0.76]), for patients aged ≥60 years, the degree of decline was significantly lower (RR 0.88 [95% CI 0.86–0.91]; χ^2 $p<0.001$). Finally, although the incidence increased only moderately among those 10–19 years of age (RR 1.02 [95% CI 0.98–1.07]) and 20–59 years (RR 1.04 [95% CI 1.03–1.06]) from 2005 through 2007, greater increases were observed for those 0–9 years (RR 1.12 [95% CI 1.08–1.17]) and those ≥60 years (RR 1.33 [95% CI 1.29–1.36]). During the surveillance period, therefore, the incidence among patients ≥60 years of age, compared with the incidence among younger patients, increased markedly (RR 0.45 [95% CI 0.44–0.47] in 1990 to RR 1.17 [95% CI

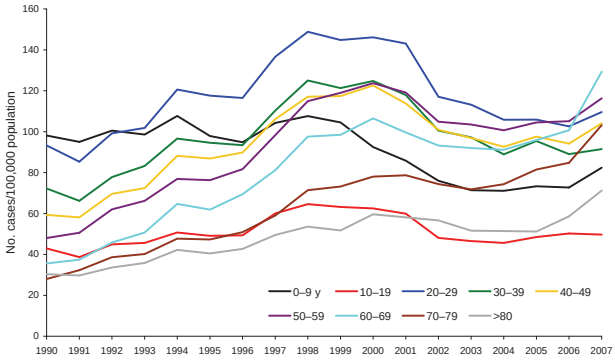

Figure 1. Incidence of laboratory-reported campylobacteriosis, England and Wales, by age group, 1990–2007.

1.15–1.19] in 2007). This effect was observed for campylobacteriosis in both sexes, in 3 geographic areas of England and Wales, and in all 4 seasons, but was not observed for nontyphoidal salmonellosis or cryptosporidiosis (Figure 2; online Appendix Table, available from www.cdc.gov/EID/content/16/1/2046-appT.htm).

We report a striking change in the population at risk for campylobacteriosis in England and Wales, which is independent of gender, geography, or season. The absence of a similar change in the age distribution of laboratory-reported salmonellosis or cryptosporidiosis from the same population suggests that this is unlikely to be a surveillance artifact.

Campylobacter infections are rarely typed beyond the genus level in England and Wales, so infections are routinely reported as *Campylobacter* species. Therefore, changes in the incidence of the various species that constitute this broad case definition could possibly explain some of the altered disease pattern reported. For example, infections caused by certain species (e.g., *C. fetus*) are more often associated with coexisting conditions that might occur more frequently in the elderly. This circumstance was unlikely to affect the results of this study, however. First, isolation methods used in England and Wales favor the growth of *C. jejuni* and *C. coli* (*4*,*5*) over that of other species, including *C. fetus*. Furthermore, *C. fetus* normally causes systemic infections detected through blood culture, and the proportion of blood isolations of *Campylobacter* species in patients ≥60 years of age reported in England and Wales remained constant from 1990 through 1999 (275/58,139 fecal isolations; 0.47%), from 2000 through 2004 (185/44,349; 0.42%), and from 2005 through 2007 (135/31,637; 0.42%) (Health Protection Agency, unpub. data).

When disease incidence was ranked according to specific population group, children 0–9 years of age had the highest ranking in 1990, but by 2007, incidence for this age group ranked seventh of 9 age groups. Incidence among children <10 years declined most rapidly from 1998 to 2003. This finding led to the hypothesis that the introduction and increased utilization of NHS Direct (a 24-hour telephone, online, and interactive digital TV service, which provided health advice and information) at this time had a "triage effect" on primary care presentation for this age group. The 2 events are correlated in time (initial NHS Direct pilot sites began taking calls in March 1998; by April 1999, 40% of the population of England had access, and by November 2000, the service was available throughout England and Wales [*6*]), NHS Direct has had a demonstrable negative effect on the use of general practice (*7*), and infants and young children are overrepresented among calls to NHS Direct about gastrointestinal conditions (*8*). The second Infectious Intestinal Disease study in England, currently under way (*9*), will provide further information upon which to assess this hypothesis, which is not readily testable by using laboratory data.

By far the most striking finding of this study is the emergence of older persons as the population most at risk for campylobacteriosis in England and Wales. Although the elderly were not the only group at risk in 2007 (because of increasing incidence), the overall trend singles them out

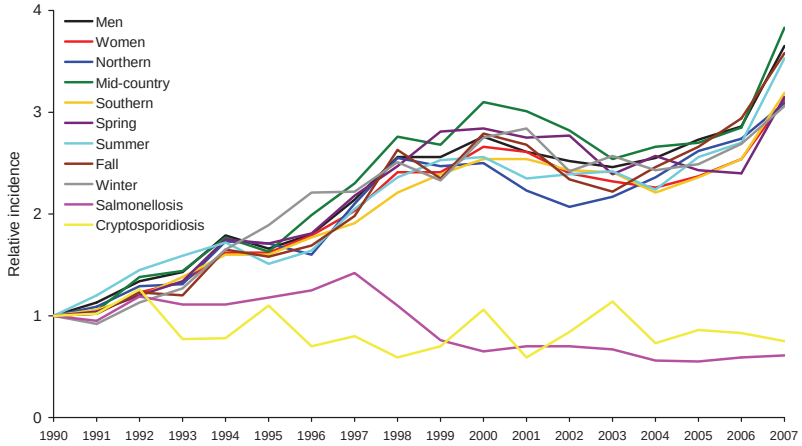

Figure 2. Relative incidence of campylobacteriosis by sex, region, and season, compared with rates of salmonellosis and cryptosporidiosis, among patients ≥60 years of age, England and Wales, 1991–2007. Northern, northwest, northeast, as well as Yorkshire and the Humber regions; mid-country, Wales, West Midlands, East Midlands, and East of England regions; southern, London as well as southeast and southwest regions. Salmonellosis includes nontyphoidal salmonellae, with age data available for 356,270 of 380,915 case-patients (94%); cryptosporidiosis includes age data for 76,462 of 79,808 case-patients (96%).

as the main emerging at-risk group (the increase in other age groups requires continued monitoring, however). The pattern of infection in older patients is perhaps predictable, given the similar pattern for incidence of listeriosis in England and Wales since 2001 (*10*). As life expectancy increases in the United Kingdom, the number of persons living with chronic conditions is likely to increase; these factors suggest that the incidence of campylobacteriosis in older persons will continue to increase in the future. Therefore, risk factors for *Campylobacter* infection specific to older UK residents must be identified.

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Identification of *Francisella tularensis* Cluster in Central and Western Europe

Paola Pilo,[1] Anders Johansson,[1] and Joachim Frey

We conducted a molecular analysis of *Francisella tularensis* strains isolated in Switzerland and identified a specific subpopulation belonging to a cluster of *F. tularensis* subsp. *holarctica* that is widely dispersed in central and western continental Europe. This subpopulation was present before the tularemia epidemics on the Iberian Peninsula.

Tularemia is a classical zoonosis caused by the facultative intracellular bacterium *Francisella tularensis*; it is transmissible to humans at infectious doses as low as 10–50 bacteria when inhaled in aerosols or by inoculation of the skin. Traditionally, tularemia is thought of as a disease contracted by persons performing outdoor activities such as hunting or farming, but it can also be acquired from pets, for example, hamsters or prairie dogs, which are occasionally traded internationally (*1*). During the past 15 years, the reemergence of tularemia has been reported in several European countries (*2*–*4*). Spain is a notable example, reporting 916 human infections from 1997 through 2007 in the Castilla and León regions alone (*5*). However, tularemia is rarely diagnosed in central Europe. In Switzerland, *F. tularensis* infection was first described in the 1950s, but the pathogen was not isolated until 1996, when *F. tularensis* infection began to reappear sporadically. To better understand the genetic diversity of Swiss *F. tularensis* strains and their relationship to strains from other geographic areas, we analyzed strains from Switzerland by using several methods that had been previously demonstrated to resolve genetic differences between *F. tularensis* subsp. *holarctica* strains: multilocus tandem repeat analysis (MLVA), canonical *F. tularensis* insertion deletion element (Ftind) analysis, and region of difference (RD) 23 analysis (*3*,*6*,*7*).

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The Study

Thirteen *F. tularensis* isolates collected over the past 10 years in Switzerland (Figure 1) were subjected to extensive genetic characterization. The species and subspecies designations of all strains were confirmed by real-time PCR that targeted the *fopA* gene and by amplification of the RD1 region (*8*), which showed that all strains were *F. tularensis* subsp. *holarctica*. A reference panel of 12 *F. tularensis* subsp. *holarctica* strains (*7*) and the genome sequence of the strain isolated in France, FTNF002 (GenBank accession no. NC_009749), were included in the study to represent the currently known genetic subpopulations within the subspecies. All strains from Switzerland were genetically characterized at 6 highly variable loci (by MLVA) and 14 more stable loci that indicate the classification *F. tularensis* subsp. *holarctica* strains into genetic subpopulations (by Ftind analysis) (*3*,*6*,*7*). The RD analysis was also performed because a 1.59-kb deletion marker, RD23, was reported to be restricted to strains from France and Spain (*3*). The MLVA markers (M3, M6, M20, M21, M22, and M24) and Ftind markers (Ftind 25–38) were amplified by PCR and then sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and the BigDye Terminator cycle sequencing kit (Applied Biosystems). DNA fragment sizes were calculated from the nucleotide sequences of the MLVA and Ftind markers and used to compare the isolates with previously analyzed strains from the United States, Japan, France, and Russia (*3*,*7*). The RD23 marker was assayed by using standard PCR and agarose gel methods as previously described (*3*). A cluster analysis based on the MLVA and indel size data was performed by using BioNumerics version 3.5 (Applied Maths, Kortrjik, Belgium).

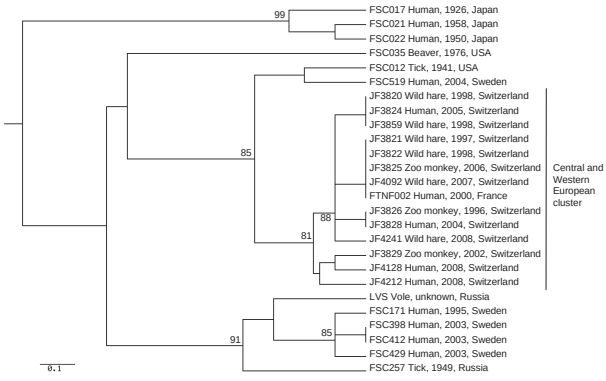

Figure 1. Genetic relationships between *Francisella tularensis* subsp. *holarctica* strains isolated in Switzerland and strains of wider geographic origin. The unweighted pair group method with arithmetic mean phylogram is based on the combined Ftind and multiple-locus variable-number tandem repeat analysis. Bootstrap values >80% are given at the respective nodes and were calculated by using 10,000 iterations. Scale bar indicates genetic distance.

[1]These authors contributed equally to this work.

As expected, the indel markers served to place each strain into major branches of the cluster tree, and the more variable MLVA markers provided the fine resolution at the tips of the tree. The Switzerland strains belonged to the same genetic cluster as the *F. tularensis* strain FTNF002 from France (Figure 1) that in a previous work clustered with strains from Spain (*3*,*9*). Moreover, all the Swiss strains exhibited the 1.59-kb genomic deletion at the RD23 locus and the unique 464-bp size at MLVA marker M24, which confirmed their close relationship to the French strain FTNF002 as well as to other strains from France and Spain (*3*).

The finding of *F. tularensis* strains in Switzerland represent sporadic occurrences of tularemia without any obvious epidemiologic connection. The strains originated from 6 hares, 3 monkeys, and 4 persons and were collected at different locations in Switzerland over a period of 10 years (Figure 2). The human infections most likely occurred through direct contact with wild animals, through rodent bites, and through consumption of a hare cooked at low temperature. Those isolates could be resolved into 7 different genotypes (Figure 2). Four Swiss strains displayed a genetic profile identical to that of the representative French strain FTNF002 (*3*). The other 6 genotypes were closely related to FTNF002, and all corresponded to the subclade B.Br:FTNF002–00 as defined by canonical single nucleotide polymorphisms by Vogler et al. (*9*). This cluster, which also contained the strains from the Iberian Peninsula, seems to have spread throughout central and western Europe. Moreover, all the Swiss strains were susceptible to erythromycin (MICs 0.25 μg/mL to 1 μg/mL), which is a phenotypic marker that has previously been suggested to divide *F. tularensis* subsp. *holarctica* strains into 2 taxonomic groups (*10*–*12*).

Figure 2. Geographic distribution of *Francisella tularensis* subsp. *holarctica* strains of the central and western European genetic cluster isolated in Switzerland. Dots represent the geographic origin of the isolates (from 7 Swiss cantons). The dashed line indicates the Alps. Strains of the subclade B.Br:FTNF002–00 are known to be present in France and Spain.

Conclusions

Strains of *F. tularensis* from Switzerland (central Europe) genetically clustered with strains from France and Spain (western Europe) as determined by the unique 464-bp genetic marker M24 and a specific deletion at marker RD23. Furthermore, strains within the cluster differed at only 2 MLVA markers,and 4 other MLVA and 14 Ftind markers were identical. In a previous study that included strains from the 1997–1998 tularemia outbreak in Spain, the specific M24 allele and the RD23 deletion were found in 49 of 49 strains from Spain and France but in only 1 of 189 strains from 7 northern and eastern European countries and Japan (*3*). The tularemia outbreak of 1997–1998 in Spain, which resulted in >500 human cases (*5*), was thus caused by *F. tularensis* strains that were genetically closely related to strains recovered in Switzerland from 1996 onwards, before the beginning of the outbreaks in Spain. This genetic relationship shows that factors other than the presence or introduction of a specific clone of the infectious agent per se determined the magnitude of the tularemia outbreaks in Spain. For epidemiologists to understand the distribution of *F. tularensis* (and other rare disease agents) in the environment and their propagation across national and geographic borders, surveillance programs that include molecular analyses of these agents should be undertaken in multiple countries, and the resulting data should be shared internationally.

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New Adenovirus in Bats, Germany

Michael Sonntag, Kristin Mühldorfer, Stephanie Speck, Gudrun Wibbelt, and Andreas Kurth

We tested 55 deceased vespertilionid bats of 12 species from southern Germany for virus infections. A new adenovirus was isolated from tissue samples of 2 *Pipistrellus pipistrellus* bats, which represents the only chiropteran virus isolate found in Europe besides lyssavirus (rabies virus). Evidence was found for adenovirus transmission between bats.

Since the recent discoveries of Ebola virus, Henipavirus, and severe acute respiratory syndrome–associated coronavirus infections, interest in the role of bats as hosts for pathogens has markedly increased (*1*). With the exception of worldwide studies on bat lyssaviruses (*2*), most virologic investigations in bats have been limited to a particular zoonotic agent implicated in a geographically localized disease outbreak (*3*–*5*). In the remaining studies, various medically less important viruses have been discovered in bats in the Americas, Africa, Asia, and Australia (*1*,*6*). As a result of increasing research efforts regarding bats and infectious diseases in Europe, 2 new virus groups were recently detected, namely beta- and gammaherpesviruses in organ tissue (*7*) and group I coronaviruses in feces of European vespertilionid bats (*8*). However, because all bat species in Europe/Germany are protected by strict regulations, the acquisition of suitable samples for virus isolation is rather challenging in comparison to most other parts of the world.

The Study

We performed an extensive search for unknown viruses in 55 German vespertilionid bats based on both generic PCR assays and virus isolation techniques, as part of a broader study investigating histopathologic changes in German bats in association with infectious pathogens. Dead or moribund bats of 12 species (*Barbastella barbastellus, Eptesicus nilssoni, E. serotinus, Myotis daubentonii, M. mystacinus, Nyctalus leisleri, N. noctula, Pipistrellus kuhli, P. nathusii, P. pipistrellus, Plecotus auritus*, and *Vespertilio murinus*) were collected at certified bat rehabilitation centers in southern Germany and were investigated macroscopically, bacteriologically, and histologically.

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For virologic examination, homogenized organ tissue was inoculated onto VeroE6/7 cells and monitored daily for cytopathic effects. Remaining tissue material was used for RNA/DNA extraction and further molecular analysis by generic PCR assays to detect members of several virus families including flaviviruses, hantaviruses, coronaviruses, orthomyxoviruses, and paramyxoviruses. The species of bat involved was determined by amplification and sequencing of the cytochrome B (*cytB*) gene, a standard technique for species identification (*9*).

Of the tested samples from 55 bats, virus was initially detected in only 2 adult common pipistrelles (*P. pipistrellus*, nos. 198/07 and 199/07). A cytopathic effect was detected in Vero E6/7 cells after the second passage, indicating the presence of virus in the cell culture. Purified supernatant of these cell cultures was subjected to negative-staining electron microscopy, which showed numerous adenovirus-like particles (Figure 1, panel A). The family *Adenoviridae* was

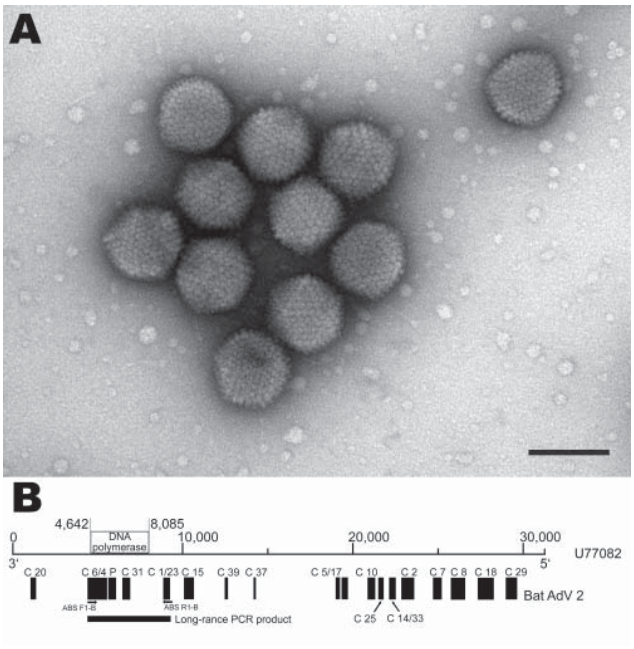

Figure 1. A) Electron micrograph of adenovirus particles isolated from *Pipistrellus pipistrellus* bat 199/07, Germany. Negatively stained with 1% uranyl acetate. Virus particles were 70–90 nm in diameter with an icosahedral shape. Scale bar = 100 nm. B) Schematic representation of the genomic fragments obtained from bat adenovirus 2 (GenBank accession no. FJ983127) in correspondence to canine adenovirus 2 strain Toronto A26/61 (GenBank accession no. U77082). Genomic fragments were generated by generic adenovirus-specific PCR (*10*) and a virus discovery based on cDNA–amplified fragment length polymorphism PCR method (*11*). Partial sequence of the DNA polymerase gene was generated from LongRange PCR product. Purified PCR products were directly sequenced by using the BigDye Terminator Cycle Sequencing ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3770 automatic sequencer (Applied Biosytems). C, clone; P, ≈550-bp nested PCR product.

