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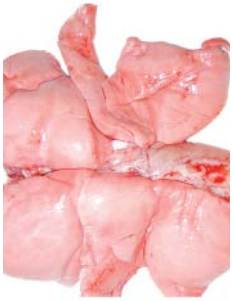

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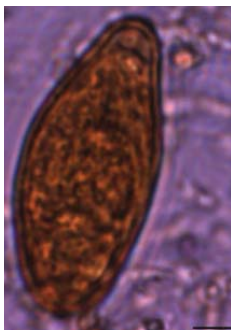

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Highly Pathogenic Avian Influenza Virus (H5N1) Infection in Red Foxes Fed Infected Bird Carcasses

Leslie A. Reperant, Geert van Amerongen, Marco W.G. van de Bildt, Guus F. Rimmelzwaan, Andrew P. Dobson, Albert D.M.E. Osterhaus, and Thijs Kuiken

Eating infected wild birds may put wild carnivores at high risk for infection with highly pathogenic avian influenza (HPAI) virus (H5N1). To determine whether red foxes (*Vulpes vulpes*) are susceptible to infection with HPAI virus (H5N1), we infected 3 foxes intratracheally. They excreted virus pharyngeally for 3–7 days at peak titers of $10^{3.5}$–$10^{5.2}$ median tissue culture infective dose ($TCID_{50}$) per mL and had severe pneumonia, myocarditis, and encephalitis. To determine whether foxes can become infected by the presumed natural route, we fed infected bird carcasses to 3 other red foxes. These foxes excreted virus pharyngeally for 3–5 days at peak titers of $10^{4.2}$–$10^{4.5}$ $TCID_{50}$/mL, but only mild or no pneumonia developed. This study demonstrates that red foxes fed bird carcasses infected with HPAI virus (H5N1) can excrete virus while remaining free of severe disease, thereby potentially playing a role in virus dispersal.

Influenza A viruses rarely infect species of the order Carnivora. However, since 2003, highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 have infected a wide range of carnivore species. Within the past 30 years, and before the emergence of HPAI viruses (H5N1), 5 documented outbreaks of influenza virus infections occurred in 2 carnivore species—the harbor seal (*Phoca vitulina*) (*1*–*4*), and the American mink (*Mustela vison*) (*5*). In both species, the infection resulted in respiratory disease. In addition, influenza virus infection has been detected by virus culture or serologic examination in other carnivores, namely, domestic dogs (*Canis lupus familiaris*) (*6*,*7*), domestic cats (*Felis catus*) (*8*,*9*), and bears kept in captivity (species not stated) (*9*); however, these animals did not show clinical signs of disease. Also, recently, outbreaks of equine influenza virus (H3N8) infections resulted in respiratory disease in domestic dogs (*10*,*11*). In contrast, within the past 5 years, HPAI viruses (H5N1) have infected and killed carnivores belonging to 7 species: captive tigers (*Panthera tigris*) and leopards (*P. pardus*) (*12*,*13*); domestic cats (*14*–*17*); captive Owston's palm civets (*Chrotogale owstoni*) (*18*); a domestic dog (*19*); a free-living stone marten (*Martes foina*) (*20*); and a free-living American mink (*21*). In these species, the infection resulted in both respiratory and extrarespiratory lesions, demonstrating systemic infection beyond the respiratory system. The most frequently reported clinical signs for all species were respiratory distress, neurologic signs, or both.

The sources of most HPAI virus (H5N1) infections in carnivores were traced to infected birds eaten by the animals (*12*–*15*,*19*). Until 2005, carnivores infected with HPAI virus (H5N1) were either wild carnivores kept in captivity or domestic carnivores that ate infected domestic or peridomestic birds (*12*–*14*,*19*). Since 2005, and after the spread of HPAI virus (H5N1) of the Qinghai sublineage (clade 2.2) outside Southeast Asia in poultry and wild bird populations, carnivores infected with HPAI virus (H5N1) included for the first time free-living wild carnivores, which presumably ate infected wild birds (*20*,*21*).

The occurrence of HPAI viruses (H5N1) in wild bird populations is likely to result in the exposure and infection of free-living wild carnivore species. In particular, abundant and widespread species of wild carnivores that have opportunistic feeding habits and that feed on wild birds may be at high risk for exposure. The red fox (*Vulpes vulpes*) is one of the most abundant and widespread species of wild carnivores in Eurasia. Partly because of rabies eradication (*22*,*23*), fox populations in western Europe have increased

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drastically since the mid-1980s (e.g., >140% in Germany; *24*). The red fox is also an opportunistic carnivore species and has a diverse diet, which includes small mammals and birds (*24*,*25*). Therefore, it may likely hunt or scavenge wild birds infected with HPAI viruses (H5N1). However, the susceptibility of this species to infection with influenza viruses is unknown.

In this study, we asked 2 questions: 1) Are red foxes susceptible to infection with a wild bird isolate of HPAI virus (H5N1) from clade 2.2? and 2) Can red foxes become infected by the presumed natural route of infection, i.e., after feeding on infected bird carcasses? To answer these questions, we experimentally assessed the excretion pattern (based on route, duration, and concentration of virus excretion) and pathogenicity (based on clinical signs, death rates, and distribution of lesions and virus) of a wild bird isolate of clade 2.2 HPAI virus (H5N1) in red foxes infected intratracheally and in red foxes fed infected bird carcasses.