Table 1. Sequence identities (%) of partial DNA polymerase gene (3,408 bp) between bat AdV-2 and selected adenoviruses, Germany*

Virus	Canine AdV-2	Canine AdV-1	Porcine AdV-5	Human AdV-26	Simian AdV-7	Bovine AdV-2	Tree shrew AdV-1
Bat AdV-2 PPV1	74	72	70	69	69	68	68

*AdV, adenovirus; PPV1, *Pipistrellus pipistrellus* virus 1.

verified by the first reaction of a generic adenovirus-specific nested PCR (*10*). The obtained sequence of a fragment of the DNA polymerase gene (≈550 bp) indicated that the viruses were a novel virus type within the genus *Mastadenovirus* and was tentatively named bat adenovirus 2 (bat AdV-2) strain *P. pipistrellus* virus 1 (PPV1).

To obtain additional sequence information of bat AdV-2, a random PCR method (virus discovery based on cDNA–amplified fragment length polymorphism) (*11*) was applied, which showed >20 adenovirus sequences distributed over the genome (Figure 1, panel B). The partial sequence of the bat AdV-2 DNA polymerase (3,408 bp; GenBank accession no. FJ983127) was obtained after LongRange PCR by using the Expand Long Range dNTPack (Roche, Mannheim, Germany) according to the manufacturer's directions and the following 2 primers: ABS F1-B (5′-AAAAgAggCAAAgCAAgACAgTgg-3′) and ABS R2-B (5′-ggCgggCAACAAAgACCTCA-3′). After repeated sequence analysis of the partial DNA polymerase gene for validation, we found that the identities of bat AdV-2 PPV1 to closely related adenoviruses ranged from 68% to 74% on nucleic acid level (Table 1), with the closest relationship found to canine adenoviruses 1 and 2. So far, the only other adenovirus in bats has been accidentally isolated from primary bat kidney cells of a healthy Ryukyu flying fox (*Pteropus dasymallus yayeyamae*) (*12*), which proved to have a rather distant phylogenetic relationship to bat AdV-2 (Figure 2).

On the basis of newly acquired sequence information, we designed a specific real-time TaqMan PCR to detect bat AdV-2 (ABS forward 5′-CACAAgTgg TgTCTTTgAgAgCA-3′, ABS reverse 5′-AgAgggATAC AAACTgATggAAACA-3′, ABS TM 6FAM-CTAACTTggCTggTggAgTgCgAAAC-q). Cycler conditions were as follows: predenaturation (95°C for 10 min), 45 amplification cycles (95°C for 30 s, 61°C for 30 s, 72°C for 30 s), and final extension (72°C for 10 min).

After screening all 55 bats from Germany of 12 species, comprising an additional 11 common pipistrelles, an identical adenovirus was detected in 1 additional common pipistrelle. Moreover, the tissue tropism of bat AdV-2 was investigated in all 3 infected bats (Table 2). Of all tested organs, bat AdV-2 was detected in high DNA copy numbers in the intestine of all 3 bats with lesser DNA copy numbers in liver and kidneys, whereas the other organs contained little or no adenovirus DNA. Unfortunately, due to advanced tissue decomposition in most of the organs, including liver, kidneys, and intestines, thorough histopathologic examination of the 3 bats was markedly impaired.

Conclusions

In contrast to Maeda et al. (*12*), who postulated the necessity of primary bat cells to isolate DNA virus from chiroptera, our isolation of a DNA virus from an European bat in a permanent cell line (monkey kidney cells) proved the opposite. We believe that the rare detection and isolation of viruses might be attributed to the fast natural degradation of bats of the suborder Microchiroptera in comparison to that of other animal carcasses, most likely due to their extremely low weight (2–10 g). Although viruses were not detected by various generic PCR assays from homogenized frozen tissue samples, we isolated a novel virus from a hibernating

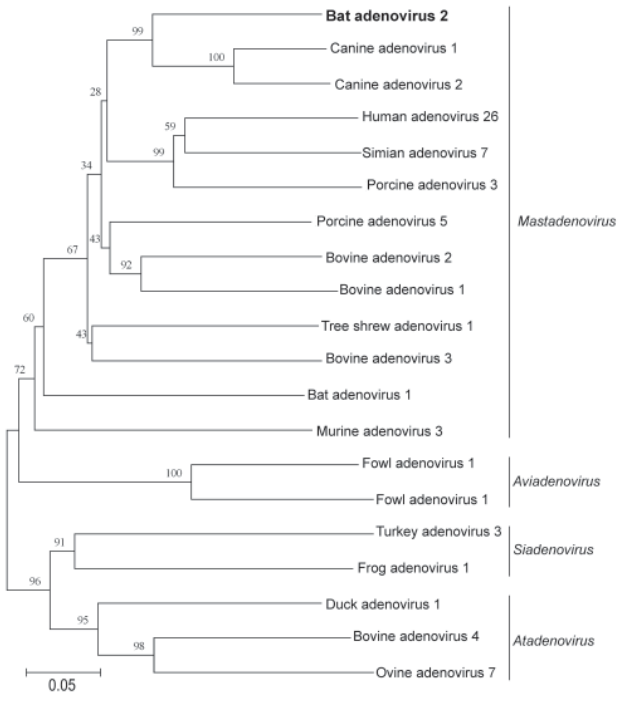

Figure 2. Phylogenetic tree constructed by using a multiple alignment of ≈550-bp amplicons, consisting of the partial DNA polymerase gene of the novel bat adenovirus 2 strain *Pipistrellus pipistrellus* virus 1 (in **boldface**; GenBank accession no. FJ983127) and selected members of the family *Adenoviridae*, Germany. Alignment was analyzed with the neighbor-joining method and p-distance model in MEGA4 (www.megasoftware.net). Bootstrap values (1,000 replicates) >35% are indicated at the branch nodes. Branch length is proportional to evolutionary distance (scale bar). Adenovirus genera are indicated.

Table 2. Distribution of DNA copy numbers of bat AdV-2 in different organs of similar size of infected *Pipistrellus pipistrellus* bats, Germany*†

Bat no.	Intestine	Liver	Kidney	Lung	Heart	Brain	Spleen
198/07	+++++	+++	++++	+	–	–	ND
199/07	++++++	++++	+++	+	+	–	ND
200/07	+++++++	+++	++	+	–	–	++

*AdV, adenovirus; +, positive; –, negative; ND, not determined.
†Analyzed by bat AdV-2 PPV1–specific TaqMan PCR. Increasing virus DNA copy numbers are indicated by the number of plus signs, which are equivalent to multiples of 3 cycle threshold values, starting with values from 37.0 to 40.0.

insectivorous bat species. This virus was detected in high DNA copy numbers in the intestine of 3 bats that died of natural causes.

The fact that no other viral or bacterial agents were detected in these animals suggests a clinical correlation to the isolated adenovirus. Moreover, all 3 bats belonged to the same species and were of similar age. Several days before their death, they were found moribund and subsequently admitted together to the rehabilitation center, which highlights the strong likelihood of infection in the colony of origin. Cross-contamination during tissue preparation can be excluded because sterilized instruments were used for each animal, and after every incision, instruments were cleaned with 70% ethanol and a Bunsen burner flame to destroy adhering tissue remnants.

Various adenovirus types of the genus *Mastdenovirus* infect a range of different mammals and cause respiratory, ocular, and gastrointestinal diseases. Here, in all 3 infected bats, the highest copy number of adenovirus DNA was detected in the intestine, which suggests a correlation with a gastrointestinal disease. The host range of mastadenoviruses is known to be limited to a single (or a few closely related) mammalian species (*13*) with a probable co-evolution between virus and their hosts (*14*). The acquired partial sequence of the bat AdV-2 DNA polymerase with the closest relation to canine adenovirus (only 74% at the nucleic acid level) and the isolation from a new animal host suggests that this virus is a new adenovirus species within the genus *Mastadenovirus*. A comparison to the only other adenovirus found in a bat (flying fox, order Megachiroptera) with the available sequence information of a ≈550-bp fragment of the DNA polymerase gene showed their distant relationship. This strict separation reflects either the co-evolutionary development between the 2 adenoviruses (bat AdV-1 FBV1, bat AdV-2 PPV1) and their host families Pteropodidae and Vespertilionidae or a host switch of the virus originating from a yet-undetermined vertebrate host. To elucidate this problem, further research will be necessary.

In conclusion, we isolated a new virus from free-ranging vespertilionid bats, which represents the only chiropteran virus isolate besides lyssavirus (rabies) found in Europe. Moreover, the detection of this chiropteran virus can be connected with its transmission between individual bats living in close proximity to other bats.

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Human Trichinosis after Consumption of Soft-Shelled Turtles, Taiwan

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In 2008, an outbreak of human trichinosis associated with ingestion of raw soft-shelled turtles was identified and investigated in Taiwan. The data suggested that patients were likely infected with *Trichinella papuae.*

Trichinosis is a zoonotic disease caused by species of the nematode *Trichinella*. In eastern Asia, human trichinosis has been reported in the People's Republic of China, Japan, and Korea (*1*–*4*). Trichinosis among humans and animals has not been reported in Taiwan (*1*,*5*,*6*). Although the major source of human infection is the meat of mammals, reptiles recently have been found to serve as hosts for certain *Trichinella* species. *T. zimbabwensis*, detected in the muscles of Nile crocodiles (*Crocodylus niloticus*) in Zimbabwe in 1995, is the first species of *Trichinella* found in a reptile host naturally infected with *Trichinella* (*7*). No human infection has been documented. Another species, *T. papuae*, was detected in a farmed saltwater crocodile in Papua New Guinea in 2004 (*8*). A trichinosis outbreak in humans caused by *T. papuae*, associated with eating wild boar meat, occurred in Thailand (*9*). Trichinosis in humans related to consumption of reptile meat was first described in Thailand; the source was turtle and brown lizard meat (*10*). We report an outbreak of human trichinosis in Taiwan in which eating raw soft-shelled turtles (*Pelodiscus sinensis*) was the suspected mode of infection.

The Study

In July 2008, four teaching hospitals in northern Taiwan consecutively reported to the Department of Health of Taipei City Government (DHTCG) and the Centers for Disease Control (CDC) 8 patients in 2 groups in whom fever, myalgias, and eosinophilia of unknown cause developed after they shared a common food source in May 2008. Group A, comprising 20 Taiwanese, participated in a festive meal in Taipei City at a Japanese food restaurant, and were served raw meat, blood, liver, and eggs of 3 of the 5 soft-shelled turtles provided by the host, a supplier of soft-shelled turtles. The other 2 soft-shelled turtles were refrigerated at 4°C and served at the same restaurant to a group of 3 Japanese customers (group B) 6 days later.

DHTCG and Taiwan CDC jointly investigated this outbreak. The restaurant had never previously served raw or undercooked soft-shelled turtles. Restaurant patrons other than those in groups A and B did not eat raw or undercooked soft-shelled turtles. Five of the 20 Taiwanese in group A and the 3 Japanese in group B exhibited signs and symptoms 1–3 weeks after eating at the restaurant and were defined as case-patients (Table 1). The 15 asymptomatic persons were defined as controls.

The most common symptoms were myalgia (88%), fever (88%), malaise (63%), and periorbital swelling (38%). Seven case-patients whose blood was analyzed all had eosinophilia and increased serum creatine phosphokinase and alanine aminotransferase levels. Five case-patients were hospitalized. Two patients underwent extensive serologic testing for helminths by ELISA, including tests for *Dirofilaria immitis, Toxocara canis, Ascaris lumbricoides, Anisakis* spp., *Gnathostoma spinigerum, Strongyloides stercoralis, Paragonimus westermanii, Paragonimus miyazakii, Fasciola hepatica, Clonorchis sinensis, Spirometra erinacei, Taenia solium, and Trichinella* spp. Both patients had weakly positive results for *A. lumbricoides, G. spinigerum,* and *S. stercoralis* and strongly reactive results for *Trichinella* spp.

Serum samples from 5 patients during the acute phase (3–5 weeks postexposure) and from all 8 patients during the convalescent phase (7–9 weeks postexposure) were sent to the Department of Parasitology, Gifu University, Gifu, Japan, for *Trichinella* serologic diagnosis, with ELISA and immunohistochemical staining. Of the 15 controls, none consented to give a blood sample. Briefly, the ELISA microtiter plates were sensitized with excretory-secretory (ES) antigen from *T. spiralis* or *T. pseudospiralis*, probed with a diluted human serum sample (1:200–1:6,400), and incubated with 100 μL of 1:10,000-diluted goat antihuman immunoglobulin G (Fab specific) peroxidase-conjugate (Sigma Chemical Co., St. Louis, MO, USA). Absorbance at 414 nm was monitored with a plate reader. All samples were analyzed in duplicate. The cutoff point was 3× the mean values of the A_{414} for the negative controls. Immunohistochemical staining was performed by incubating skeletal muscle tissues from *T. spiralis*–infected mice with the serum specimens (1:200 dilution) for 1 h at 37°C and

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Table 1. Clinical characteristics and results of serologic assays of 8 case-patients with *Trichinella* infection, Taiwan, 2008

Patient no./group	Age, y/ sex	Incubation period, d	Symptoms	Eosinophils, cells/μL	Acute-phase titer†		Convalescent-phase titer‡	
					T. spiralis	*T. pseudospiralis*	*T. spiralis*	*T. pseudospiralis*
1/A	60/M	7	Fever, myalgia, malaise	6,825	25,600	25,600	51,200	51,200
2/A	52/M	6	Fever, myalgia, malaise, periorbital swelling, leg swelling	3,815	800	400	51,200	51,200
3/A	57/M	14	Myalgia, malaise, trismus, tremor	2,713	NA*	NA	12,800	12,800
4/A	57/F	8	Fever, chills, dyspnea, myalgia, malaise, trismus, tremor, periorbital swelling	5,055	NA	NA	51,200	51,200
5/A	62/M	15	Fever, myalgia, malaise	1,421	3,200	1,600	51,200	51,200
6/B	52/M	8	Fever	4,461	12,800	6,400	51,200	25,600
7/B	57/M	7	Fever, myalgia, leg swelling, periorbital swelling, skin rash	8,505	12,800	12,800	25,600	12,800
8/B	47/M	8	Fever, myalgia	NA	NA	NA	25,600	25,600

*NA, not applicable.
†Weeks 3–5 postexposure.
‡Weeks 7–9 postexposure.

processing the sections with the HistoStain SP kit (Zymed Laboratories Inc., San Francisco, CA, USA).

In both the acute and convalescent phases, all serum samples reacted to *T. spiralis* and *T. pseudospiralis* ES antigen and were positive in immunohistochemical staining (Table 1). The diagnosis of trichinosis was confirmed. Mebendazole or albendazole was prescribed for all patients, and their symptoms gradually resolved.

We conducted semistructured interviews with the 8 case-patients and 15 controls in both groups to learn which food items they had eaten at the restaurant. None had eaten raw or undercooked soft-shelled turtles before this outbreak (Table 2). In univariate analysis, consumption of raw soft-shelled turtle meat was strongly associated with infection ($p = 0.003$). Trichinosis developed in 8 (62%) of the 13 persons who ate raw soft-shelled turtle meat.

We performed an environmental study of the restaurant and the soft-shelled turtle farm. No leftover food was available from the restaurant for analysis. The soft-shelled turtles were bred artificially and hatched on a farm in Taiwan. They were fed only indigenous fish and shellfish. The farm used neither imported feed nor feed containing any mammals or reptiles. Microscopic inspection, with a meat-digesting method, of the soft-shelled turtles obtained from the farm 2 months after the outbreak did not show *Trichinella* spp. After the investigation, Taiwan CDC issued a press release to describe the outbreak and alert the public of the risk for trichinosis from eating raw or undercooked soft-shelled turtles.

Conclusions

The incubation period, clinical features, and laboratory findings in this outbreak are similar to those of other reported trichinosis outbreaks associated with eating mammals (*11*,*12*). *T. papuae* and *T. zimbabwensis* are the most likely parasites causing this outbreak because of their abil-

Table 2. Results of univariate analyses of selected food items in an outbreak of trichinosis, Taiwan, 2008*

Ingested food items	Case (n = 8)		Control (n = 15)		OR (95% CI)†	p value‡
	Ate	Did not eat	Ate	Did not eat		
Soft-shelled turtles						
Raw meat	8	0	5	10	–	0.003
Fried meat	6	2	14	1	0.21 (0.003–5.22)	0.269
Raw liver	7	1	8	7	6.13 (0.51–314.71)	0.176
Fresh blood	6	2	7	8	3.43 (0.40–43.28)	0.379
Raw eggs	7	1	10	5	3.50 (0.28–188.78)	0.369
Raw intestines	3	5	2	13	3.90 (0.32–56.52)	0.297
Cooked soup	7	1	13	2	1.08 (0.05–72.50)	1.000
Rice with cooked eel	7	1	15	0	–	0.348
Raw abalone	6	2	12	3	0.75 (0.07–11.43)	1.000

*OR, odds ratio; CI, confidence interval.
†Significant at $\alpha = 0.05$.
‡By Fisher exact test.

ity to infect mammals and reptiles (*13*). The ELISA method has limited specificity because of cross-reactions with non-*Trichinella* helminths (*14*). Moreover, because of similar antigen patterns among all *Trichinella* spp., the antigens prepared with 1 species can be used to detect specific antibodies in patients infected with any species (*1*). Therefore, although we detected strongly reactive antibodies to *T. spiralis* and *T. pseudospiralis*, we could not determine the etiologic *Trichinella* sp. in this outbreak without parasitic diagnosis.

A recent study demonstrated that the 53-kDa recombinant proteins in larval ES products could provide species-specific antibody responses in *Trichinella*-infected mice (*15*). We assessed the absorbance at 414 nm with a 1:200-diluted serum sample in our patients by using the 53-kDa recombinant proteins expressed from 5 *Trichinella* species (*T. spiralis, T. britovi, T. nativa, T. pseudospiralis, and T. papuae*). Our preliminary results showed that convalescent-phase serum specimens from 6 of the 8 case-patients reacted most strongly to the 53-kDa recombinant protein of *T. papuae*. Although application of this method in species-specific human diagnosis requires further studies, the data suggest our patients were likely to be infected with *T. papuae.* Because we have not yet determined how soft-shelled turtles were infected by *T. papuae* in this outbreak, further investigation of the potential infectious source is warranted.

Persons in many parts of the world typically consume raw or uncooked reptile meat. Further investigations are urgently needed to assess the epidemiology of reptile trichinosis and the human risk for trichinosis from reptiles.

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Extracorporeal Membrane Oxygenation for Pandemic (H1N1) 2009

To the Editor: As the world struggles with the challenges of influenza A pandemic (H1N1) 2009, it is clear that treatment options for critically ill infected patients are suboptimal because deaths continue to be reported in otherwise young and healthy patients. Extracorporeal membrane oxygenation (ECMO) is an established therapeutic option for patients with medically refractory cardiogenic or respiratory failure. We describe the successful use of ECMO in a patient with complicated pneumonia and influenza A pandemic (H1N1) 2009 virus infection.

Our patient, a 21-year-old woman who was 4 months postpartum, had poorly controlled insulin-dependent diabetes (hemoglobin A1C level 13.2 mg/dL). She sought treatment at another hospital after 3 days of respiratory symptoms, a productive cough after working in her garden, and a fever ≥103°F. Her condition rapidly deteriorated, and she required mechanical ventilation, vasoactive medications, and drotecogin-α (Xigris; Eli Lilly and Company, Indianapolis, IN, USA) for profound shock.

The patient was then transferred to Ohio State University Medical Center on August 24, 2009; at admission she exhibited hypotension (83/43 mm Hg) and tachycardia (159 bpm), despite having received high doses of vasoactive medications (norepinephrine 1.0 μg/kg/min, phenylephrine 2.0 μg/kg/min). A transthoracic echocardiograph showed severe biventricular failure (ejection fraction 5%–10%); peak tropinin level was 6 mg/dL. Arterial blood gas confirmed metabolic acidosis (pH 7.12, partial carbon dioxide pressure [pCO_2] 48 mm Hg, pO_2 117 mm Hg, HCO_3 15.3 mmol/L). Despite fluid resuscitation and administration of epinephrine (0.06 μg/kg/min), her condition failed to improve, and she was given femoral vein–femoral artery ECMO.

A comprehensive search for infectious causes was undertaken. Treatment with broad-spectrum empiric antimicrobial drugs such as linezolid (Pfizer, Inc, New York, NY, USA), piperacillin/tazobactam (Wyeth, Madison, NJ, USA), and doxycycline (Pfizer, Inc) and the antiviral drug oseltamivir (Tamiflu; Roche Laboratories Inc., Nutley, NJ, USA), 150 mg 2×/d, was started. Respiratory cultures were positive for methicillin-sensitive *Staphylococcus aureus* and *Aspergillus glaucus*. Naficillin and voriconazole were added to the treatment regimen. PCR of a bronchoalveolar lavage specimen later identified pandemic (H1N1) 2009 virus. The patient was weaned from ECMO on hospital day (HD) 10 and extubated on HD11. Repeat cardiovascular evaluation showed normal biventricular function and no coronary disease. She was discharged from hospital for rehabilitation on September 15, 2009 (HD 22), with an oxygen saturation of 98% on room air and is now fully recovered.

The use of ECMO is an established option for patients with medically refractory acute and reversible cardiopulmonary failure (*1*–*3*) (Table). For isolated respiratory failure, veno–veno support can be used by femoral vein to femoral vein or femoral vein to right internal jugular vein cannulation. With concomitant cardiogenic shock, veno–arterial cannulation may be required with cannulation of the right internal jugular or femoral vein for outflow, and for inflow, the femoral artery directly or the axillary artery by a surgically placed side graft. Central venous (right atrium) and arterial (ascending aorta) cannulation is an option but requires median sternotomy.

This case is not the first reported use of ECMO for respiratory failure secondary to viral pneumonia (*4*), and recently, ECMO was used with

Table. Relative indications and contraindications for extracorporeal membrane oxygenation*

Characteristics
Cardiac support (*1*)
Cardiac index <2.2 L/min/m^2
Systolic pressure <90 mm Hg
Pulmonary capillary wedge pressure >20 mm Hg
Central venous pressure >20 mm Hg
Two high-dose inotropic medications (Including intraaortic counter pulsation)
Respiratory support (*2*)
Murray score >3.0 based on:
PaO_2/FiO_2 ratio
No. infiltrated quadrants on chest radiograph
Positive end-expiratory pressure requirement
Pulmonary compliance
Uncompensated hypercapnea (pH<7.2)
Contraindications
Advancing age (>70 y)
Prolonged mechanical ventilation (>7 d)
Surgically correctable causes
Pneumothorax, effusions, endoluminal obstructions
Intracardiac shunts, valvular pathology, incomplete revascularization
Medical problems incompatible with prolonged survival
Advanced malignancies
Contraindications to anticoagulation
Irreversible neurologic dysfunction (dementia, stroke, hemorrhage)
Medical futility (i.e., prolonged CPR, multiorgan failure)

*CPR, cardiopulmonary resuscitation; PaO_2, partial pressure of oxygen in arterial blood; F_1O_2, concentration of inspired oxygen.

limited success for complications of pandemic (H1N1) 2009 (*5*). Its broader use in treating critically ill patients has been limited, however, because ECMO requires substantial institutional and multidisciplinary commitment for implementation and is typically only available at major medical centers offering cardiovascular surgery.

Although we cannot say specifically why our patient survived, clearly, aggressive and comprehensive empiric treatment, physiologic support, and close multidisciplinary communication were vital to managing the condition of this critically ill patient. ECMO may have assisted in organ recovery and patient survival. However, further studies should be conducted to critically evaluate ECMO in the armamentarium of therapeutic options for severe pandemic (H1N1) 2009 respiratory failure.

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Respiratory Disease in Adults during Pandemic (H1N1) 2009 Outbreak, Argentina

To the Editor: We report a mild to moderate respiratory disease in patients seeking treatment for influenza-like illness (ILI) within the first 8 weeks of an outbreak of influenza A pandemic (H1N1) 2009 virus (pandemic [H1N1] 2009) infection in the Province of Buenos Aires. The first cases of pandemic (H1N1) 2009 in Argentina were reported in early May 2009 in travelers returning from Mexico and the United States. In mid-June, a sharp increase was reported in the number of patients with acute respiratory symptoms who were seeking treatment in emergency rooms. By July 9th, the Argentinean Ministry of Health had confirmed 2,677 cases and 82 deaths; most of those infected were residents of Buenos Aires and the surrounding area (*1*). When the World Health Organization raised the pandemic level to 6, >80% of circulating influenza A virus in Argentina was subtype H1N1 (*2*). At the Hospital Central de San Isidro, a tertiary hospital of 160 beds in the Province of Buenos Aires, initial clinical evaluation of patients with ILI symptoms included physical examination and, eventually, chest radiograph and pulse oximetry. Because diagnostic resources were limited, patients with ILI were eligible to receive oseltamivir with no prior sampling for respiratory pathogens. A standardized form was used for prescription and data collection, including demographic data, history of influenza vaccination, date symptoms began, and coexisting illnesses. From June 16 to July 5, a total of 2,135 patients with ILI were evaluated. The age of patients ranged from 14 to 82 years of age (median, 35 years); 854 patients (40 %) were male. Because of lower respiratory disease, a total of 166 (8%) of 2,135 patients were admitted to the internal medicine ward (n = 139) or to the intensive care unit (n = 27). At admission, patients had ≥1 of the following: diffuse pulmonary infiltrates, room air oxygen saturation <95%, crackles on auscultation. Other clinical manifestations were not statistically different from those already reported in patients with pandemic (H1N1) 2009 in the United States, including cough, fever, and sore throat (*3*). The most common radiology pattern was basal, and bilateral interstitial infiltrates were consistent with primary viral pneumonia. Notably, some patients had clinical radiologic dissociation characterized by cough and pulmonary infiltrates in the absence of fever. Median time of hospitalization was 36 hours (range 1–25 days). No significant differences were observed between the groups of patients that were admitted versus outpatients in terms of age, sex, number of days from initiation of fever to first hospital visit, and history of influenza vaccination. A total of 163 (98%) of 166 patients admitted to the hospital during the observation period were discharged with no further complications.

Patients admitted to the hospital with pulmonary infiltrates were empirically treated with high dose oseltamivir (150 mg 2×/d) for 5 days, other antimicrobial drugs, and, eventually, steroids. In 2 patients, the respiratory disease progressed initially but they eventually recovered; 2 patients (1.2% of admissions to hospital) with acute respiratory failure died. Despite improvement in clinical symptoms at discharge, chest radiographs performed on a limited number of patients showed no substantial changes at 72–96 h after admission.

Clinical manifestations of pandemic (H1N1) 2009 have not yet been fully characterized. We observed a mild to moderate lower respiratory disease in ≈8% of consecutive patients with ILI during the current pandemic in Argentina. A more severe respiratory disease was observed in Mexico during the current pandemic (*4*) In contrast, early reports indicated that pandemic (H1N1) 2009 disease might be similar in severity to seasonal influenza (*3*). A lack of microbiologic confirmation may bias our observation. Because pulmonary infiltrates are uncommon in previously healthy persons with ILI, a simultaneous circulation of other respiratory pathogens may explain our observation. Furthermore, early empirical use of antimicrobial drugs could overshadow clinical features of bacterial pneumonia.

We observed an unexpectedly high rate of lower respiratory disease in adults with ILI during an outbreak of pandemic (H1N1) 2009 in Argentina. This finding suggests that a unique pattern of virulence, pulmonary tropism, or both may characterize the current influenza A (H1N1) infection, although we could not rule out co-infection with other viral or bacterial respiratory pathogens. Considering the evolving nature of influenza viruses, the wide clinical spectrum of pandemic (H1N1) 2009 should be further investigated.

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Susceptibility of Poultry to Pandemic (H1N1) 2009 Virus

To the Editor: During April 2009, cases of acute respiratory disease in humans caused by influenza A pandemic (H1N1) 2009 virus in Mexico were reported (*1*). By August 21, 2009, a total of >182,166 human cases, including 1,799 deaths, had been reported from 177 countries (www.who.int/csr/don/2009_08_21/en/index.html).

The origin of the new virus appears to be a reassortant event of a virus from swine in North America that contained the classic swine, human, and avian influenza genes and a virus of unknown origin that contributed neuraminidase and matrix genes of swine in Europe. On May 2, 2009, the first nonhuman infections were detected in a swine operation in Canada (www.who.int/csr/don/2009_06_24/en/index.html).

Historically, human seasonal influenza A viruses have not been reported to infect poultry, but clinical cases of respiratory disease or reduction in egg production have been reported for domestic turkeys after infection with subtypes H1N1, H1N2, and H3N2 swine influenza viruses and for multiple poultry species with subtype H1N1 avian influenza virus (*2*–*4*). The presence of avian and swine influenza virus genes in pandemic (H1N1) 2009 virus increases the potential for infection in poultry after exposure to infected humans or swine.

To determine infectivity potential, 3-week-old chickens (*Gallus domesticus*) (n = 11), 2-week-old domestic ducks (*Anas platyrhynchos*) (n = 11), 73-week-old reproductively active turkey hens (*Meleagris gallopavo*) (n = 9), 3-week-old turkey poults (n = 11), and 5-week-old Japanese quail (*Coturnix japonica*) (n = 11) were intranasally inoculated with 10^6 mean chicken embryo infectious doses of A/Mexico/4108/2009(H1N1). Five uninfected chickens, ducks, turkey poults, and quail, and 3 uninfected turkey hens were contact exposed to intranasally inoculated birds to assess transmission potential. Cloacal and oropharyngeal swabs were taken on 2, 4, 7, and 10 days postinoculation (DPI) from all birds, and internal tissues were taken from 2 birds on 2, 4 and 7 DPI for virus detection by quantitative real-time reverse transcription–PCR (qRRT-PCR) assay

specific for the influenza virus matrix gene (*5*).

To improve sensitivity because of several primer mismatches, we updated the reverse primer to 3′-CAGAGACTGGAAAGTGTCTTT GCA-5′. Virus isolation in embryonating chicken eggs was used on a subset of samples to verify qRRT-PCR results at 4 DPI. Serum samples were collected on 15 DPI for antibody testing by hemagglutination inhibition. Shams were intranasally inoculated with culture media and sampled on 4 and/or 7 DPI. We inoculated ten 4 week-old chickens intravenously to determine pathotype by using the intravenous pathogenicity index (IVPI). All animal studies were conducted under BioSafety Level 3 enhanced conditions with approval by Institutional Animal Care and Use and BioSafety committees.

During the 15-day observation period, clinical signs did not develop in any of the birds; none of the birds died. An IVPI of 0.00 indicated the virus was not of high pathogenicity for chickens. No virus was detected by qRRT-PCR or isolated in chicken eggs from swabs or tissues from chickens, turkeys, or ducks. All chickens and turkeys were negative for antibodies to the virus on 15 DPI, but 1 intranasally inoculated duck had a hemagglutination inhibition (HI) antibody titer of 16. Virus was detected in oropharyngeal swabs at 2 and 4 DPI from intranasally (IN)–inoculated quail (Table), and these quail had antibodies against influenza A at 15 DPI. The intranasally inoculated quail had heterophilic-to-lymphocytic rhinitis, and influenza virus was visualized by immunohistochemical analysis of epithelium and macrophages within the mucosa of the nasal cavity; neither lesions nor antigen were identified in other respiratory and nonrespiratory tissues. Virus was not isolated from contact-exposed quail (Table), and they lacked antibodies on 15 DPI.

Table. Results of testing for influenza A pandemic (H1N1) 2009 virus in oropharyngeal swabs of experimental quail

	Sampling day (days postinoculation) for oropharyngeal swab*			
Group	2	4	7	10
Intranasally inoculated	2/5 ($10^{0.9}$)	5/5 ($10^{2.8}$)	0/5	0/5
Contact	0/5	0/5	0/5	0/5

*Number virus positive/total sampled (average titer of positive samples, mean chicken embryo infectious doses). Test results for all cloacal swabs were negative.

Infection with swine influenza viruses in turkeys has been frequently reported, and experimental intranasal inoculation studies using 5 such viruses have produced infection and disease with associated contact transmission to uninfected turkeys (*3*,*4*,*6*). However, infection of chickens by swine influenza viruses has been rare in the field, and experimental studies have shown limited respiratory replication after intranasal inoculation but no transmission (*3*,*6*–*8*). Experimental inoculation of ducks failed to produce infection or transmission (*8*).

Recently, subtype H3N2 swine influenza A virus infection with respiratory disease in Japanese quail has been reported in Canada, and such infections have been experimentally reproduced by intranasal inoculation (*9*,*10*). However, in our studies, pandemic (H1N1) 2009 virus was biologically distinct from swine influenza viruses, failing to produce infection in experimentally inoculated turkey hens or chickens, and only 1 serologically positive IN-inoculated domestic duck. In addition, Japanese quail were infected by high dose IN exposure, but replication and shedding was limited to the respiratory tract, and the virus did not transmit to quail by contact, suggesting low potential of poultry involvement as an amplification host for current pandemic (H1N1) 2009 virus. Pandemic (H1N1) 2009 virus is unlikely to produce sustained outbreaks in poultry unless the virus mutates or reassorts with existing avian influenza viruses. Since the submission of this report, the virus has been detected in 2 turkey flocks in Chile (www.oie.int/wahis,/public.php?page=single_report&pop=1&reportid=8404). Currently, only limited data are available, and it is unknown if pandemic (H1N1) 2009 has changed and acquired the ability to infect and transmit in turkeys or if the 2 cases are isolated events without epidemic potential in turkeys.

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Oropouche Fever Outbreak, Manaus, Brazil, 2007–2008

To the Editor: Oropouche virus (OROV) is an arbovirus, *Orthobunyavirus*, transmitted among sloths, marsupials, primates, and birds by the mosquitoes *Aedes serratus* and *Culex quinquefasciatus*. Notably, this virus has adapted to an urban cycle involving man, with midges (*Culicoides paraensis*) as the main vector (*1*). Oropouche fever is the second most frequent arboviral disease in Brazil, surpassed only by dengue. OROV causes large, explosive outbreaks of acute febrile illness in cities and villages in the Amazon and central regions of Brazil. An estimated 500,000 cases of OROV infection have occurred in Brazil in the past 48 years. In addition to outbreaks, OROV can also cause sporadic human infections (*2*).

The Tropical Medicine Foundation of Amazonas State (TMF-AM) is a tertiary care center specializing in tropical and infectious diseases and is located in the city of Manaus. Syndromic surveillance for acute febrile illness has been conducted by TMF-AM since 1998. During January 2007 through November 2008, we obtained blood samples from 631 patients who had acute febrile illness for ≥5 days but who had negative results at initial screening for malaria (thick blood smear) and dengue (MAC-ELISA). Blood samples were tested for OROV immunoglobulin (Ig) M antibodies by an indirect enzyme immune assay using infected cells as antigen, as previously reported for dengue (*3*).