Materials and Methods

Virus Preparation

A virus stock was prepared of influenza virus A/whooper swan/Germany/R65-1/2006 (H5N1), which was isolated from a wild whooper swan (*Cygnus cygnus*) found dead on Rügen Island, Germany, in February 2006. (The isolate was kindly provided by Dr Martin Beer, Friedrich-Loeffler-Institute, Greifswald–Insel Riems, Germany.) It was propagated twice in MDCK cells and titrated according to standard methods (*26*). The stock reached an infectious virus titer of $10^{6.9}$ median tissue culture infectious dose ($TCID_{50}$) per mL. It was then diluted in phosphate-buffered saline (PBS) to obtain a concentration of 10^4 $TCID_{50}$/mL.

Experimental Design

Eight juvenile (6–10 months of age) red foxes were obtained from a control program involving the fox population in the Netherlands. All were negative for antibodies against influenza viruses according to a commercially available nucleoprotein-based ELISA test (European Veterinary Laboratory, Woerden, the Netherlands) and for antibodies against canine distemper virus according to a virus neutralization assay. The foxes had been treated against helminthic infections with 50 mg of fenbendazole when they were 2.5 months old and with 22.7 mg of praziquantel, 22.7 mg of pyrantel base as pyrantel pamoate, and 113.4 mg of febantel 2 months later. One month before the start of the experiment, transponders (Star-Oddi, Reykjavik, Iceland) that record body temperature every 15 minutes were placed in the peritoneal cavity of each fox, after the animal had been anesthetized with intramuscular injections of ketamine (5 mg/kg) and medetomidine (0.05 mg/kg). During the experiment, 6 foxes (nos. 1–3 and 5–7) were singly housed in negatively pressurized isolation units. Two negative-control foxes (nos. 4 and 8) were housed separately in an indoor enclosure.

To determine susceptibility to infection, we infected 3 foxes (nos. 1–3) intratracheally with a clade 2.2 HPAI virus (H5N1). Each anesthetized fox received 2.5×10^4 $TCID_{50}$ of virus in a volume of 2.5 mL through a catheter. One anesthetized fox (no. 4) was sham-infected intratracheally with 2.5 mL of PBS and served as a negative control. To determine whether red foxes can become infected by the presumed natural route of infection, we fed infected birds to 3 foxes (nos. 5–7). The infected birds were 1-week-old chicks that had been infected intratracheally with 2.5×10^4 $TCID_{50}$ of the HPAI virus (H5N1) in a volume of 0.5 mL. At 24 hours postinoculation, the chicks were euthanized by cervical dislocation and fed to foxes nos. 5–7 (2 whole chicks/fox). Homogenates of liver, lung, kidney, and brain from infected chicks contained $10^{6.3}$ to $>10^{9.3}$ $TCID_{50}$/g tissue; pharyngeal and cloacal swabs reached titers of $10^{4.5}$ to $10^{7.2}$ $TCID_{50}$/mL. On the basis of the relative weight of the lungs, liver, kidneys, and brain of 1-week-old chicks weighing 50 to 55 g (*27*,*28*), foxes fed 2 chick carcasses received a minimal titer of 10^{10} $TCID_{50}$. Virus titers in internal organs of dead wild or domestic birds naturally infected with HPAI virus (H5N1) have been reported sparingly; however, an article from Japan reported high virus titers, e.g., as high as $10^{7.5}$ $TCID_{50}$/mL, in the lung of a naturally infected large-billed crow (*Corvus macrorhynchos*) (*29*). High titers were also detected in internal organs of 10-week-old chickens and in highly susceptible species of wild swans, geese, and ducks that were experimentally infected with a clade 2.2 HPAI virus (H5N1), e.g., whooper swans, mute swans (*Cygnus olor*), bar-headed geese (*Anser indicus*), common pochards (*Aythya ferina*), and tufted ducks (*Aythya fuligula*) (*30*–*32*). For example, virus titers in internal organs of common pochards and tufted ducks infected with a low dose of clade 2.2 HPAI virus (H5N1) reached $>10^6$ $TCID_{50}$/mL (*32*). Our experimental design thus likely reproduces natural exposure after ingestion of dead or moribund birds infected with the virus. Our negative control was 1 fox (no. 8) that was fed 2 whole chicks that had been sham infected with PBS.

Before inoculation and at 1, 2, 3, 5, and 7 days postinoculation (dpi), all foxes were anesthetized with ketamine-medetomidine, after which they were weighed, and nasal, pharyngeal, and rectal swabs were collected and placed in 3 mL of virus transport medium (Hank's balanced salt solution containing 10% glycerol, 200 U/mL penicillin, 200 μg/mL streptomycin, 100 U/mL polymyxin B sulfate, and 250 μg/mL gentamicin). Each day, foxes were observed for clinical signs; observers were ≈2 m from the isolation units. At 7 dpi, all foxes were anesthetized with ketamine-medetomidine and euthanized by exsanguination. Experi-

mental procedures were approved by an independent animal care and use committee.

Postmortem and Immunohistochemical Examinations

Necropsy examinations and tissue sample collection were performed according to a standard protocol. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin for histologic evaluation, or they were processed according to an immunohistologic method that used a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody for detection of influenza viral antigen (*33*). Lung tissue of an experimentally infected cynomolgus macaque (*Macaca fascicularis*) experimentally infected with influenza virus A/Hong Kong/156/97 (H5N1) served as a positive control. Negative controls were created by omitting the primary antibody or replacing the primary antibody with an irrelevant antibody, immunoglobulin G2 (clone 20102; R&D, Abingdon, UK). The following tissues were examined by these 2 methods: conjunctiva, nasal concha, nasal septum, trachea, lung (6 specimens/fox), tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, tonsil, tracheobronchial lymph node, mesenteric lymph node, spleen, thymus, heart, liver, pancreas, kidney, adrenal gland, urinary bladder, olfactory bulb, cerebrum (at level of hippocampus), cerebellum, and brain stem.