For the indirect enzyme immune assay using infected cells as antigen, C6/36 *A. albopictus* cells were grown in 96 well microplates; these cells were infected with OROV (BeAn 1991 strain). After 4 days, the cells were fixed in the wells with 7% formalin buffered at pH 7.0. The microplate was blocked with 5% skim milk and, after washing the wells, 100 μL of serum diluted 1:400 was added into infected and uninfected wells. After incubation and washing the wells, a peroxidase-conjugated goat anti-human IgM was added; finally, the ABTS substrate (KPL, Inc., Gaithersburg, MD, USA) was added into the wells. The plates were incubated and read on a spectrophotometer at 405 nm. The cutoff for the test was determined to be the mean of optical densities read in all wells containing uninfected cells plus 3 standard deviations.

Of the 631 patients in the study, 128 (20.3%) had IgM antibodies to OROV. The age range was 2–81 years (mean 29.5 ± 14 years), and 77 (60.2%) were women or girls. Most of the cases occurred November through March during the rainy season. In addition to fever, the patients had headache (93 [72.7%]), myalgia (90 [70.3%]), and arthralgia (74 [57.8%]). Rash was observed in 54 patients (42.2%), and hemorrhagic phenomena (petechiae, epistaxis, and gingival bleeding) were observed in 20 patients (15.5%). All patients recovered without sequelae and were not hospitalized.

Despite the knowledge of the occurrence of several arboviruses in the Amazon region, most cases of arboviral diseases remain undiagnosed, probably because of their generally mild and self-limited clinical manifestations. Patients usually recover completely after a couple of days. However, even more severe cases may remain undiagnosed, especially because of long distances to health care facilities, difficulties in sample transportation, and lack of laboratory facilities capable of conducting the diagnostic assays. With regard to OROV infections, diagnosis of OROV may be easily confused with other acute febrile illness, including malaria and dengue, both of which are highly endemic in Manaus.

In the present study, an inhouse enzyme immune assay for IgM using infected cell culture as antigen was

found suitable for the diagnosis of OROV infections in the acute phase. Thus, a combination of a systematic surveillance for acute febrile illnesses and efficient laboratory diagnosis for OROV resulted in the discovery of an outbreak, which would probably have been overlooked if it had occurred in any region simultaneously with large dengue outbreaks or in the absence of laboratory diagnosis. The cases of OROV fever reported here likely represent a small portion of the cases; a much higher number of cases probably occurred in Manaus during the study period.

The clinical characteristics of most cases of OROV fever in this outbreak were similar to previously reported descriptions of the illness. Notably, however, 20 (15.5%) patients from Manaus had spontaneous hemorrhagic phenomena (petecchiae, epistaxis, and gingival bleeding) that had not previously been described in OROV fever (*4*–*6*). Moreover, symptoms of involvement of the central nervous system were not observed.

In recent years, the area of circulation and the epidemic potential of OROV have increased, and this virus has emerged as a public health problem in Brazil and other countries in the Americas. Presently, OROV is the most common of the Brazilian zoonotic arboviruses infecting humans (*7*). Further evidence of the spread of OROV was its isolation in 2003 from a small primate, a marmoset (*Callithrix*), in the state of Minas Gerais in southeast Brazil, far from the Amazon region (*8*). Considering that midges (*Culicoides paraensis*) occur in most low altitude areas of the Americas, it is conceivable that environmental destruction and climate changes could result in OROV outbreaks in the large cities of Brazil, as well as in other parts of the Western Hemisphere (*9*).

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Identical Strains of *Borrelia hermsii* in Mammal and Bird

To the Editor: On August 5, 1994, a northern spotted owl, *Strix occidentalis caurina*, was found dead in Kittitas County, Washington, USA (*1*). A thorough investigation and necropsy identified the probable cause of death to be a spirochete infection. The organisms were seen in sections of the bird's liver with use of modified Steiner silver stain and microscopy. DNA was extracted from the infected liver, and PCR–DNA sequencing of the 16S ribosomal RNA (rRNA) locus identified the bacterium as a relapsing fever spirochete related most closely to *Borrelia hermsii* (*1*). The lack of additional data surrounding this case precluded Thomas et al. from concluding that this spirochete

infecting the owl was *B. hermsii* (*1*). Yet, in a subsequent analysis using the intergenic spacer region, the owl spirochete was included with isolates of *B. hermsii* (*2*).

To investigate the distribution and prevalence of *B. hermsii* , during the summer of 2008, we began a study at Flathead Lake, Lake County, Montana, USA, where 9 persons have contracted relapsing fever since 2002 (*3*–*5*). A blood smear from 1 pine squirrel (*Tamiasciurus hudsonicus*) captured July 9 at Yellow Bay on the east shore of the lake (elevation 887 m; geographic coordinates 47°52′35″N, 114°01′54″W) contained spirochetes detected when stained with Giemsa and examined by microscopy (600× brightfield with oil immersion). Whole blood from the squirrel contained live spirochetes visible by dark-field microscopy, and ≈50 μL of this blood was injected intraperitoneally into a laboratory mouse. The next day, a few spirochetes were observed in the peripheral blood of the mouse, and during the next 3 days, the density of spirochetes increased. We used intracardiac puncture to collect blood from the mouse for spirochete isolation in BSK-H medium (Sigma-Aldrich, St Louis, MO, USA) and for analysis by PCR and DNA sequencing of multiple bacterial loci as described elsewhere (*4*,*6*).

The spirochetes observed in the squirrel's blood failed to grow in BSK-H medium after passage in the laboratory mouse; however, we acquired DNA sequences from infected squirrel and mouse blood from PCR amplicons for 6 spirochete loci including *16S rDNA*, *flaB*, *gyrB*, *glpQ*, IGS, and *vtp*. Sequences for the loci were each aligned with homologous sequences from other borrelia in our collection, and each locus grouped the spirochete within the 2 genomic groups of *B. hermsii* described previously (*4*,*6*). The unique squirrel spirochete differed from all other *B. hermsii* identified in our previous studies; deep branches in each phylogram grouped the spirochete more closely with *B. hermsii* genomic group I than with genomic group II (data not shown).

Next, we compared the sequences from the squirrel spirochete with those available in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov), including those sequences reported for the spirochete found in the spotted owl (AY515269.1, AF116903.1, AF116904.1) (*1*,*2*). The 3 trimmed and aligned sequences for the *16S rDNA* (1,290 bases), *flaB* (467 bases), and IGS (665 bases) from the squirrel spirochete were identical to those of the owl spirochete; no base differences were found among the 2,422 bases compared. We also examined DNA extracted from the spotted owl's liver during the first investigation (*1*) (provided by Alan G. Barbour). We successfully PCR amplified most of the *16S rDNA* and the complete *flaB*, *gyrB*, *glpQ,* and *vtp* genes from the owl spirochete DNA and determined their sequences. The complete sequences of the first 4 loci from the owl and squirrel spirochetes were identical and differed from all other *B. hermsii* sequences. A phylogram of the concatenated sequences totaling 5,188 bases demonstrated that the owl and pine squirrel spirochetes were identical and were divergent members of *B. hermsii* genomic group I (Figure).

Finding the same strain of *B. hermsii*, separated by ≈525 km, in a pine squirrel and a spotted owl demonstrates a broader geographic distribution and host range for this spirochete than what could have been envisaged previously. The possible role of birds as hosts for the vector *Ornithodoros hermsi* ticks has been demonstrated elsewhere (*4*). Given the ecologic overlap of pine squirrels and coniferous forest-dwelling birds, we believe that the previous finding of the infected spotted owl is likely not an isolated event. Instead, it may represent a tick–spirochete cycle for *B. hermsii* that includes a broader host range for this group of relapsing fever spirochetes than previously appreciated.

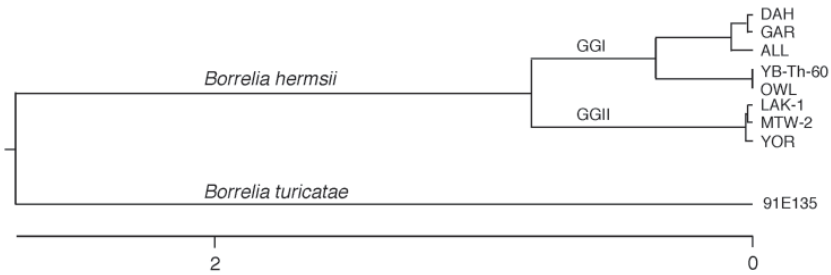

Figure. Phylogram based on the alignment of the concatenated DNA sequences containing the *16S rDNA*, *flaB*, *gyrB*, and *glpQ* loci for 6 isolates (DAH, GAR, ALL, LAK-1, MTW-2, and YOR) and infected tissues from the owl (OWL) and pine squirrel (YB-Th-60) of *Borrelia hermsii*. The same loci from *B. turicatae* 91E135 were used for the outgroup. New DNA sequences determined for the owl and pine squirrel spirochetes are available in GenBank (accession nos. GQ175059–GQ175068). Scale bar indicates number of base substitutions (×100).

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Mycobacterium bovis and *M. tuberculosis* in Goats, Nigeria

To the Editor: Documentation of possible tuberculosis (TB) in goats in Nigeria was reported by Ojo (*1*) on the basis of gross lesions without culture confirmation. Livestock owners in Nigeria normally graze cattle and goats together, and this practice poses a high risk for transmission of bovine TB among these animals (*1*). This practice is especially a threat to goats in Nigeria because several reports have described bovine TB in cattle in Nigeria (*2*–*5*). However, reports of diagnosis of TB in goats in Nigeria are lacking.

Molecular epidemiologic techniques such as deletion typing and spoligotyping have been used to characterize members of the *Mycobacterium tuberculosis* complex (MTC) and to provide information on transmission of mycobacterial diseases between animals and humans (*6*). However, because of limited resources and lack of expertise, these techniques are not commonly used in most developing nations such as Nigeria, where TB is endemic (*3*).

Because slaughterhouses provide excellent opportunities for detecting diseases of economic and public health importance, we investigated the presence of mycobacteria in slaughtered goats with lesions suggestive of TB. The investigation was conducted at the Bodija Municipal Abattoir in Ibadan in southwestern Nigeria over a period of 6 months. Slaughtered goats were obtained from local herds and herds in northern Nigeria. A total of 10,389 male and female goats of 2 breeds (West African Dwarf and Red Sokoto) and 1–2 years of age were slaughtered; 1,387 were inspected for gross lesions of TB.

Of 1,387 animals screened, 62 (4.47%) had lesions suggestive of TB in the liver, lungs, and mesenteric lymph nodes. Five (0.36%) goats were confirmed positive by culture as described (*2*). Deletion typing (*6*) with the RD9 deletion was used to distinguish *M. tuberculosis* from other members of the MTC. Those isolates with a deletion in this region were further investigated with primers specific for RD4. This reaction distinguishes between *M. bovis*, *M. caprae*, and other members of the MTC. Spoligotyping was performed as described (*7*) to type an *M. tuberculosis* isolate from a goat after identification of this bacterium by deletion typing.

We isolated 4 strains of *M. bovis* and 1 strain of *M. tuberculosis* from the goats (Table). Spoligotyping identified the *M. tuberculosis* isolate as belonging to the East African Indian (EAI)–5 family in the SpolDB4 database. All *M. bovis* isolates were *M. bovis bovis*, not *M. bovis caprae*, according to their deletion typing profile (*6*). One *M. bovis* isolate was obtained from a male goat; the 3 remaining *M. bovis* isolates and the *M. tuberculosis* isolate were obtained from female goats.

Epidemiologic inferences can be made from the results of our study. First, *M. bovis*, which is naturally found in cattle, was isolated from 4 slaughtered goats. Although *M. bovis caprae* was the *M. bovis* variant most frequently isolated from goats in some areas (*8*), in our study, only *M. bovis bovis* was isolated. This finding is consistent with results reported by Crawshaw et al. (*9*), and suggests transmission from cattle, rather than transmission from the goat reservoir. Second, because the infected goats were adult females, infection may be transmitted to their offspring. Third, *M. tuberculosis* was isolated from a goat. Its presence in this goat may have been caused by direct transmission from humans because this bacterium may be a natural pathogen of humans.

Transmission caused by close cohabitation of goats and humans

Table. Results of deletion typing for *Mycobacterium tuberculosis* and *M. bovis* in goats, Nigeria*

Region of difference	*M. tuberculosis*	*M. bovis*
RD1	+	+
RD4	+	–
RD9	+	–
RD12	+	–

*+, present; –, absent.

with advanced TB may occur, given the endemic nature of TB in humans in Nigeria (*10*). TB cases caused by EAI strains have been found in humans in southwestern Nigeria (*4*; S.I. Cadmus et al., unpub. data), a finding that supports zoonotic transmission of this organism from humans to goats. However, different lineages of *M. tuberculosis* may vary in host range, and EAI genotype strains may be adapted to human and animal hosts. Conversely, human-to-animal transmission of *M. tuberculosis* has been reported in Nigeria relative to infection in cattle (3,*4*). Thus, confirmation of TB in goats supports the possibility of risk for TB transmission between humans and animals in Nigeria.

This study should be interpreted in the context of its limitations. Because the sources of the animals were unknown, we could not determine whether the organisms were imported from a neighboring country (*3*). In addition, we lacked information on the breed and condition of the animals. However, we have identified *M. tuberculosis* and TB in goats in Nigeria. Additional studies of other slaughterhouses in Nigeria are needed to confirm our results.

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Streptococcus suis Meningitis, Hawaii

To the Editor: *Streptococcus suis* is a swine pathogen and zoonotic agent responsible for septicemia and meningitis (*1*). *S. suis* is in emergence in some Asian countries. Indeed, this pathogen has been described as the most and second-most common cause of adult meningitis in Vietnam and Thailand, respectively (*2*,*3*). Moreover, during an outbreak in People's Republic of China in 2005, 39 of 215 patients died from *S. suis* diseases (*4*). On the other hand, only 2 human *S. suis* cases have been reported in the United States (*5*,*6*). Here, we describe a first case of human *S. suis* meningitis in Hawaii.

The patient, a 34-year-old Tongan male with no medical history who worked as a coconut tree trimmer, was singing in his church choir when he developed an acute-onset, global headache and emesis. Upon hospital admission, he described a week of

antecedent nonspecific symptoms for which he had taken nonsteroidal anti-inflammatory drugs without relief.

On examination, he was afebrile, tired-appearing but alert, and with stable vital signs. He presented mild meningismus and photophobia; no rash was observed. Blood tests showed 27,600 leukocytes/μL with 65% neutrophils; 168,000 platelets/μL; hemoglobin 17.3 g/dL; and creatinine 1.4 mg/dL. A computed tomography scan of the head was read as showing substantial motion artifact and a possible cerebral mass. Nuclear magnetic resonance imaging (MRI) of the head showed no mass, but T2-weighted images (postgadolinium) suggested both increased grey/white matter contrast consistent with diffuse cortical edema, and vascular congestion/inflammation of the sulci.

Cerebrospinal fluid (CSF) obtained from a lumbar puncture had 2,770 leukocytes/μL with 94% neutrophils; glucose 30 mg/dL; and protein 230 mg/L. A Gram stain of the CSF showed numerous gram-positive cocci, mostly in pairs and short chains (Figure). Empiric intravenous therapy with dexamethasone, vancomycin, and ceftriaxone was administered for possible pneumococcal meningitis.

Blood cultures grew a *Streptococcus* species, later identified by 16S rRNA gene sequencing as being *S. suis*, sensitive to penicillin, vancomycin, and ceftriaxone. The isolate was assigned to serotype 2 by the coagglutination test (*7*) and shown by Western blot to produce suilysin, extracellular protein factor and muramidase-released protein, which are virulence markers often associated with highly virulent strains of Eurasian, but not North American, origin (*1*,*8*). A strain of this phenotype was responsible for a previous US *S. suis* meningitis case, but the patient had been infected in the Philippines (*5*; unpub. data).

Upon identification of the *S. suis* isolate, the patient was questioned about swine contact. He described slaughtering by hand several noncommercially raised pigs over the preceding several weeks for a church-related luau. The patient did not recall any clear incident of mucosal exposure to pig blood or secretions. The exact route of *S. suis* infection for humans is not known. However, most cases have been linked to accidental inoculation through skin injuries (*1*). The patient did not wear gloves, masks, or any other protective equipment during the prolonged process of butchering the pigs, and his exposure to pig blood, skin, and internal organs was extensive. He sustained multiple small cuts on his hands during butchering. No other church members who participated in preparing pigs for the luau became ill.

The patient was treated with ceftriaxone and a 4-day course of dexamathasone. His headache and meningismus improved progressively, and he was discharged after 6 days to complete a 2-week course of intravenous ceftriaxone. However, 1 day after discharge, the patient complained of headaches and mild-to-moderate bilateral hearing loss. He was readmitted; a repeat lumbar puncture showed resolving CSF pleocytosis, and an MRI showed that his prior radiographic findings had normalized. The symptoms, attributed to residual meningeal/cerebral edema, resolved quickly after the reintroduction of steroids. Audiometric testing suggested mild sensorineural hearing loss in the right ear. The patient completed the remainder of his intravenous ceftriaxone course and was discharged on a 2-week course of amoxicillin and oral steroids.

He was again admitted 2 days after completing treatment, with disabling dizziness. On exam he showed new torsional nystagmus, more pronounced with left lateral gaze, consistent with a right peripheral vestibulopathy. An MRI of the head was again normal. Oral dexamethasone promptly resolved his vestibulopathy, and the patient was discharged on a slow steroid taper. After a month, dexamethasone was discontinued. The patient has been asymptomatic since, and his hearing loss has resolved fully.