Virus Titrations

The same tissues examined for histopathologic changes were also sampled for viral titration. Tissue samples were weighed and homogenized in 3 mL of transport medium with a homogenizer (Kinematica Polytron, Lucerne, Switzerland). Serial dilutions (10-fold) of the tissue homogenates and swabs were inoculated into MDCK cells in triplicate as described previously (*26*). The minimal detectable titer was $10^{0.8}$ $TCID_{50}$/mL. All experiments were performed under BioSafety Level 3 conditions.

Results

Clinical Signs

Clinical signs were not observed in foxes infected intratracheally or in foxes fed infected bird carcasses. However, body temperature of 2 of the 3 foxes infected intratracheally (nos. 1 and 2) and of 1 of the 3 foxes fed infected bird carcasses (no. 5) rose from 38.5°C–39°C (reference range) to 40°C–40.5°C at 2 to 4 dpi. No clinical signs and no rise in body temperature were observed for the negative-control foxes (nos. 4 and 8).

Virology

The virus was isolated from pharyngeal swabs from all infected foxes and from nasal and rectal swabs from 1 fox and rectal swabs from another (Figure 1). Foxes infected intratracheally excreted the virus through the pharynx from 1 dpi on, up to 3–7 dpi; peak titers of pharyngeal excretion were $10^{3.5}$–$10^{5.2}$ $TCID_{50}$/mL at 1–3 dpi. Foxes that had been fed infected bird carcasses excreted the virus through the pharynx from 1 dpi on, up to 3–5 dpi; peak titers of pharyngeal excretion were $10^{4.2}$–$10^{4.5}$ $TCID_{50}$/mL at 1 dpi. Student *t* test showed no significant difference in the patterns of pharyngeal excretion between the 2 groups of foxes according to areas under the curve ($t = -0.667$, df = 4, $p = 0.54$). No virus was isolated from any swabs from any negative-control foxes.

The virus was isolated from the trachea ($10^{2.6}$ $TCID_{50}$/g tissue) and lung ($10^{3.3}$ $TCID_{50}$/g) of 1 of 3 foxes infected intratracheally (no. 2), and from the tonsil (10^{2} $TCID_{50}$/g) of another fox infected intratracheally (no. 3). No virus was isolated from any of the organs of the foxes fed infected bird carcasses or of the negative-control foxes.

Gross Examination

Of the 3 foxes infected intratracheally, 2 (nos. 1 and 2) had extensive multifocal or coalescing pulmonary lesions, which were dark purple and slightly firm (Figure 2). The

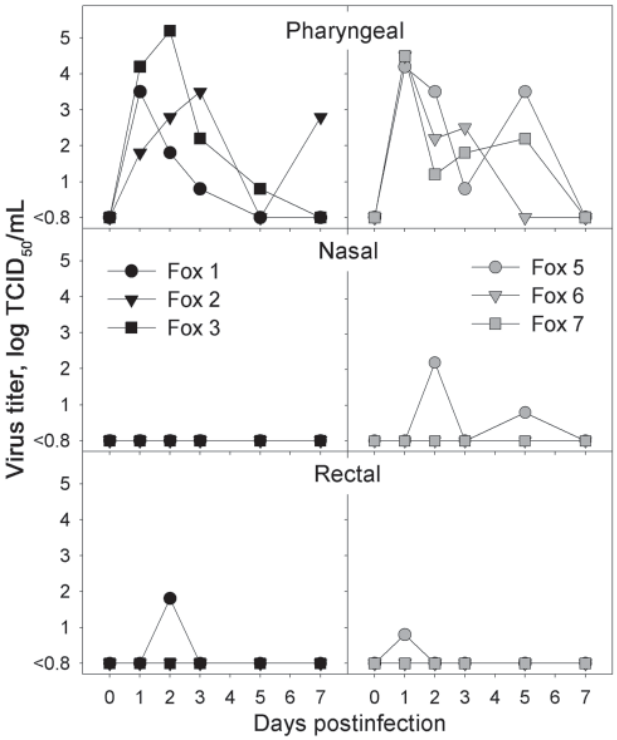

Figure 1. Infectious virus titers obtained from pharyngeal, nasal, and rectal swabs of foxes infected intratracheally with highly pathogenic avian influenza (HPAI) virus (H5N1) (left, black symbols) or fed chicks infected with HPAI virus (H5N1) (right, gray symbols) at various time points after infection. No virus was isolated from any swabs of the negative-control foxes. $TCID_{50}$, median tissue culture infectious dose.