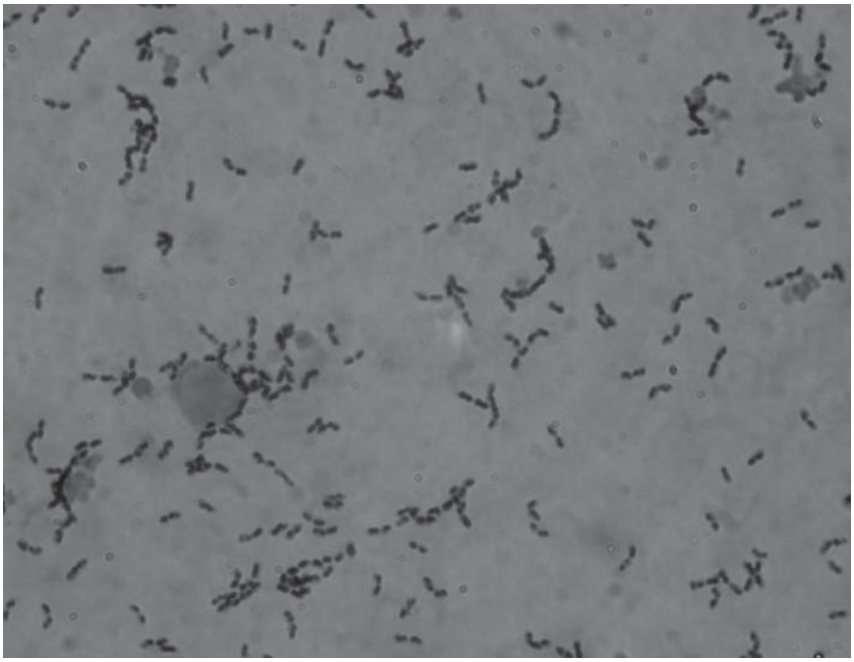

Figure. Gram-positive cocci, mostly in pairs and short chains, found in cerebrospinal fluid from a 34-year-old man with *Streptococcus suis* meningitis. The sample was not centrifuged before staining. Original magnification ×1,000.

The role of steroids in treating patients with *S. suis* infection remains unclear, although this case illustrates that the inflammation associated with this infection can be profound and can require prolonged steroid therapy. Since at least 2 cases of relapse have been reported after 2 and 4 weeks of treatment (*1*), prolonged therapy should be considered for infections caused by this pathogen. Hearing loss from *S. suis* meningitis occurs frequently and can be irreversible (*1*). Hawaii's swine industry is characterized by small herds and a high degree of concentration (*9*). This case of human *S. suis* meningitis in Hawaii emphasizes the need for these data to be generated and made available. Indeed, this bacterium is increasingly recognized as a significant zoonotic agent in Asia; although it remains a relatively rare cause of human infection elsewhere, persons in close occupational contact with pigs or pork products are at higher risk than others (*1*). Increasing awareness of this disease is expected to help counter human *S. suis* infections.

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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.

Chorioamnionitis and Neonatal Sepsis from Community-associated MRSA

To the Editor: Chorioamnionitis is a common cause of maternal and neonatal illness and death (*1*), but chorioamnionitis attributed to *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), is reported infrequently (*2–5*). In the context of the rising incidence of community-associated MRSA (CA-MRSA) infections (*6*), we report an apparent case of CA-MRSA chorioamnionitis.

The patient, a 31-year-old woman with polycystic ovary syndrome and hypothyroidism, had 1 prior pregnancy but no viable offspring. After a clomiphene-assisted conception, routine ultrasound at 21 weeks' gestation identified a shortened cervix (5 mm). The patient declined amniocentesis for cerclage and was treated with pelvic rest and vaginal progesterone. Five days later, she arrived at the emergency department with foul-smelling vaginal discharge. At this time, the patient was afebrile and hemodynamically stable, had no abdominal pain, and had a leukocyte count of 9.5×10^3 cells/mm^3.

Premature rupture of membranes was diagnosed, and the patient was admitted and administered intravenous ampicillin and azithromycin. Nine days into treatment, at 23 weeks' gestation, 210 hours after membrane rupture, a 415-g live-born girl was delivered spontaneously in footling breech with Apgar scores of 1 (1 min) and 5 (5 min). During admission, the mother was never febrile and did not complain of abdominal tenderness or chills. The highest leukocyte count was 12.4×10^3 cells/mm^3. The mother was discharged the day after delivery without further complications. At 6-week follow-up, she remained well, with no signs of infection.

Pathologic examination of the placenta demonstrated focal acute funisitis, acute chorioamnionitis with fetal surface acute arteritis and acute deciduitis. Cultures from the maternal and fetal sides of the placenta grew predominantly MRSA and rare colonies of methicillin-susceptible *S. aureus*. The MRSA antimicrobial drug profile, including trimethoprim/sulfamethoxazole and clindamycin susceptibility, was characteristic of CA-MRSA (*6*).

The neonate, who died on day 16, was culture-positive for CA-MRSA from blood, 2 umbilical swabs, and a tracheal aspirate. The antibiogram of these isolates was identical to the placental cultures, including absence of inducible clindamycin resistance. Postmortem examination showed hemorrhagic necrotizing pneumonia and gram-negative bacilli. Culture of lung tissue grew *Escherichia coli*. Isolates from the placenta and neonate were identified phenotypically, without molecular testing.

Maternal complications of chorioamnionitis include endometritis, bacteremia, hemorrhage, and cesarean delivery (*1*). Clinically, chorioamnionitis can be diagnosed by maternal fever (>38°C) and 2 of the following: maternal leukocytosis (>15 × 10^3cells/mm^3), maternal tachycardia (>100 bpm), fetal tachycardia (>160 bpm), uterine tenderness, and foul-smelling amniotic fluid (*1*). This patient had none of these signs, except foul-smelling amniotic fluid, and fetal tachycardia was absent. In this case, chorioamnionitis was diagnosed by histology.

Amniotic fluid cultures from pregnancies complicated by chorioamnionitis have shown multiple organisms from the vaginal flora, such as *Streptococcus agalactiae*, *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, anaerobes, and *E. coli* (*1*). Chorioamnionitis associated with *S. aureus* is uncommon (*2*,*3*), and MRSA chorioamnionitis is rare (*4*,*5*). The first 2 reports of MRSA chorioamnionitis appeared in 1998 (*4*) and 2002 (*5*). In both instances, the patients worked in the healthcare industry, and the authors considered the MRSA to have been nosocomial strains. The patient in our report was a restaurant manager, had no prior recorded hospital admissions, and was not previously known to be colonized by MRSA.

CA-MRSA strains are epidemiologically and clonally unrelated to hospital-associated MRSA (HA-MRSA) strains and can be differentiated by the presence of staphylococcal cassette chromosome *mec* type IV and the absence of multidrug resistance seen with HA-MRSA (*6*). Recently, the incidence of CA-MRSA infections increased in community settings, including outbreaks in settings in which CA-MRSA is endemic, with manifestations ranging from skin and soft tissue infections to necrotizing pneumonia (*6*). Genital colonization with MRSA recently has been reported with a frequency of 0.5%–3.5% in pregnant women (*7*,*8*). In 1 study, most (93%) of these isolates were CA-MRSA (*7*).

Eckhardt et al. described a patient with chorioamnionitis in whom CA-MRSA bacteremia developed (*9*). However, this descriptor was used to specify multidrug-resistant MRSA not acquired in a hospital. Moreover, neither placental nor amniotic fluid cultures were described. Laibl et al. reported 2 patients with CA-MRSA infections in whom chorioamnionitis developed (*10*). Again, placental and amniotic fluid culture results were not reported, nor was chorioamnionitis listed as an infection caused by CA-MRSA in their cohort. However, these latter 2 patients might represent additional cases of CA-MRSA chorioamnionitis.

Although the incidence of CA-MRSA infections continues to increase, CA-MRSA chorioamnionitis appears to remain rare. Nevertheless, the prevalence of MRSA genital colonization among pregnant women creates an opportunity for this agent to cause ascending gestational infection. This finding is meaningful because recommended empirical antimicrobial drug treatments may not cover CA-MRSA, increasing the likelihood of infectious complications (*1*). However, culture results when available can provide therapeutic guidance. We hope this report raises awareness of the possibility of CA-MRSA chorioamnionitis and encourages reports from other authors so this entity can be better established, characterized, and monitored.

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Methicillin-Resistant *Staphylococcus aureus* in Marine Mammals

To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) is emerging as an important cause of illness and death in animals and has been found in an impressive variety of species. However, to date, only 2 studies have reported the isolation of MRSA from marine mammals, 1 seal (*1*) and 3 bottlenose dolphins (*2*). We describe an investigation that was conducted after MRSA was isolated from a dolphin at a marine park in North America.

In November 2006, a 20-year-old, male, captive, bottlenose dolphin, suspected of having pneumonia, was treated empirically with ciprofloxacin and itraconazole. Despite treatment, the dolphin died in December 2006. A necropsy was performed, and a culture swab specimen of the blowhole was submitted for bacteriologic examination; MRSA was then isolated. The clinical relevance of this finding was unclear. Pulsed-field gel electrophoresis (PFGE) was conducted (*3*), and results indicated that the MRSA strain isolated was the Canadian epidemic MRSA (CMRSA)2 (USA100) strain, the predominant hospital- and community-associated MRSA strain found in persons in Canada (*4*). To determine the extent of MRSA colonization in this marine park, blowhole swab specimens were collected from dolphins, orcas, and beluga whales, and nasal swab specimens were collected from walruses, sea lions, harbor seals, gray seals, and park personnel, excluding 4 employees in January 2007. Selective culture for MRSA was performed, and strains were typed with PFGE (*3*) and *spa* typing (*5*). All MRSA strains were investigated for the Panton-Valentine leukocidin (PVL) toxin genes (*6*).

In January 2007, MRSA was not isolated from personnel (0/22), sea lions (0/12), harbor seals (0/2), gray seals (0/2), orcas (0/4), or beluga whales (0/23); it was isolated from dolphins (2/6, 33.3%) and a walrus (1/6, 16.7%). To reduce the risk for MRSA transmission among the marine mammals and to personnel, the following steps were recommended: colonized animals were isolated, contact with colonized animals was restricted, all park personnel were required to wear gloves and masks when handling colonized animals, and routine hand hygiene was emphasized. Colonized walruses were isolated in a separate facility until May 2007. Because of space limitations, colonized dolphins could not be isolated. Although the park instituted a strict policy that required personnel to wear gloves and masks, this policy ceased during the summer months due to the park's exhibition schedule.

Because we knew from our observations of other animal species that natural decolonization with MRSA is common, as well as lacking information about antimicrobial drug efficacy for MRSA decolonization in marine mammals, and had concerns regarding the emergence of further antimicrobial drug resistance, we recommended that no attempt be made to decolonize the animals with antimicrobial agents. After these recommendations were made and implemented, follow-up testing for MRSA colonization was performed on the dolphins and walruses throughout 2007 and 2008 (Table). In October 2007, testing conducted on all sea lions, harbor seals, gray seals, orcas,

Table. MRSA colonization status of dolphins and walruses during 2007–2008*

Date	No. (%) dolphins MRSA positive	Identification nos. of MRSA-positive dolphins	No. (%) walruses MRSA positive	Identification nos. of MRSA-positive walruses
2007 Jan	2/6 (33.3)	2, 3	1/6 (16.7)	1
2007 Feb	2/6 (33.3)	2, 4	2/5 (40)	2, 3
2007 Apr	2/5† (40)	3, 5	0/6 (0)	NA
2007 May	2/3 (66.7)	3, 5	0/6	NA
2007 Oct	1/5 (20)	3	0/5	NA
2008 May	1/5 (20)	3	NT	NA
2008 Jul	0/5	NA	NT	NA
2008 Oct	0/5	NA	NT	NA

*MRSA, methicillin-resistant *Staphylococcus aureus;* NA, not applicable; NT, not tested.
†Dolphin 2 died due to unknown circumstances.

and beluga whales showed that none of these animals were colonized with MRSA. Overall, MRSA was isolated on ≥1 occasions from 5 dolphins (n = 6, 83.3%) and 3 walruses (n = 6, 50%) (Table). All strains were indistinguishable on PFGE and were consistent with the CMRSA2 (USA100) strain. They were also *spa* type t002 and did not possess the PVL toxin genes.

This report of MRSA shows colonization in several dolphins and walruses, with apparent transmission between species. The direction of transmission cannot be determined because of the sampling method; however, a human origin is suspected because the clone that was isolated is a predominant human clone. The failure to identify a concurrently colonized person does not preclude a human source. Since the time MRSA was introduced into the facility is unknown, the source of infection may have been decolonized by the time of sampling or was not sampled. Furthermore, park visitors occasionally have contact with these animals so the origin could have been from the general public. Whether colonization of multiple animals was due to repeated instances of human-to-animal transmission or whether animal-to-animal transmission may have occurred is not clear. For the dolphins, the second scenario is most likely, considering the social nature of these animals and the inability to isolate colonized dolphins. These factors may have resulted in the circulation of MRSA among these animals. Although no water samples were obtained for testing, waterborne transmission cannot be dismissed.

Colonization was eliminated without antimicrobial agents; however, long-term (15 months) MRSA colonization was found in 1 dolphin. With patience and continued use of infection control measures, MRSA was apparently eradicated from this facility without the need for active decolonization. This study shows the impressive ability of MRSA to colonize diverse animal species and provides further evidence suggesting that interspecies transmission of human epidemic clones can occur between persons and animals. This study also provides evidence suggesting that MRSA colonization in many animal species can be transient and that application of appropriate infection control and hygiene measures may be critical control tools for the management of MRSA in animals.

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Parachlamydia and *Rhabdochlamydia* in Premature Neonates

To the Editor: New members have recently been recognized in the order Chlamydiales (*1*). The family *Rhabdochlamydiaceae* includes *R. porcellionis* (a parasite of *Porcellio scaber*) and *R. crassificans* (a pathogen of the cockroach *Blatta orientalis*) (*2*,*3*); their pathogenic role in humans has not yet been investigated. *Parachlamydia acanthamoebae* and *Protochlamydia naegleriophila* belong to the family *Parachlamydiaceae* (*1*,*4*). Increasing evidence indicates that these obligate intracellular bacteria infecting free-living amebae may cause respiratory diseases in humans (*1*). Recent findings also suggest a role for *Parachlamydia* in miscarriage, stillbirth, and preterm labor (*5*–*7*). Whether these bacteria may contaminate the newborns of infected mothers is unknown.

The aims of this study were to 1) develop a real-time PCR for detecting *Rhabdochlamydia* spp. and 2)

apply this PCR, and those previously described for *Parachlamydia* and *Protochlamydia* (*4*,*8*), to respiratory samples from premature neonates. Using the GenBank database (www.ncbi.nlm.nih.gov), we selected primers RcF (5′-GACGCTGCGTGAGTGATGA-3′) and RcR (5′-CCGGTGCTTCTTTACGCAGTA-3′), and probe RcS (5′-6 carboxyfluorescein-CTTTCGGGTT-GTAAAACTCTTTCGCGCA-Black Hole Quencher 1-3′), which amplify parts of the 16S rRNA encoding gene, to specifically amplify *Rhabdochlamydia* spp. The 5′-FAM probe (Eurogentec, Seraing, Belgium) contained locked nucleic acids (underlined) to improve specificity. Reactions were performed with 0.2 μM of each primer, 0.1 μM of probe, and iTaq Supermix (Bio-Rad, Rheinach, Switzerland). PCR products were detected with ABI Prism 7000 (Applied Biosystems, Rotkreuz, Switzerland). Inhibition, negative PCR mixture, and extraction controls were systematically tested.

To enable quantification, a plasmid containing the target gene was constructed as described (*4*,*9*). The analytical sensitivity of the real-time PCR for *Rhabdochlamydia* spp. was ≤10 copies DNA/μL. No cross-amplification was observed when the analytical specificity was tested with human, amebal (*Acanthamoeba castellanii* ATCC 30010), and bacterial DNA (online Technical Appendix, available from www.cdc.gov/EID/content/15/12/2072-Techapp.pdf). Intrarun and interrun reproducibility were excellent (online Technical Appendix).

This PCR and those previously described for *Parachlamydia* and *Protochlamydia* (*4*,*8*) were retrospectively applied to 39 respiratory samples from 29 neonates admitted in the neonatology unit of our institution (median 1 sample per patient, range 1–4 sample). All but 1 patient had a gestational age at birth ≤36 weeks (median 28.6, range 24.6–41.2 weeks). Respiratory

Table. Characteristics of 29 newborns with positive PCR results for *Parachlamydia acanthamoebae* or *Rhabdochlamydia* spp. and controls*

Characteristics	Positive PCR result, n = 12	Negative PCR result, n = 17	p value†
Sex, M/F	8 (67)/4 (33)	6 (35)/11 (65)	0.14
Gestational age at birth, wk, median (range)	27 (24–36)	30 (25–41)	0.16
Weight <10th percentile	4 (33)	4 (24)	0.68
Height <10th percentile	3 (25)	6 (35)	0.69
Primary adaptation			
First Apgar score (1 min), median (range)	2.5 (0–7)	8 (2–9)	0.0017
First 3 Apgar scores,‡ median (range)	18.5 (8–27)	27 (17–29)	0.0023
Cardiac massage in first 48 h	6 (50)	0 (0)	0.002
Endotracheal intubation in first 48 h	11 (92)	8 (47)	0.019
Respiratory distress syndrome	11 (92)	14 (82)	0.62
Hyaline membranes disease	9 (75)	8 (47)	0.25
Bradypneic syndrome	7 (58)	11 (65)	1.00
Bronchopulmonary dysplasia	8 (67)	9 (53)	0.70
Amniotic fluid aspiration	1 (8)	3 (18)	0.62
Invasive mechanical ventilation, d, median (range)	12 (2–50)	3 (0–14)	0.005
Endotracheal intubation during hospital stay	12 (100)	11 (65)	0.028
Infectious complications			
Lung infection	5 (42)	7 (41)	1.00
Other systemic infection	7 (58)	5 (29)	0.15
Other complications			
Intraventricular hemorrhage	4 (33)	6 (35)	1.00
Persistent artery canal	7 (58)	6 (35)	0.27
Necrotizing enterocolitis	2 (17)	1 (6)	0.55
Congenital malformations	1 (8)	1 (6)	1.00
Hospitalization			
Stay in neonatology ward, d,§ median (range)	113.5 (9–435)	48 (7–131)	0.003
Death	3 (25)	0 (0)	0.06
Pregnancy			
Premature membranes rupture	5 (42)	6 (35)	1.00
Placental detachment	2 (17)	1 (6)	0.55
Preeclampsia	4 (33)	2 (12)	0.20
Systemic infection	7 (58)	9 (53)	1.00
Cesarean delivery	9 (75)	10 (59)	0.45

*Results are given as no. (%) except as indicated.
†Fisher exact test and nonparametric Mann-Whitney rank sum test were used for the analysis of proportions and continuous variables, respectively.
‡Sum of the 3 scores at 1, 5, and 10 min after birth.
§If survived.

distress syndrome was present in 25 (86%) of these 29 neonates. Samples had been drawn a median of 14 days (range 1–229 days) after birth, when clinically indicated. Results of PCR for *Parachlamydia*, *Protochlamydia*, and *Rhabdochlamydia* were positive for 9 (31%), 0 (0%), and 4 (14%) neonates, respectively. Positive results were obtained on the first sample drawn after birth for all but 2 neonates (initial negative results). One patient had positive PCR results for *Parachlamydia* and *Rhabdochlamydia*. These 12 newborns with positive PCR results for *Parachlamydia* and/or *Rhabdochlamydia* were compared with the 17 who had negative PCR results (Table).

Newborns with a *Chlamydia*-related organism documented in the respiratory tract had a significantly worse primary adaptation score (Apgar). These patients experienced more resuscitation maneuvers at birth. Durations of invasive mechanical ventilation and hospital stay were also longer among them. Three newborns died, compared with no deaths among the 17 with negative PCR results ($p = 0.06$). Pneumonia was documented in 5 of the 12 patients with positive *Parachlamydia* and/or *Rhabdochlamydia* PCR results but was concomitant to PCR positivity for only 3 of them. An alternative etiology was documented in all 3 (online Technical Appendix).

Parachlamydia and *Rhabdochlamydia* have thus been detected in a population of premature neonates. Most of these patients had severe respiratory distress syndrome, and the role of these bacteria as a causal agent of pneumonia could not be clearly assessed. The longer duration of mechanical ventilation for newborns with positive PCR results may suggest an occult superinfection with a *Chlamydia*-related bacterium contributing to the severity of the initial respiratory disease.

Our results also raise a question about the mode of acquisition of these microorganisms. A recent study reported a higher seroprevalence of *Parachlamydia* in women experiencing miscarriage (*5*,*6*), and DNA of this bacterium has been detected in the amniotic fluid of a woman with premature delivery (*7*). Whether neonatal infection results from a systemic infection during pregnancy or an inoculation at delivery is unknown. Because of the retrospective design of the study, no samples from the mothers were available for additional molecular or serologic analyses. Hospital water supplies are an important reservoir of free-living amebae and may represent another mode of acquisition because patients undergoing mechanical ventilation are exposed to aerosolized particles (*10*). Simultaneous detection of Parachlamydia and Rhabdochlamydia in 2 patients with initial negative results and their simultaneous detection in 1 neonate supports the latter hypothesis.

In conclusion, *Parachlamydia* and *Rhabdochlamydia* DNA were detected in respiratory secretions of premature newborns with more severe conditions at birth, more mechanical ventilation requirements, and a trend toward a higher mortality rate. The pathogenic role of these *Chlamydia*-related bacteria in neonates deserves further investigations.

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Porcine Kobuvirus in Piglets, Thailand

To the Editor: To date, the genus *Kobuvirus* has consisted of 2 officially recognized species, *Aichi virus* and *Bovine Kobuvirus* (*1*). Aichi virus has been shown to be associated with acute gastroenteritis in humans (*2*–*4*), and bovine kobuvirus has been detected only in cattle (*5*,*6*). Most recently, a third candidate species of *Kobuvirus* has been described in pigs by 2 different groups of investigators from Hungary and the People's Republic of China (*7*,*8*). This new candidate species was serendipitously recognized in stool specimens from pigs when PCR products (≈1,100 bp) were amplified by using a primer pair for the detection of caliciviruses (*7*).

Nucleotide sequences of these nonspecific PCR products were similar to those of the U-1 bovine kobuvirus and Aichi virus A846/88 reference strains; sequence identities ranged from 73% to 79% at the nucleotide level and from 69% to 70% at the amino acid (*7*). The representative strain of a new candidate species of porcine kobuvirus, S-1-HUN (Porcine kobuvirus/swine/S-1-HUN/2007/Hungary), has been analyzed to determine its complete genome sequence and genetic organization (*9*). The RNA genome of the S-1-HUN strain comprises 8,210 nt, with a genome organization analogous to that of picornaviruses. Therefore, this strain is tentatively classified as a new species of the genus *Kobuvirus*, and named porcine kobuvirus (*7*,*9*).

Currently, 2 reports have described the epidemiologic feature of porcine kobuvirus in healthy piglets. Thirty-nine (65%) of 60 stool samples collected from pigs in Hungary were positive for porcine kobuvirus by reverse transcription–PCR (RT-PCR) (*9*). Another report from China found that the prevalence of porcine kobuvirus was 30% (97 of 322 piglets) (*8*). These findings suggested that porcine kobuvirus infections are common in piglets. However, whether this agent is associated with particular diseases, including gastroenteritis, in piglets was not clear.

We conducted an epidemiologic survey of porcine kobuvirus and report the detection of this virus in the stool specimens of piglets with diarrhea. Sequence and phylogenetic analyses of the porcine kobuvirus strains were carried out to determine their evolutionary relationships with kobuvirus strains previously reported.

A total of 98 stool specimens were collected from piglets with diarrhea from 6 farms in Chiang Mai Province, Thailand, during 2001–2003. Age of the piglets ranged from 7 to 49 days old. Porcine kobuvirus was detected in fecal specimens by RT-PCR (*9*). The representative strains of porcine kobuvirus detected in our study were analyzed further by direct sequencing of their PCR amplicons (216 bp) by using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences of these fragments were compared with those of reference strains available in the NCBI GenBank database by using BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA 4 (*10*). Nucleotide sequences of porcine kobuvirus strains described in this study were deposited in GenBank under accession nos. GQ152093–GQ152122.

Prevalence of porcine kobuvirus was exceptionally high in piglets with diarrhea, 99% (97 of 98 specimens). Thirty representative strains of porcine kobuvirus detected in this study were randomly selected, sequenced, and analyzed to determine their evolutionary relationships with other kobuvirus reference strains. The partial 3D region among all 30 porcine kobuvirus strains was highly conserved, with nucleotide sequence identities >90%. In addition, our strains were most closely related to 2 porcine kobuvirus reference strains (S-1-HUN and Swine/2007/CHN) available in GenBank, with the nucleotide sequence identity ranging from 91.5% to 96.3%. Phylogenetic analysis of partial 3D nucleotide sequences of our porcine kobuvirus strains, together with published sequences of porcine kobuvirus reference strains (and those of Aichi virus and bovine kobuvirus), is shown in the Figure. The phylogenetic tree confirmed that all strains we identified belonged to the porcine kobuvirus species and formed a tight cluster in a monophyletic branch with the other 2 porcine kobuvirus reference strains (S-1-HUN and Swine/2007/CHN). These strains are also distantly related to standard strains of Aichi virus and bovine kobuvirus. Recently, 18 sequences of partial 3D region of the porcine kobuvirus strains detected in China have been deposited in GenBank. Unfortunately, the specific position of PCR amplification of the strains found in China was different from that of our strains (*8*). Therefore, the relationship between these strains could not be analyzed.

Porcine kobuviruses have previously been reported only in healthy pigs (*7*–*9*). In our study, the exceptionally high prevalence of porcine kobuviruses (99%) has been observed in piglets with acute gastroenteritis; those samples were negative for rotavirus infection as determined previously by RT-PCR. However, associations of this agent with enteric diseases in pigs

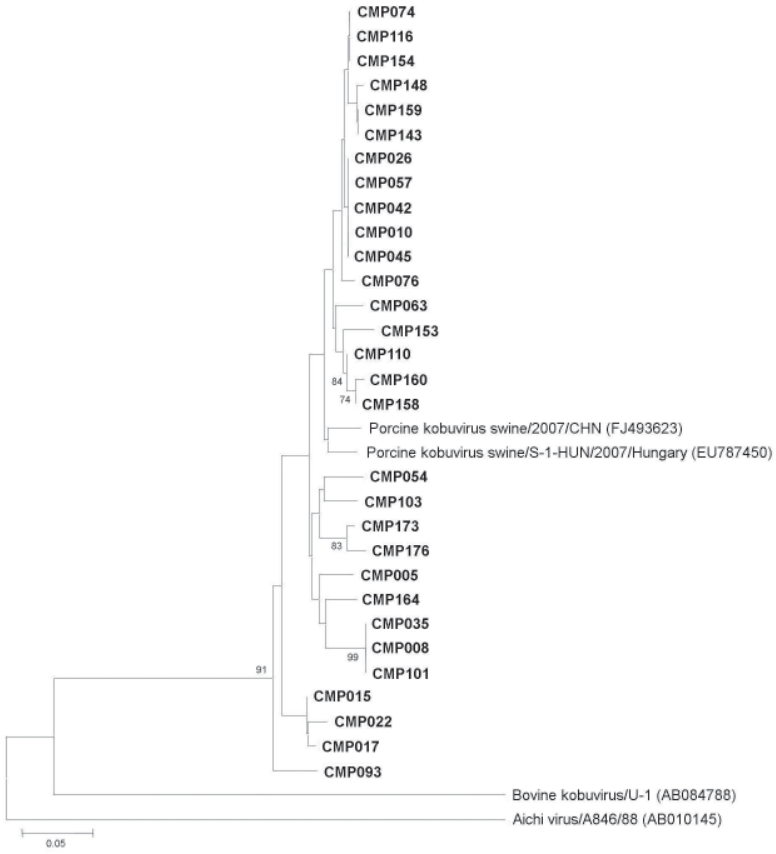

Figure. Phylogenetic analysis of the partial nucleotide sequence encoding the 3D region of porcine kobuviruses (in **boldface**) isolated in Thailand, 2001–2003, and other reference strains. The tree was generated on the basis of the neighbor-joining method by using the MEGA4 program (*10*). Scale bar indicates nucleotide substitutions per site.

remains unclear because no data were available that tested for porcine kobuvirus in pigs without gastroenteritis from the farms in the same area. In addition, infection with other pathogens that may cause diarrhea in pigs, such as bacteria or other porcine caliciviruses, needs to be ruled out. Further extensive epidemiologic surveillance and comprehensive characterization of porcine kobuvirus strains from other areas may help clarify the distribution, heterogeneity, and association of porcine kobuviruses with enteric diseases in pigs.

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Rickettsia slovaca in *Dermacentor marginatus* ticks, Germany

To the Editor: *Dermacentor* spp. ticks are found in many countries in Europe, and are vectors for several pathogens, including *Francisella tularensis*, *Coxiella burnetii*, *Rickettsia* spp., and *Babesia canis* (*1*,*2*). Because *Dermacentor marginatus* ticks require warm dry habitats, these ticks are found in only a few areas in southern Germany, mainly in the Rhine and Main valleys (*3*). However, these ticks may spread northwards because of increasing temperatures. In contrast, *D. reticulatus* ticks are present throughout Germany.

Fourteen *Rickettsia* spp. are currently identified as human pathogens. The severity of human diseases differs among these species, ranging from mild to lethal illness (*4*). On the basis of serologic and genotypical characteristics, rickettsiae are divided into typhus and spotted fever groups. Within each group, antigenic differences are small, resulting in cross-reactivity that complicates differentiation of *Rickettsia* spp. by serologic methods. Therefore, PCR of eschar biopsy samples is a useful tool for diagnosis of rickettsial diseases (*5*).

Little information exists regarding the prevalence of *Rickettsia* spp. in *D. marginatus* and *D. reticulatus* ticks in Germany. *R. raoultii* was recently detected in 23% of *D. reticulatus* ticks (*6*). In 1977, Rehacek et al. identified *R. slovaca* in 12% of *Dermacentor* spp. ticks from southern Baden-Wuerttemberg (*7*). Since then, this pathogen has not been detected in Germany. *R. slovaca*, a member of the spotted fever group, causes tick-borne lymphadenopathy, a relatively mild rickettsiosis (*8*). We report detection of *R. slovaca* in 5 of 666 *Dermacentor* spp. ticks from southern Germany. Moreover, we identified a case of tick-borne lymphadenopathy from Rhineland-Palatinate.

We collected 666 adult *Dermacentor* spp. ticks by blanket-dragging; 26 were collected along the Main River near Aschaffenburg (Bavaria), and 640 from the Rhine Valley near Lörrach (Baden-Wuerttemberg). Ticks were homogenized, and the DNA was isolated by using the Maxwell 16 Instrument (Promega, Madison, WI, USA). For detection of rickettsiae, a TaqMan real-time PCR with the LightCycler system (Roche Diagnostics, Mannheim, Germany) was performed according to Wölfel et al. (*9*). A primer pair amplified a 70-bp fragment of the citrate synthase (*gltA*) gene. All positive samples were also tested with a PCR specific for the outer membrane protein A (*rOmpA*) gene (*10*). This amplification yielded an *rOmpA* fragment of 532 bp. Amplified products were analyzed by agarose gel electrophoresis.

For identification of *Rickettsia* spp., amplification products of the *rOmpA* PCR were sequenced by using fluorescence-labeled dideoxynucleotide technology (Applied Biosystems, Darmstadt, Germany). Separation of sequenced fragments and data collection were performed by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). All obtained sequences were analyzed and compared by using BLAST (www.ncbi.nlm.nih.gov/BLAST).

Rickettsia spp. was detected by both PCR methods in 31% of 666 *Dermacentor* spp. ticks examined. Sequencing of part of the *rOmpA* gene showed that sequences of 5 samples (0.75%) from Aschaffenburg were *R. slovaca*, showing 100% similarity with a sequence deposited in GenBank (accession no. U43808). Only *D. marginatus* ticks from Aschaffenburg were infected with *R. slovaca*, and 202 ticks from Lörrach were infected with *R. raoultii*.

We also identified a case of *R. slovaca* infection in southern Rhineland-Palatinate. The patient reported a tick bite; the tick was identified as *Dermacentor* spp. Fever, lymphadenopathy of submandibular lymph nodes, and exanthema at the site of the tick bite developed 7 days later. Serologic examinations by using an immunofluorescent test (Focus Diagnostics, Cypress, CA, USA) showed antibody titers of 64 for immunoglobulin (Ig) M and 1,024 for IgG against rickettsiae of the spotted fever group. These results indicated an acute rickettsial infection. Because of strong cross-reactivity among all species in the spotted fever group, we cannot differentiate between antibodies against *R. slovaca* and other species in this group.

However, another immunofluorescent test for typhus group rickettsiae showed negative results, confirming that the patient was infected with spotted fever group rickettsiae. Results of PCRs specific for *gltA* and *rOmpA* of the patient's tick were positive. We identified *R. slovaca* by sequencing the *rOmpA* gene. The sequence obtained showed 100% similarity with sequences in ticks from Aschaffenburg. Clinical symptoms, serologic results, and detection of *R. slovaca* in the tick from the patient strongly indicate that the patient had tick-borne lymphadenopathy caused by *R. slovaca*.

The high prevalence of *R. raoultii* in *Dermacentor* spp. ticks is of concern because this species can also cause tick-borne lymphadenopathy, although *R. raoultii* is less pathogenic than *R. slovaca* (*8*). Tick-borne lymphadenopathy should be considered in the differential diagnosis of tick-borne diseases. The extent of the distribution of *R. slovaca* and *R. raoultii* in Germany remains to be elucidated.

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Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies

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In Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies, Jens Kuhn presents a complete review of every paper published on the subject, as well as hundreds of unpublished reports. In addition, most of the world's known experts on filoviruses contributed personal data and anecdotes. A CD-ROM with a searchable list of all quoted references (≈4,500) is a useful addition to the book.

The author details the history of all Marburg and Ebola outbreaks during the 40 years from the discovery of Marburg in 1967 to the latest Marburg outbreak in 2007 in Uganda, including difficult-to-find information. The clinical and pathologic presentations of Ebola and Marburg diseases in human and animal models contain a substantial number of black and white and color illustrations. With the range of the Ebola and Marburg viruses still unknown and the search for the animal reservoirs ongoing, antibody serosurveys in humans and animals have been conducted in most countries in Africa and in the Philippines after the Ebola Reston outbreaks; Kuhn dedicates a long chapter to this subject. All animal species tested are presented in tables but, more interestingly, also indexed at the end of the book. This information is particularly valuable for ecologists and epidemiologists searching for the reservoir of filoviruses.

Kuhn explains the structure and replication of the filoviruses, with the actual role of each gene of the virus from entry into the target cells to production of infectious virus. He lists diagnosis techniques and experimental treatments of the Marburg and Ebola diseases, from the traditional healers to the molecular antisenses RNA approaches. In the past 10 years, preexposure and postexposures vaccinations have resulted in tremendous progress in schedules, routes of administration, and more importantly, understanding mechanisms of action. Lack or efficacy of inactivated and live-attenuated constructs is reviewed of all testing in available animal models.

This book is not, and is not supposed to be, a critical review of the literature but is rather a compilation of all known information on filoviruses by subject matter experts that is presented for filovirologists. Nonspecialist virologists, scientists, epidemiologists, clinicians, and students interested in the subject will also find the book useful, but they will have to digest and analyze the information and then weigh the values and relevance of this incredible compendium of data.