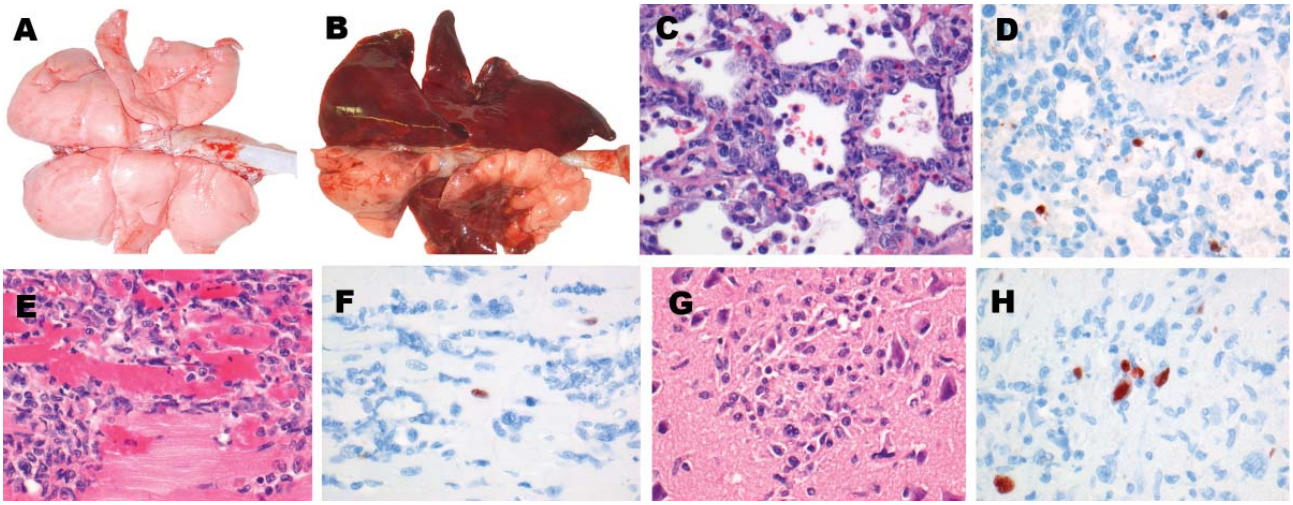

Figure 2. Lesions and associated expression of influenza virus antigen in respiratory and extrarespiratory organs of foxes infected intratracheally with HPAI virus (H5N1), at 7 days postinoculation. A) Lungs of control fox sham-inoculated with phosphate-buffered saline. B) Lungs of intratracheally inoculated fox presenting extensive consolidated lesions (darkened areas), characterized by C) diffuse alveolar damage and regeneration (type II pneumocyte hyperplasia) and D) expression of influenza virus antigen in the nucleus and, to a lesser extent, cytoplasm of mononuclear and epithelial cells. E) Focus of inflammation and cardiomyocytic necrosis in the heart, associated with F) expression of influenza virus antigen in the nucleus of cardiomyocytes. G) Focus of gliosis and neuronal necrosis in the cerebrum, associated with H) expression of influenza virus antigen in the nucleus and, to a lesser extent, cytoplasm of glial cells and neurons. Panels C–H, original magnification ×40.

estimated percentage of affected lung tissue was 20% (no. 1) and 80% (no. 2). In contrast, 1 of the 3 foxes infected intratracheally (no. 3) and all foxes fed infected bird carcasses had ≥2 small multifocal lesions (1–5 mm), which affected <5% of the lungs. In addition, 2 of the 3 foxes fed infected bird carcasses (nos. 5 and 6) had randomly distributed petechial hemorrhages throughout the lungs. Moderate enlargement of the spleen, tonsils, and/or tracheobronchial lymph nodes was observed in all foxes, whether infected intratracheally or fed infected bird carcasses. Negative-control foxes had no respiratory or extrarespiratory lesions.

Histopathologic Findings

Histologic lesions were found in foxes infected intratracheally and in foxes fed infected bird carcasses. However, the lesions were more severe in foxes infected intratracheally (Table). The 2 most severely affected foxes (nos. 1 and 2, infected intratracheally) had severe hemorrhagic bronchointerstitial pneumonia with extensive coalescing lesions of inflammation and necrosis, characterized by macrophage and neutrophil infiltration of the alveolar walls and loss of histologic architecture. The alveolar and bronchiolar lumina were filled with alveolar macrophages, neutrophils, and erythrocytes, mixed with fibrin and cellular debris. In both foxes, sloughing of the alveolar and bronchiolar epithelia indicated necrosis, and type II pneumocyte and bronchiolar epithelial hyperplasia indicated regeneration (Figure 2). The other foxes (no. 3, infected intratracheally, and all foxes fed infected bird carcasses) had minimal to mild bronchointerstitial or interstitial pneumonia. They had small- to medium-sized foci of inflammation in the lungs, located mostly around the bronchioles and characterized by thickened alveolar walls that were infiltrated with macrophages and neutrophils. Type II pneumocyte and bronchiolar epithelial hyperplasia was observed in the lungs of fox no. 7. Respiratory organs of negative-control foxes had no lesions.

Extrarespiratory histologic lesions were seen only in foxes infected intratracheally, namely, in the heart of fox no. 2 and in the cerebrum of foxes nos. 1 and 2. Fox no. 2 had multiple inflammatory and necrotic lesions in the myocardium, characterized by infiltration of macrophages and neutrophils and necrotic cardiomyocytes (Figure 2). The cerebrum of foxes nos. 1 and 2, infected intratracheally, had multiple lesions of acute to subacute encephalitis, from mild to severe, characterized by perivascular cuffing, foci of gliosis or neuronal necrosis, or a combination of these lesions; their cerebellum and brain stem did not show any lesions (Figure 2). No relevant lesions were seen in other organs, including organs of the digestive tract, of any other foxes.