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Erratum—Vol. 15, No. 11

A word was missing from a sentence in the article Fatal Case of Enterovirus 71 Infection, France, 2007 (Vallet S, et al.). The second sentence of the article text should read: "The virus is a leading cause of hand, foot, and mouth disease and, above all, is an emerging agent of acute central nervous system disease (aseptic meningitis, flaccid paralysis, encephalitis)." The article has been corrected online (www.cdc.gov/eid/content/15/11/1837.htm).

A Groundhog, a Novel *Bartonella* Sequence, and My Father's Death

Edward B. Breitschwerdt, Ricardo G. Maggi, Maria Belen Cadenas, and Pedro Paulo Vissotto de Paiva Diniz

During the summer of 2007, migratory joint pain developed in my (E.B.B.) 86-year-old father, previously an ironworker, farmer, and World War II veteran. Because of occasional tick attachments, a *Borrelia burgdorferi* ELISA was performed; antibodies were not detected, and no treatment was instituted. In the fall, subtle memory loss developed, and he fell twice a few weeks apart. Dad jokingly blamed the falls and the memory loss on "old timer's disease." Subsequently, episodes of subtle confusion and more frequent memory loss generated family concern as to what the future might hold. On December 15, he broke his left femur during a fall while climbing 2 stairs to enter our home. Despite having successfully climbed those stairs thousands of times in the past, he would never climb those or any other stairs again.

Retrospectively obvious, a pattern of insidious illness characterized by joint pain, memory loss, and incoordination, not recognizable by my father or other family members, had begun before that summer. Medically stable historical problems included coronary artery disease, atherosclerosis, carotid artery occlusion, hypertension, and atrial fibrillation. During the previous year, a normocytic, normochromic, nonregenerative anemia persisted. Despite normal serum iron, total iron binding capacity, ferritin, and vitamin B12 values, anemia was attributed to intestinal blood loss. When examined in May 2007, before anesthesia for endoscopy, mood and affect were appropriate, recent and remote memory were intact, insight and judgment were good. A hiatal hernia, mild antral gastritis, and duodenitis were visualized.

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Initial Hospitalization

When my father was hospitalized December 15, 2007, with a broken femur, a resting pill roll tremor and cogwheel rigidity were suggestive of Parkinson disease. Preoperative neurologic consultation identified severe confusion, inattention, and an inability to answer questions. Short-term memory and problem-solving abilities were decreased. There was mild ptosis of the right eye, normal cranial nerves, mild asterixis, and hand weakness. Laboratory abnormalities included anemia, hypercreatinemia, an elevated aspartate aminotransferase level, and hyperglobulinemia. Due to the severity of the femoral fracture, the femoral head was excised and replaced with a bipolar femoral prosthesis.

Postoperatively, poor mentation was considered a sequela of general anesthesia and peri-operative analgesics. For more than a week, dementia persisted. He did not recognize family members and had near constant hallucinogenic activities, including agitation, tying knots, sawing motions, and constantly pulling covers, bed clothes, and fluid lines. Severe hematuria developed after he pulled an inflated Foley catheter from his urethra. Concurrent gastrointestinal bleeding of undetermined cause necessitated multiple blood transfusions. Other complications included difficulty swallowing and paralytic ileus. Repeat abdominal radiographs, in conjunction with stool softeners and laxatives, failed to alleviate gastrointestinal complications. Eventually, he refused food and became severely bloated. Endoscopy performed on December 26 identified severe necrotizing esophagitis, multiple plaques, and a stricture attributed to *Candida albicans* and herpes zoster. *C. albicans* esophagitis is known to accompany HIV infection, leukemia, or an unidentified source of immune suppression (*1*,*2*). Shingles, caused by herpes zoster, occurred during the previous Thanksgiving and can be associated with

immunosuppression, stress, or an aging immune system (*3*,*4*). Mentation and gastrointestinal abnormalities improved after starting treatment with fluconazole, acyclovir, and symptomatic medications for erosive esophagitis. However, confusion, lack of orientation, hyponatremia, hypokalemia, and hyperglycemia remained problematic until discharge to a physical therapy center on December 31.

Second Hospitalization

During the next week, strength and mental capacities improved rapidly and discharge to the home environment was scheduled for January 9. On that morning and while driving to Maryland to build entry ramps, I was informed by cell phone that my father fell out of a chair and became nonverbal and that a stroke was suspected. For me, the roller coaster illness ultimately leading to his death would take an unbelievable turn of events. Upon his transfer to the neurology service, encephalopathy, asterixis, Parkinsonian-type tremor, hypoactive reflexes, pinpoint and minimally reactive pupils, and cogwheel rigidity were found. Verbal communication was absent, but he would grimace with pain whenever extremities were manipulated. An urgent computed tomography scan did not identify intracranial abnormalities.

When I was a boy, my father used the expression "something is fishy in Denmark" to imply that something was astray. Based upon historical events, I suspected something was being missed. Laboratory findings included anemia (hemocrit 34%), a normal leukogram (leukocytes 9,100 cells/μL, 2% band neutrophils), mild hypokalemia, hypoalbuminemia, hyperglycemia, increased serum alkaline phosphatase level, erythrocyte sedimentation rate of 79, and hypergammaglobulinemia. Thoracic radiographs identified mild bilateral pleural effusion. Because an undefined infectious source of immunosuppression seemed plausible, and fever (maximum temperature 38.6°C) occurred 24 hours after neurologic decompensation an infectious etiology was pursued. Nasal methicillin-resistant *Staphylococcus aureus*, blood, urine, and cerebrospinal fluid (CSF) cultures were negative. CSF results, including those for special stains, were unremarkable. Serologic results for *Treponema pallidum, Borrelia burgdorferi*, *Rickettsia rickettsii, Bartonella henselae, Bartonella quintana*, and HIV were negative. Results of CSF herpes simplex PCR and a test for *T. pallidum* antibodies were negative.

On January 11, results of magnetic resonance imaging and magnetic resonance angiography were interpreted as a left posterior stroke with no active bleeding. Initial treatment included intravenous acyclovir, fluconazole, vancomycin, ceftriaxone, ampicillin, and dexamethasone.

Translational Research and the Practice of Medicine

Because I direct the Intracellular Pathogens Research Laboratory (IPRL) at the North Carolina State University College of Veterinary Medicine, aseptically obtained blood and CSF samples were kindly provided for testing. Results of PCR (*5*,*6*) specific for *Anaplasma, Ehrlichia*, and *Rickettsia* species were negative. *Bartonella* 16S–23S intergenic spacer primers (*7*) repeatedly generated amplicons of different sizes from blood and CSF, respectively. Compared with GenBank sequences, the blood amplicon was most similar (434/465bp) to *Candidatus* Bartonella volans (strain FSq-1, EU294521) isolated from a southern flying squirrel (*Glaucomys volans*) and *Candidatus* Bartonella durdenii (391/422bp) amplified from *Orchopeas howardi* (GenBank accession no. DQ 336386), a flea found on eastern US gray squirrels (*Scinrus carolinensis*), and a *Bartonella* sp. (446/492bp, EF125214) identified in ground squirrels (*Spermophilus danricus*) in the People's Republic of China. The novel rodent *Bartonella* sequence obtained from my father's blood had an 18-bp insert at positions 2047 or 334 in EU294521 and DQ336386, respectively. Previously, our laboratory had never worked with rodent *Bartonella* species and had never amplified a 300-bp internal transcribed spacer region amplicon from >3,000 animal or human blood samples. After several unsuccessful cloning attempts, the CSF amplicon was most similar (393/394 bp) to *B. henselae* (NC-005956). Blood and CSF, cultured by using *Bartonella* α Proteobacteria growth medium (BAPGM) (*8*), did not result in the growth of a *Bartonella* species.

Clinicians and Scientists Working Together

After *Bartonella* PCR results became available, treatment with piperacillin and tazobactam were continued for 3 weeks, until discharge. Levetiracetam was added to the patient's treatment because a generalized seizure occurred shortly after antimicrobial drugs were given. On January 18, severe dependent edema of the right elbow resulted in fluid leakage through intact skin. For 3 weeks, Dad remained semicomatose, disoriented, agitated, and encephalopathic. Hallucinations continued, accompanied by frequent involuntary motor movements. Diabetes mellitus and a large decubital ulcer on the right heel developed. During the fourth week, mentation improved, and he could rise and stand for brief periods. On January 28, he was discharged to a rehabilitation facility, with instructions to receive doxycycline and rifampin 2×/d for 13 days. Blood samples, obtained aseptically before discharge, were again submitted to the IPRL. After BAPGM pre-enrichment and subculture, *B. vinsonii* subsp. *berkhoffii* genotype II was isolated, and sequential serologic testing identified a rising titer to

Table. PCR, blood, and CSF *Bartonella* spp. culture and serologic results from an 86-year-old man with recent onset arthritis, memory loss, and encephalopathy*

Date	Location and sample type	Serologic results (titers)		BAPGM enrichment platform		
		B. henselae	*B. vinsonii* subsp. *berkhoffii*	Direct extraction	Pre-enrichment culture	*Bartonella* isolate
2005 Sep 23	Home/blood	NA	NA	Neg	Neg	NIO
2006 Aug 19	Home/blood	NA	NA	Neg	Neg	NIO
2008 Jan 11	Hospital 2/blood	NA	NA	*B. volans*–like	Neg	NIO
	Hospital 2/CSF	NT	NT	*B. henselae*	Neg	NIO
2008 Jan 28	Hospital 2/EDTA blood	16	16	Neg	Neg	*Bvb* II
	Hospital 2/ACD blood	NA	NA	Neg	Neg	*Bvb* II
2008 Feb 10	Home/blood	<16	128	Neg	Neg	NIO
2008 Mar 11	Hospital 3/blood	<16	64	Neg	Neg	NIO
2008 Apr 4	Hospital 3/blood	<16	32	*B. volans*–like	Neg	Neg
2008 Apr 28	Home/blood	16	64	Neg	*Bvb* II	Neg

*CSF, cerebrospinal fluid; BAPGM, *Bartonella* α Proteobacteria growth medium; NA, no available serum; Neg, DNA was not amplified by using *Bartonella* 16S–23S intergenic spacer primers; NIO, no isolate obtained by subculture after BAPGM pre-enrichment culture; Hospital 2, second hospitalization; NT, not tested; ACD, acid-citrate-dextrose; *Bvb*II, *B. vinsonii* subsp. *berkhoffii* genotype II; Hospital 3, third hospitalization.

B. vinsonii subsp. *berkhoffii* but did not detect *B. henselae* antibodies (Table). After a brief, emotionally traumatic stay at the rehabilitation facility, Dad returned home to be cared for by 4 sons, his wife of 60 years, and other family members. Each week a different son slept by his bed, which was relocated to the family living room.

Home Again at Last

During the next 3 weeks, there was substantial and progressive improvement in physical capabilities and a return of normal mental capabilities, including exceptional short- and long-term memory. Appetite normalized, and despite severe atrophy, muscle strength increased so he could stand, walk with assistance, and, although a daily struggle, access the bathroom. A February 10 blood sample obtained while he was receiving oral antimicrobial drugs was *Bartonella* PCR negative, and no bacteria were isolated in BAPGM. During this precious 3 weeks, our father joked, laughed, and vividly recalled wartime friends and other experiences. On March 1, 2008, he opened Christmas presents with our family. I should have been there.

Third and Final Hospitalization

On March 4, ≈2 weeks after the course of oral antibiotics was completed, agitation and disorientation returned, and mental status deteriorated. Within 24 hours, Dad was hospitalized, where fine motor tremors of the right hand and wrist, asymmetric edema involving the right leg, and edema of the penis and scrotum were noted. He was afebrile and nonverbal and could not follow simple commands. Hematocrit was 30.2%, platelet count 557,000 cells/μL, and leukocyte count 7,800 cells/μL with a normal differential count. Serum biochemical abnormalities included hyperglycemia (glucose 218 mg/dL), hyperglobulinemia (3.6 g/dL), and increased alkaline phosphatase activity (169 IU/L). Urinalysis abnormalities included proteinuria with occasional hyaline casts.

Lorazepam was administered to control the agitation and restlessness. The warfarin dose was increased and heparinization initiated for a potential cerebrovascular accident. Due to the prior documentation of *Bartonella* infection, intravenous doxycycline, rifampin, and gentamicin were administered, and total parenteral nutrition was instituted. Again, shortly after initiation of antimicrobial drugs, a seizure occurred. *Bartonella* spp. were not amplified or isolated from a 1-mL blood sample obtained 4 days after initiation of treatment with antimicrobial drugs. During the next 3 weeks, while intravenous antimicrobial drugs were administered, our father again remained encephalopathic, with frequent hallucinations, severe agitation, and near-constant mental confusion. On March 14, intravenous methylprednisolone for potential immune-mediated vasculitis elicited no improvement in mental status. Similar to the previous treatment course, improvement in mental status, coherent communication, and renewed ability to recognize family members occurred during the fourth hospitalization week.

A Battle Lost

On April 4, Dad was discharged to our home and oral antimicrobial drugs (doxycycline and rifampin) were dispensed. Despite all efforts by medical professionals, members of our family, and our tough 86-year-old father, protracted illness and prolonged hospitalizations had resulted in mental and physical debilitation, severe muscle wasting, and profound weakness. More important, he had lost his desire to live. After discharge, there was minimal neurologic improvement. Before the availability of April 4 IPRL test results, he began to refuse all medications. The identical rodent *Bartonella* DNA sequence was again amplified from his blood, but no bacteria were isolated.

Four weeks later my father died, on Friday, May 2, at 5 PM, around quitting time for an old iron worker. My mother and youngest brother were at his side. After his

death, blood culture results from another sample obtained by the hospice nurse on April 28, 2008, became available. *B. vinsonii* subsp. *berkhoffii* genotype II was amplified and sequenced from the enrichment BAPGM blood culture. As direct extraction of DNA from blood was negative, growth of viable bacteria in liquid culture was implicated (*8*,*9*).

Groundhogs, Fleas, and the Genus *Bartonella*

Following our father's death, I recalled a small, 0.5-cm, raised, firm lesion within his right eyebrow that developed during the summer of 2007 and would spontaneously hurt or burn, causing him to rub or squeeze the lesion. The mass disappeared after he began taking antimicrobial drugs in 2008. Retrospectively, I suspected a rodent flea bite above the eye had transmitted a novel *Bartonella* species, which we sequenced from his blood after each hospitalization. All known *Bartonella* spp. have preferential animal reservoir hosts, and each uses arthropods or animal bites and scratches as the primary modes of transmission (*10*–*12*). Dad would occasionally capture mice, rats, skunks, and groundhogs in the barns. Groundhogs were transported in the car trunk to a distant location for release, potentially leaving behind fleas. Therefore, 3 *Candidatus* Bartonella spp. isolates were provided by Dr. William Nicholson, a colleague at the Centers for Disease Control and Prevention in Atlanta. After sequencing, the 16S–23S intergenic spacer region of a ground squirrel (*Candidatus* Bartonella durdenii), a flying squirrel (*Candidatus* Bartonella volans), and a groundhog (*Candidatus* Bartonella monaxi) isolate, the most similar GenBank sequence was *Candidatus* Bartonella volans. There was no perfect match with these 3 isolates. However, sequences from Dad's blood clustered with a squirrel *Bartonella* subgroup. This observation supports the presence of a novel *Bartonella* species on the eastern shore of Maryland, an as yet undefined animal reservoir, and an unknown arthropod vector.

Regardless of the mode(s) of transmission, repeated molecular documentation of a novel rodent *Bartonella* sp. and *B. vinsonii* subsp. *berkhoffii* supports the unexpected failure of 2 intensive courses of intravenous and oral antimicrobial drugs to eliminate these fastidious, intravascular bacteria. During the first two hospitalization periods, there was similar and progressive improvement in neurologic signs and mental capabilities that began during the fourth week of antimicrobial drug administration. Pre-enrichment BAPGM growth of *B. vinsonii* subsp. *berkhoffii* from blood obtained 4 days before death supports persistence of viable organisms. Recently, antimicrobial drug resistance genes have been characterized in *B. bacilliformis*, *B. henselae*, and *B. quintana* by in vitro serial passage (*13*–*15*). Retrospectively, the relapse in encephalopathic signs might have been avoided if antimicrobial drugs were continued for a larger interval after discharge from hospital 2, and blood cultures were optimally obtained and sequentially tested to confirm therapeutic elimination.

Elimination of *Bartonella* spp. by antimicrobial drugs in immunocompetent patients may be more difficult to achieve than is currently appreciated (*16*). Although coinfection with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* has been previously reported, DNA of 3 *Bartonella* spp. was detected in our father. Based on repeatable PCR testing, a small quantity of *B. henselae* DNA was in the January CSF sample. Because PCR amplicon contamination was never detected in any negative control, laboratory error is considered unlikely. Although the BAPGM enrichment approach has improved molecular detection and isolation of some *Bartonella* spp. from human patient samples (*9*,*16*–*18*), a rodent *Bartonella* sp. isolate was not obtained. Unfortunately, 8 weeks can be required from inoculation of BAPGM until a subculture agar plate isolate is characterized by DNA sequencing. Therefore, IPRL test results were often not available to Dad's physicians in a timely manner.

Age, *Bartonella* spp., and Immune Suppression?

Suspicion of an undetermined source of immune suppression and recent tick exposures were primary factors motivating testing in the IPRL. Previously, *B. vinsonii* subsp. *berkhoffii* was shown to induce immunosuppression in experimentally infected dogs (*19*,*20*). In retrospect, occult infection with *Bartonella* spp. may have contributed to shingles at Thanksgiving and necrotizing *C. albicans* esophagitis after hospitalization for the fractured femur. Recently, *B. quintana* lipopolysaccharide was found to have antiinflammatory properties (*21*). Immune suppressive factors may facilitate persistent intravascular *Bartonella* infection without inducing obvious infection indicators, such as fever, tachycardia, leukocytosis, and CSF pleocytosis. Fever was documented once and mild neutrophilia for 3 of 48 blood counts. Thrombocytosis, previously associated with *B. henselae* (*22*), was documented 14 times.

Ecologic Complexity of *Bartonella* spp.

Because of my father's long-standing atherosclerosis and because BAPGM will grow a spectrum of seemingly difficult to isolate bacteria (*8*,*23*), pre-enrichment blood cultures and *Bartonella* internal transcribed spacer region PCR had been performed in September 2005 and August 2006 (Table). *Bartonella* spp. were not amplified or isolated, which suggests infection occurred after the summer of 2006. Transmission of *B. henselae, B. vinsonii* subsp. *berkhoffii*, and *B. alsatica* can occur as a result of a scratch from a cat, a dog, or a wild rabbit, respectively (*17*,*24*–*26*). Cats are the primary reservoir for *B. henselae*, whereas dogs and coyotes are the only reported reservoir hosts for *B. vinsonii* subsp. *berkhoffii* genotype II in North America (*27*). Recently, *B. vinsonii* subsp. *berkhoffii* genotype II was

isolated by BAPGM blood culture from a cat with recurrent osteomyelitis (E.B. Breitschwerdt, unpub. data), which suggests that a bacteremic cat might facilitate transmission of this subspecies. My parents had an old (≈21 years of age) exclusively outdoor barn cat that would occasionally scratch. The cat could not be tested because it died in 2007. *B. henselae* and *B. clarridgeae* have been transmitted experimentally by transfusion to cats (*24*). Because *B. vinsonii* subsp. *berkhoffii* seroconversion occurred during hospitalization, transfusion-associated transmission is also possible. The exact timing and mode of transmission of *B. henselae, B. vinsonii* subsp. *berkhoffii*, and the rodent *Bartonella* sp. to our father cannot be established. However, his illness serves to illustrate the medical and ecologic complexity of this genus.

Occult Infection and Chronic Illness

Reconstructing the history of a chronic illness is always difficult and remains an unexacting science due to known, unknown, and undetermined factors that influence disease expression over time. Experimental studies that used rodent models have emphasized the ability of *Bartonella* spp. to invade erythrocytes and vascular endothelial cells (*28*). In vitro *studies* indicate that *B. henselae* can infect macrophages, microgial cells, dendritic cells, and CD34+ progenitor cells (*29*). *B. henselae* and *B. vinsonii* subsp. *berkhoffii* have been amplified from dog lymph node aspiration samples (*30*). Thus in a given patient, *Bartonella* organisms likely infect a substantial number of cellular targets. *B. henselae* infection induces chronic arthritis in a subset of cat scratch disease (CSD) patients, and atypical CSD manifestations are more likely to develop in elderly patients (*31*,*32*). In the context of arthritis, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* were repeatedly isolated from joint fluid from a dog in which repeated antimicrobial drug therapy was not successful (*33*). Although a spectrum of acute and generally self-limiting neurologic manifestations have been historically described in CSD patients, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* were only recently isolated from patients with chronic neurologic and neurocognitive abnormalities (*16*).

We propose that the initial arthritic signs, short-term memory loss, and incoordination were premonitory signs of *Bartonella* spp. infection, and that persistent infection contributed to localized edema, nonregenerative anemia, thrombocytosis, hyperglobulinemia, and a protracted debilitating illness accompanied by hallucinations, agitation, seizures, and death. Agitation, disorientation, and combative behavior have been reported in association with CSD and physicians have implicated *Bartonella* spp. as contributors to agitation and treatment-resistant depression (*34*,*35*). Memory loss and a spectrum of neurocognitive complaints have also been reported in immunocompetent persons infected with *B. vinsonii* subsp. *berkhoffii* and *B. henselae* (*9*,*16*,*17*).

My Father and "One Medicine"

In recent years, there has been renewed interest in the concept of "One Medicine" (*36*). I hope that lessons from my father's death can reinforce the importance of "One Medicine." However, as in the past, rhetoric may not result in needed increases in resource allocation to enhance educational, research, service, and public health capabilities of the veterinary profession (*37*). In the context of vector-borne infectious diseases, zoonotic diseases, food safety, zoologic medicine, and environmental medicine and ecosystem health, to name a few areas, veterinarians continue to make major contributions that ensure and enhance the daily health of animals and humans. Because of a research focus in comparative infectious diseases and knowledge of the biologic, immunologic, and pathophysiologic behavior of *Bartonella* spp. in a spectrum of animal species, a veterinary research laboratory was able to assist with the management of my father's illness.

As is often true of research at the bedside and the laboratory bench, new lessons and challenges arose from the collective efforts of doctors, nurses, veterinarians, research scientists, and others attempting to heal my father. I and my family remain sincerely grateful to the many doctors, nurses, and other caregivers who contributed to the management of my father's surgical and medical problems. Because of the severe encephalopathic and combative nature of his behavior, this was frequently not an easy or pleasant task. During his illness, I was struck by several items: 1) most nurses are absolutely amazing, caring, and dedicated professionals; 2) in human medicine, unlike veterinary medicine, no physician claimed or accepted the responsibility to be my father's doctor; and 3) for many reasons, I found the human healthcare system to be frayed, if not broken. Whether blame lies with the insurance companies, our litigious society, the profit-based motivations of hospital administration, the increased complexity of medical technology, or the medical education of physicians, it really does not matter. As my father would say, "It is no way to do business." I have taught internal medicine at a College of Veterinary Medicine for 32 years. During that time, every sick animal on our medical service had at least 2 doctors (1 being a student), who were directly responsible for the animal's care, for frequent communications with the owner, and for communications with the referring veterinarian. In our increasingly complex hospital environments, every patient needs a personal advocate or designated doctor to represent his or her interests.

Epilogue

Some years ago in a conversation with my mother I suggested that the term natural death may well represent an

oversimplification of the processes that end a person's life. My father and our family were substantially affected during his illness. Each day, from initial hospitalization until his death, there was at least 1 family member at his side. In the eyes of family and friends, my father was a great man in so many respects. He was a loving husband, a caring father, diligent worker, and a friend and supporter to many persons. Potentially, his illness illustrates a complex interaction between intravascular *Bartonella* infection and complex disease expression, provides documentation for an as yet uncharacterized zoonotic rodent *Bartonella* sp., and offers disconcerting evidence supporting antimicrobial drug ineffectiveness and clinical evidence supporting the concept that persistent infection with ≥1 *Bartonella* spp. may lead to immunosuppression and opportunistic infections with organisms such as herpes zoster and *C. albicans*. As in his life, Dad would want this story to benefit others after his death. We hope that it does.

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Rosa Bonheur (1822–1899) Plowing in Nivernais (1850) Oil on canvas (133.4 cm × 259.1 cm) SN433 Collection of the John and Mable Ringling Museum of Art, the State Art Museum of Florida, a Division of Florida State University

"I think I could turn and live with animals"

—Walt Whitman

Polyxeni Potter

"I wed art. It is my husband—my world—my life-dream—the air I breathe," said Rosa Bonheur, explaining her life choices. "Art is absorbent—a tyrant. It demands heart, brain, soul, body, the entireness of its votary." A native of Bordeaux, France, Bonheur was betrothed to art early in life, born into a family of artists. They moved to Paris when she was still a young child. Precocious and headstrong, she had to be coaxed to learn how to read by her mother, who had her select and draw an animal for each letter of the alphabet. Bonheur later attributed her love of drawing animals to this early practice.

During her early days in Paris when the family lived in an apartment, she kept a small menagerie of ducks, rabbits, squirrels, and a sheep that had to be carried up and down the stairs regularly. Expelled from traditional schools for rebelliousness by age 12 and refusing to apprentice with a seamstress, another conventional option, she was turned over to her father Raimond Bonheur, a painter, sculptor, and educator, for instruction. She showed herself a diligent and conscientious art student and soon began copying masterpieces at the Louvre. She assisted him with painting commissions and excelled as sculptor. She started visiting Paris abattoirs and the École nationale vétérinaire d'Alfort to learn animal anatomy by dissecting carcasses. "Oh! You've got to be devoted to art to live in pools of blood, surrounded by butchers."

She also attended horse fairs and farmers' markets to observe animals' emotions and behavior. During these outings, "I was forced to recognize that the clothing of my sex was a constant bother. That is why I decided to solicit the authorization to wear men's clothing from the prefect of police. But the suit I wear is my work attire and nothing else." Despite her protestations, Bonheur's independent thinking, original approach to societal restrictions, and bohemian lifestyle created an aura of notoriety about her that at times eclipsed her artistic accomplishments.

She met and became friends with Étienne Geoffroy Saint-Hilaire and his son, Isidore, renowned anatomists and zoologists. Her sympathetic portrayal of animals was influenced by their studies in natural history, particularly the father's unity of composition principle. He believed that all organisms shared the same underlying design and that diversity in external form was simple variation. If birds and reptiles are built on a single plan, "an accident that befell

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one of the reptiles … could develop in every part of the body the conditions of the ornithological type."

Bonheur's style was part of the realist movement of the mid-1800s, led by Gustave Courbet and Jean-François Millet. In this style, which relied on direct observation and meticulous draftsmanship, naturalism was aligned with social causes and the labor movement. Despite prevailing trends, which favored rural life, the plight of peasants, and the ills of growing industrialization, she chose animals as her subjects. Even in rural scenes, her focus was on them. Her affinity to animals and her devotion to showing them in their natural environment established her as the foremost animalière of her century, one of the best of all time.

Although she was aware of the impressionists, Bonheur did not adopt their style. For inspiration she turned instead to the Parthenon friezes and the romantic paintings of horses by Théodore Géricault and Eugène Delacroix. She was also influenced by her friend, the acclaimed English animal painter Edward Landseer.

Her work attracted early attention. She began exhibiting in the Salon at age 19 and continued to exhibit there successfully over many years. She stopped showing sculpture when she became aware of her brother Isidore's talent in that art. She "did not want to hinder [his] artistic career." Her painting Cows and Bulls of the Cantal received a gold medal at the Salon. After this success she received a commission from the government to create a painting of animals at work in the fields. The work, Plowing in Nivernais, was very well received.

When her father died, she succeeded him as director of the art school for girls where he had worked. At that time she also established a studio with friend and fellow artist Nathalie Micas and began to work on massive paintings of horses. One of these, The Horse Fair, became a sensation and attracted the attention of Britain's Queen Victoria. Bonheur's fame was far-reaching. She received the Cross of San Carlos of Mexico; membership in the Académie des Beaux-Arts of Antwerp, Belgium; the Commander's Cross of the Royal Order of Isabella from Spain's Alphonso XII; and the French Legion of Honor. She handled fame gracefully and wore the medals.

As her commercial success increased, she was able to move to a chateau outside Paris near the forest of Fontainebleau. On these spacious grounds, she created a small zoo, with ponies, deer, monkeys, cattle, and other animals that populated her future work. "One of her pets was a young lion whom she allowed to run about and often romped with."

Bonheur's fascination with the New World and the American West began with Buffalo Bill's Wild West extravaganza, a combination of circus and historical reenactment, during its European tour through France. She traveled to the United States and painted American themes and a famous portrait of Buffalo Bill Cody, who became her friend. Her interest in the United States led to her long connection with Anna Klumpke, an American artist, who after Bonheur's death, found hundreds of paintings and drawings unseen by anyone in her friend's studio and pulled together the artist's (auto)biography.

Plowing in Nivernais, on this month's cover, is a copy by Bonheur of the original government commission, made a year later and likely inspired by The Devil's Pool, a novel by George Sand (1804–1876). This story, about the displacement of peasants by industrialization, contained the lines, "But what caught my attention was a truly beautiful sight, a noble subject for a painter. At the far end of the flat plow land, a handsome young man was driving a magnificent team [of] oxen." Bonheur lived in Nivernais, in central France, for weeks, observing the animals and the land, the people at work, the agricultural tools. Her depiction was so accurate that the region was immediately identified when the painting was unveiled.

"Oxen that rattle the yoke and chain or halt in the leafy shade, what is that you express in your eyes?" wondered Walt Whitman in his poem Song of Myself, "It seems to me more than all the print I have read in my life." In Bonheur's own "poem," the massive beasts crisscross the good earth, plowing uphill. Slow and solid, they dominate a landscape vast as the sky, tails whisking, mucus glistening. The peasants are in the sidelines. Small and unimposing, they follow or step next to them, light-footed, like dancers. The man toward the front holds a thin stick above his head, exerting dominance, choreographing the animals' movements. He is their colleague, a member of the herd, who grooms and feeds them and lives with them. The animal in the lead squints ahead, one that follows looks directly at us. Industrialization has not yet arrived in this agrarian corner.

Nineteenth-century concerns about the spread of urbanization have only grown in our times. The percentage of residents in urban areas is projected to increase from 50% in 2008 to 70% in 2025. But along with displacement of agriculture, other fears cloud the horizon. Crowded urban areas have become uniquely vulnerable to public health crises, not least of them pandemic (H1N1) 2009. Recent outbreaks in Mexico City and New York demonstrate that surveillance efforts and management of public health communication and response demand exceptional alertness and coordination.

And the animals? "[T]hey are so placid and self contan'd, / I stand and look at them long and long," wrote Whitman when he offered to "turn and live with" them. For the poet, they held as much fascination as for Bonheur, who first brought them into her apartment and later established them in her own zoo at Fontainebleau, shunning portraiture and trendy interiors to paint them exclusively. He was attracted to their non-humanness, "They do not sweat and

whine about their condition, / They do not lie awake in the dark and weep for their sins." Furthermore, "Not one is dissatisfied, not one is demented with the mania of owning things, / Not one kneels to another, nor to his kind that lived thousands of years ago, / Not one is respectable or unhappy over the earth."

Bonheur understood animals. Her unconventional spirit was drawn to their wildness. "I too am not a bit tamed, I too am untranslatable, / I sound my barbaric yawp over the roofs of the world." But, like the poet, she did not know all we share with them. Her furry friends in the apartment or the chateau—rodents, rabbits, goats, deer, cattle, lions-and some that she did not collect, appear in the pages of this issue. For they share with us not just living space but countless infections.

How could the artist have known the litany of zoonoses that continue to complicate our relationship with animals: highly pathogenic influenza A virus (H5N1) in backyard chickens; human trichinellosis associated with ingestion of soft-shelled turtles; new adenovirus in bats; polycystic echinococcosis in jaguar hunters; *Bartonella rochalimae* in raccoons, coyotes, and red foxes; *Ehrlichia chaffeensis* in Sika Deer; *Mycobacterium bovis* and *M. tuberculosis* from goats. We have learned to live and work with animals. Now if we could also choreograph the microbes we all share ….

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Laboratory Surge Response to Pandemic (H1N1) 2009 Outbreak, New York, New York, USA

Public Health Threat of New, Reemerging, and Neglected Zoonoses in the Industrialized World

Projecting Global Occurrence of *Cryptococcus gattii* by Meta-Analysis of Published Reports

Urinary Tract Infections Caused by Foodborne *Escherichia coli*

Meningitis Caused by Novel Enterovirus, Northern Territory, Australia

Worldwide Dissemination of the bla_{OXA-23} Carbapenemase Gene of *Acinetobacter baumannii*

Recombinant Canine Coronaviruses in Dogs, Europe

Travel-associated Pandemic (H1N1) 2009 Virus Infection, Singapore

Severe Pneumonia Associated with Pandemic (H1N1) 2009 Outbreak, Mexico

Comparison of Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Infection in Dogs

Ceftiofur Use in Chicken Hatcheries and Human *Salmonella enterica* Serovar Heidelberg Infection, Canada

Healthcare-associated Viral Gastroenteritis among Children in a Large Pediatric Hospital

Actinobaculum schaalii, a Common Uropathogen in Elderly Patients, Denmark

Norovirus Gastroenteritis Outbreak with a Secretor-Independent Susceptibility Pattern, Sweden

Fluoroquinolone-Resistant *Escherichia coli* from Broiler Chicken Feed, Iceland

Human Listeriosis Caused by *Listeria ivanovi*

Pandemic (H1N1) 2009 Surveillance and Seasonal Influenza Prevalence, Singapore

Acute Encephalopathy Associated with Influenza A Infection in Adult Patients

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

Upcoming Infectious Disease Activities

December 4–6, 2009
Northeastern Ohio Universities Colleges of Medicine and Pharmacy
27th Annual Infectious Disease Seminar for the Practicing Physician
Edgewater Beach Hotel
Naples, FL, USA
http://www.neoucom.edu/ce

2010

February 19–21, 2010
2nd International Berlin Bat Meeting: Bat Biology and Infectious Diseases
Berlin, Germany
http://www.izw-berlin.de

March 18–22, 2010
Fifth Decennial: International Conference on Healthcare-Associated Infections 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.decennial2010.com

March 24–26, 2010
16th ISHEID (International Symposium on HIV & Emerging Infectious Diseases)
Marseille, France
http://www.isheid.com

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Community-associated Methicillin-Resistant *Staphylococcus aureus* in Outpatients, United States, 1999–2006

CME Questions

1. Which of the following characteristics helps to differentiate community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) from hospital-associated (HA)–MRSA?

A. Resistance to fluoroquinolones
B. Resistance to a higher number of antibiotics
C. Resistance to vancomycin
D. Resistance to beta-lactam and erythromycin only

2. Which of the following trends were noted in the epidemiology of *outpatient* MRSA in the current study?

A. The presence of MRSA was stable over the study period
B. The number of MRSA isolates resistant to at least 1 other drug increased significantly
C. *S aureus* infections that were MRSA nearly doubled
D. HA-MRSA accounted for the majority of change in the prevalence of MRSA among outpatients

3. The number of *S aureus* isolates resistant only to oxacillin increased most significantly from which anatomic site?

A. Lung
B. Blood
C. Skin and soft tissue
D. Genitourinary tract

4. Which of the following statements about the epidemiology of MRSA among *inpatients* is most accurate?

A. The proportion of *S aureus* infections that were MRSA increased by 25%
B. The prevalence of *S aureus* isolates resistant only to oxacillin decreased
C. The prevalence of HA-MRSA isolates fell sharply as CA-MRSA increased
D. There was a significant increase in lung infections with multiple-drug resistant MRSA

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

November 2009

Globally Mobile Populations

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

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

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

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

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

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

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

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

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

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.