Immunohistochemical Findings

Cells expressing the influenza virus antigen were present in the lungs, heart, and brain of 1 of 3 foxes infected intratracheally (no. 2) but in none of the foxes fed infected bird carcasses (Table). Mononuclear cells and alveolar epithelial cells in damaged parts of the lungs expressed the influenza virus antigen as diffuse red staining in their nucleus and, to a lesser extent, in their cytoplasm. Occa-

Table. Distribution of lesions and influenza virus antigen expression in experimentally infected red foxes*

	Lesions			Influenza virus antigen		
Route of inoculation, fox no.	Lungs	Heart	Brain	Lungs	Heart	Brain
Intratracheal inoculation						
1	+++	–	+	–	–	–
2	+++	+	++	++	+	+++
3	++	–	–	–	–	–
Fed infected bird carcasses						
5	+	–	–	–	–	–
6	–	–	–	–	–	–
7	+	–	–	–	–	–

*Foxes were infected either intratracheally with highly pathogenic avian influenza (HPAI) virus (H5N1) or by being fed chicks infected with HPAI virus (H5N1); they were examined at 7 days postinoculation. Respiratory lesions, extrarespiratory lesions, or influenza virus antigen expression were not observed in negative-control foxes. –, absence of lesions (no cells expressing the influenza virus antigen); +, mild and focal or multifocal lesions (few cells expressing the influenza virus antigen); ++, moderate and multifocal lesions (moderate number of cells expressing the influenza virus antigen); +++, severe and extensive lesions (numerous cells expressing the influenza virus antigen).

sional cardiomyocytes in the periphery of a lesion in the heart expressed the influenza virus antigen as granular red staining in their nucleus. Lastly, neuronal and glial cells in the periphery of lesions in the cerebrum expressed the influenza virus antigen as granular to diffuse red staining in their nucleus and, to a lesser extent, their cytoplasm (Figure 2). No influenza virus antigen was detected in any cells of other organs, including the intestinal tract, of any other foxes.

Discussion

This study demonstrates that red foxes are susceptible to infection with a wild bird isolate of HPAI virus (H5N1) from clade 2.2. Red foxes can become infected after eating infected bird carcasses, and they can excrete the virus for as many as 5 days in the absence of severe disease. Therefore, naturally infected red foxes may potentially survive infection in the wild and excrete and disperse HPAI viruses (H5N1) within their home ranges. The size of foxes' home ranges depends on the environmental conditions and availability of food resources, but typically it varies between 1 km² and 10 km² (*34*). Red foxes are highly mobile and may travel 5–20 km within their home range during a night (*35*). A juvenile fox traveled 90 km in 1 direction within 5 days during fall dispersal from its place of birth (*35*). Furthermore, red foxes have colonized most urbanized areas in Europe, resulting in increased contact with domestic and peridomestic animals (*23*). They may transmit the virus to domestic species, such as poultry. Therefore, we propose that this abundant and widespread carnivorous species be surveyed for exposure to or infection with HPAI viruses (H5N1) in influenza-endemic areas or in areas experiencing outbreaks of HPAI virus (H5N1) infections in wild bird populations. Where foxes are hunted, carcasses may be routinely sampled and tested. Where foxes are protected or not hunted, live trapping, bleeding, and pharyngeal swabbing of anesthetized foxes may be implemented.

Although red foxes fed infected bird carcasses may survive infection, severe pneumonia, myocarditis, and encephalitis may develop in those inoculated intratracheally. Frequent findings of HPAI virus (H5N1) infections in naturally infected carnivores were pneumonia associated with respiratory distress and encephalitis (in some cases associated with neurologic signs) (*12–14*,*16*,*18–21*). In most instances, the animals were either euthanized because of the severity of the disease or were found dead. Surprisingly, foxes with severe respiratory and cerebral lesions did not show any visible clinical signs. Foxes, being wild animals, were wary in the presence of humans and changed their behavior even when observed from a distance. This behavior may have prevented us from observing subtle clinical signs, notably abnormal breathing. A cat that died of HPAI virus (H5N1) infection in Germany did not show visible clinical signs 24 hours before death, despite marked respiratory lesions (*16*), which suggests that even severe respiratory lesions may not be noticed clinically. Clinical manifestations of neurologic lesions in infected foxes may have gone unnoticed because the lesions were in the cerebrum rather than in the cerebellum. Although cerebellar lesions may cause conspicuous neurologic signs, e.g., ataxia and loss of balance, cerebral lesions may cause more subtle clinical signs, e.g., altered mental attitude, which were not noticed under these experimental conditions (*36*).

Foxes may exhibit more severe disease after eating infected birds under natural conditions than under the controlled conditions of our feeding experiments, because of poorer health, possible co-infections, and poorer nutritional status of wild animals. For instance, the cats that died of HPAI virus (H5N1) infection in Germany were all infected with *Aelurostrongylus* spp., and pulmonary aelurostrongylosis was considered to have contributed to the severity of the disease in these animals (*16*). Fatal cases of HPAI virus (H5N1) infection in red foxes may have been missed after the spread of HPAI viruses (H5N1) in poultry and wild bird populations outside Asia because fox carcasses are difficult to locate and because those found may likely be routinely tested for rabies and canine distemper rather than for influenza virus infection. Therefore, we suggest that red foxes

with neurologic signs or red foxes found moribund or dead in disease-endemic areas or in areas experiencing outbreaks of HPAI virus (H5N1) infections in wild bird populations be tested for HPAI virus (H5N1) infection.

Foxes infected intratracheally and those fed infected bird carcasses exhibited similar virus-shedding patterns despite the different routes of exposure and despite marked differences in the severity and extent of associated lesions. Foxes fed infected bird carcasses likely inhaled virus particles during mastication. The differences in the severity and extent of associated lesions may have thus resulted from a difference in the respiratory inoculum received by foxes infected intratracheally and those fed infected carcasses. We did not observe influenza antigen–positive neuronal cells in the submucosal or myenteric plexi of the small intestine of foxes fed infected bird carcasses. This contrasts with findings in cats infected in the same way; in the cats, these cells were positive, which suggests the intestine as a route of virus entry (*37*). The fact that the viral shedding patterns were similar despite the marked differences in severity and extent of respiratory lesions is surprising and difficult to explain. On the basis of the absence of influenza antigen–positive cells in the respiratory tract, all but 1 fox appeared to have cleared the virus from this site by 7 dpi. Signs of regeneration in bronchiolar and alveolar epithelia were observed both in foxes infected intratracheally and in those fed infected bird carcasses.

In summary, we have shown that red foxes are susceptible to infection with a wild bird isolate of HPAI virus (H5N1) from clade 2.2, can become infected after feeding on infected bird carcasses, and can excrete the virus for as many as 5 days without severe disease developing. Surveillance and monitoring of HPAI virus (H5N1) infections may therefore be beneficially expanded to red foxes, and potentially to other free-living wild carnivores, in influenza-endemic areas and in areas experiencing outbreaks of HPAI virus (H5N1) infections in wild bird populations.

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Influenza Infection in Wild Raccoons

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Raccoons (*Procyon lotor*) are common, widely distributed animals that frequently come into contact with wild waterfowl, agricultural operations, and humans. Serosurveys showed that raccoons are exposed to avian influenza virus. We found antibodies to a variety of influenza virus subtypes (H10N7, H4N6, H4N2, H3, and H1) with wide geographic variation in seroprevalence. Experimental infection studies showed that raccoons become infected with avian and human influenza A viruses, shed and transmit virus to virus-free animals, and seroconvert. Analyses of cellular receptors showed that raccoons have avian and human type receptors with a similar distribution as found in human respiratory tracts. The potential exists for co-infection of multiple subtypes of influenza virus with genetic reassortment and creation of novel strains of influenza virus. Experimental and field data indicate that raccoons may play an important role in influenza disease ecology and pose risks to agriculture and human health.

The primary reservoirs of avian influenza (AI) are wild birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds). In these hosts, low-pathogenic forms of the virus typically cause little or no apparent disease, however, large quantities of virus are shed in fecal matter. AI virus is relatively stable in water and can remain viable for up to 200 days, depending on temperature and other environmental factors (*1*). Thus, bodies of water and adjacent shorelines that wild birds use can become potentially contaminated, increasing the likelihood of subsequent exposure of avian and nonavian species to AI virus.

The preference of influenza viruses for different cellular receptors and the presence and distribution of those receptors in the host are important factors involved in determining host range and tissue tropism (*2*). Humans are not typically infected by AI virus because receptors for this virus are distributed in tissues that are located predominantly in the lower respiratory tract. As such, these receptors are not as accessible as human type receptors found in the upper respiratory tissues and require more intimate contact for transmission. Swine are considered important intermediate hosts between birds and humans because they are frequently infected by avian and human influenza viruses (*3*). This finding underscores the potential for genetic reassortment that can create new, possibly more virulent subtypes.

Other non-avian hosts of AI virus include mink, harbor seals, pilot whales, dogs, cats, and horses (*4*). These species were found to be competent hosts only after attracting attention because of severe death or illness (*4*). Wild mammals often reside in the same habitats as waterfowl, feed in the same agricultural areas, wallow and swim in the same bodies of water, and prey on and scavenge dead birds for food. Therefore, ample opportunities exist for free-ranging wild mammals to be exposed to AI by contact with waterfowl and their environment. Many of these species are highly mobile and have large home ranges that can include agricultural operations, wetlands, and human residences. Humans are frequently unaware of their presence, and wild

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mammals have the potential to contract AI from waterfowl or their environment and to then transmit AI to domestic animals or humans. To date, no studies have systematically examined wild mammalian species, particularly peridomestic mammals, for exposure to AI, their ability to become infected, and their reassortment potential. This knowledge is critical for accurate risk assessments of low pathogenic and highly pathogenic AI to agriculture and human health.

Raccoons (*Procyon lotor*) are widespread and common in riparian, wooded, and suburban settings over much of North America (*5*). Previously, antibodies against AI virus (H4N6) were found in 1 raccoon in Pennsylvania (J. Hall, unpub. data). This finding led us to conduct the present study in which we examined wild populations of raccoons from various regions of the United States for antibodies to influenza virus. Experimental infections of raccoons with avian and human influenza viruses were performed to determine viral shedding, transmission, and immune response. The abundance and distribution of avian or human influenza virus cellular receptors in respiratory tissues was analyzed to determine potential for co-infection and possible reassortment of influenza virus strains in this host. These data provided insight into the complexity of influenza disease ecology and the overlooked, potentially important roles of peridomestic wildlife in transmission cycles.

Materials and Methods

Field Sample Collection

Blood was opportunistically collected from wild raccoons taken during population control operations in various counties/parishes in Texas, Wyoming, Louisiana, California, and Maryland. Raccoons from northwestern Georgia were sampled as part of the US Department of Agriculture Cooperative National Oral Rabies Vaccination program. Raccoons in Colorado were captured for this, and other studies, in and around Fort Collins, Colorado. Blood samples were obtained by cardiac or jugular puncture, allowed to clot, and centrifuged to separate serum from cellular blood components. Serum was transferred to fresh cryovials and stored frozen (–20°C) until transport to the National Wildlife Research Center in Fort Collins, where they were stored at –80°C until analysis.

Screening for Antibodies to Influenza Virus

Agar gel immunodiffusion is a serologic assay used to detect antibodies to influenza viruses. The antigen used in the assay was derived from the matrix and nucleoproteins of AI and is used to detect antibodies to all subtypes of AI. The procedure has been described by Beard (*6*) and was performed by using reagents and the protocol provided by the Center for Veterinary Biologics and National Veterinary Services Laboratories (Ames, IA, USA).

Determination of Antibody Subtypes for Influenza Virus

Hemagglutination inhibition and neuraminidase inhibition are used to determine subtype identity of influenza antibodies in sera. These procedures are described by Beard (*6*) and were performed at the National Veterinary Services Laboratories.

Experimental Infection of Raccoons

Ten wild raccoons were live-trapped in and around Fort Collins, Colorado. These animals were transported to the National Wildlife Research Center and held for a 2-week quarantine period, where they were observed daily and judged to be in good overall health on the basis of food intake, behavior, and absence of clinical signs of disease. After 2 weeks, the animals were anesthetized and moved into a biocontainment level 2 facility. Blood samples and nasal and rectal swabs were collected, and animals were placed in individual cages (5 per room). Four animals in each room were inoculated intranasally with 10^5 50% egg infectious dose (EID_{50}) of AI virus A/CK/AL/75 (H4N8) diluted in 100 μL of viral transport medium. The fifth animal in each room was uninoculated and monitored to determine transmission between animals. To prevent potential fomite transmission, these controls were always handled and sampled first when technicians entered the rooms, and all food and water bowls were cleaned and sanitized in hypochlorite solution each day. Each animal was provided food and water ad libitum and observed daily for illness, behavior, and general welfare. To avoid excessive handling and anesthesia, serum samples, nasal and rectal swabs, and rectal temperatures were collected from each group of 5 raccoons on alternating days for 14 days. Blood samples were obtained by jugular puncture. Nasal and rectal swabs were collected by using dacron-tipped applicators placed into viral transport media after swabbing. All samples were stored at –80°C until analyzed.

Subsequently, a second cohort of 6 raccoons was captured in Fort Collins, quarantined, and placed into biocontainment. Four raccoons were intranasally inoculated with 10^5 EID_{50} of human influenza virus (A/Aichi/2/68 [H3N2]) and were sampled and monitored as described above. Two uninoculated raccoons were housed in cages adjacent to inoculated animals to assess transmission. All animal handling, trapping, and experimental infections were performed following Institutional Animal Care and Use Committee and institutional biosafety protocols, guidelines, and approval.

Reverse Transcription–PCR

Viral RNA was extracted from nasal swabs by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), following manufacturer's instructions. Viral

RNA from rectal swabs was extracted by using the same procedure with addition of half of an Inhibitex tablet (Stool Extraction Kit; QIAGEN) to remove PCR inhibitors. Real-time reverse transcription–PCR (RT-PCR) was performed following the procedure of Spackman et al. (*7*). Samples were compared with standard curves generated from known concentrations of AI, extracted, and amplified by using the same procedures. Results are expressed as EID_{50} equivalents. These procedures were used to analyze environmental samples (feces and water) as part of the national surveillance for highly pathogenic AI (*8*).

Detection of Influenza Virus Cellular Receptors in Raccoon Respiratory Tissues

Airway tissue sections were collected from 5 adult raccoons (humanely killed in a different study) from 7 standardized locations (nasal mucosa; larynx; upper, middle, and lower trachea; bronchus; lung), fixed in formalin, and embedded in paraffin. Airway tissues were cut into 5 μm–thick sections, mounted on 3-aminopropyltrethoxy-silane–coated slides, deparaffinized in xylene, and rehydrated in alcohol. For detection of sialic acids (SAs), sections were stained with SAα2,3Gal- and SAα2,6Gal-specific lectins. Briefly, sections were incubated overnight with 250 μL of Western blocking solution (Roche Biochemicals, Indianapolis, IN, USA), washed 3× in Tris-buffered saline, pH 7.6, and incubated with 250 μL of fluorescein isothiocyanate–labeled *Sambucus nigra* lectin (Vector Laboratories, Burlingame, CA, USA) and biotinylated *Maackia amurensis* lectin (Vector Laboratories) overnight at 4°C. After 3 washes in Tris-buffered saline, sections were incubated with Alexa Fluor 594–conjugated streptavidin (Molecular Probes, Inc., Eugene, OR, USA) for 2 hours at room temperature. The sections were washed, counterstained with 4′,6-diamino-2-phenylindole dihydrochloride (Molecular Probes), washed again, and mounted on cover glass. Sections were examined with a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany). Because previous research has demonstrated that equine tracheal epithelial cells predominantly express SAα2,3Gal residues and pig tracheal cells express SAα2,3Gal and SAα2,6Gal residues (*2*), sections of equine and porcine trachea were included as positive controls for each staining procedure.

Results

Serologic Survey of Wild Raccoons for Exposure to AI Virus

We screened 730 wild raccoons from California, Texas, Louisiana, Maryland, Wyoming, and Colorado. Of these, 17 (2.4%) had antibodies to AI virus. Table 1 summarizes the raccoon serosurvey and subtyping results from these states. Four (2.4%) of 168 Maryland raccoons in 2004 had antibodies to AI virus with 3 hemagglutinin subtypes represented. Two of these raccoons had antibodies to 2 subtypes, which indicated multiple exposures to AI virus. Colorado and Wyoming also had seropositive raccoons with prevalences of 12.8% and 25%, respectively. Multiple subtypes were present in both populations, and multiple exposures in individual raccoons were observed. However, none of the raccoons from Georgia, Texas, or California showed serologic evidence of exposure to AI virus. These results indicated that wild raccoons are exposed to a variety of AI virus subtypes and seroconvert on the basis of these exposures.

Table 1. Exposure to avian influenza virus in wild raccoons in 7 states, United States*

State	Year	No. positive/no. tested (%)	Influenza antibody subtypes (no.)
MD	2004	4/168 (2.4)	H4 + H10† (1), H1 + H10† (1), H4† (2)
	2005	0/13	–
GA	2004	0/366	–
CA	2006	0/46	–
TX	2004	0/40	–
	2006	0/16	–
LA	2004	0/10	–
WY	2004	8/32 (25)	H4N6 (7), H4N2 (1)
CO	2006	5/39 (12.8)	H4N2 + N6 (3), H3† (1), H10N7 (1)

*H, hemagglutinin; N, neuraminidase.
†N subtype was not determined because of insufficient sample volume.

Experimental Infection of Raccoons with AI Virus

To determine whether raccoons are competent hosts for AI virus infection and are capable of shedding and transmitting virus, raccoons were infected with a specific subtype of AI virus (H4N8) and monitored for symptoms of infection and disease. Two of 10 wild-caught raccoons had antibodies to AI virus (Table 1). These animals were included in the infection study because the AI virus inoculum used was a different subtype, but with potential cross-neutralization as a caveat.

Eight raccoons were inoculated intranasally with $10^{5.0}$ EID_{50} of AI virus (H4N8) and monitored for 14 days postinoculation (dpi). Four (50%) of these animals became infected, as shown by nasal shedding of viral RNA detected by RT-PCR. Two of these animals (256 and 275) shed detectable amounts of virus at only 1 time point (1 dpi). Another raccoon (259) shed virus at least up to 6 dpi, and the other infected raccoon (263) shed for the entire 14 days of the study (Table 2). RT-PCR analyses of rectal swabs showed no detectable viral RNA shed by digestive tracts of infected raccoons (data not shown), which is consistent with influenza being primarily a respiratory disease in mammals (*2*).

One of the 2 uninoculated raccoons housed in cages adjacent (within ≈0.5 m) to inoculated raccoons devel-

Table 2. Nasal shedding of avian influenza virus by experimentally inoculated raccoons*

Raccoon ID	Day postinoculation														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
264	–	–		–		–		–		–		–		–	
275	–	0.2		–		–		–		–		–		–	
256	–	1.1		–		–		–		–		–		–	
253	–	–		–		–		––		–		–		–	
260†	–	–		–		–		–		–		–		–	
262†	–		0.2		0.8		0.9		0.4		–		–		–
258	–		–		–		–		––		–		–		–
257	–		–		–		–		–		–		–		–
259	–		1.1		0.1		0.4		‡						
263	–		–		0.6		0.2		0.9		0.02		0.4		0.1

*Shedding was determined by real-time reverse transcription–PCR of nasal swabs compared with standard curves generated from avian influenza virus stocks of known concentrations and expressed as $\log_{10}$ 50% egg infectious dose equivalents. –, no viral RNA detected. Raccoons 262 and 263 were seropositive for avian influenza virus before inoculation.
†Uninoculated raccoons housed in cages adjacent to infected raccoons.
‡Raccoon humanely killed 8 days postinoculation.

oped nasal shedding of virus. Every precaution was taken to prevent inadvertent transmission by handling; thus, this animal (262) probably contracted the virus by aerosol from ≥1 of its infected cohorts. This result indicated that raccoons are capable of transmitting influenza virus from one to another. Given the small amounts of AI virus shed by these raccoons and the timing of infection of this animal, we cannot rule out possible aerosolization of inoculum by adjacent raccoons and transmission by that route.

Three of the 5 raccoons that shed virus developed antibodies to the AI virus (H4N8) isolate, including raccoon 262, which was not inoculated but contracted the virus from adjacent, infected raccoons (Table 3). Raccoon 259 was humanely killed on 8 dpi because of an unrelated physical condition (tooth abscess), presumably before detectable antibodies were produced. Raccoon 256 shed virus only on 1 dpi yet developed detectable antibodies to AI virus (H4N8) by 9 dpi. However, the other raccoon that shed virus on 1 dpi (275) did not develop a detectable immune response, which indicated that virus detected in the swab was probably residual inoculum. Raccoons 263 and 262 had preexisting antibodies to a different subtype of AI virus that did not prevent infection and seroconversion to the other AI virus (H4N8) inoculum.

We observed no overt clinical signs of disease in these animals. Rectal temperatures showed no obvious trends and were probably confounded by stresses of anesthesia and handling. Most of the animals appeared lethargic, possibly because of confinement and manipulations occurring during daytime (raccoons are nocturnal). All other animals ate and drank well and most gained weight over the course of the experiment (data not shown).

Influenza Virus Receptors in Raccoons

The predominant receptor for AI virus is SA linked α2,3 to galactose. In waterfowl, these receptors are located primarily in intestinal epithelium, which is why AI is primarily a disease of the digestive tract in avian species. In contrast, humans have SA linked α2,6 to galactose that is located predominantly in the respiratory system (*2*). Tissues from raccoon respiratory tracts were examined for avian and human influenza virus receptors

Table 3. Antibody production in raccoons experimentally infected with avian influenza virus (H4N8)*

Raccoon ID	Day postinoculation															Final subtype†
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
264	N	N		N		N		N		N		N		N		
275	N	N		N		N		N		N		N		N		
256	N	N		N		N		N		WP		SP		SP		H4N8
253	N	N		N		N		N		N		N		N		
260‡	N	NS		N		N		N		N		N		N		
262‡	P		P		P		P		P		P		WP		WP	H4N8§
258	N		N		N		N		N		N		N		N	
257	N		N		N		N		N		N		N		N	
259	N		N		N		N		N		¶					–
263	SP		SP		SP		SP		SP		SP		SP		SP	H4N8§

*Antibodies were detected by agar gel immunodiffusion, and final subtyping was determined at the National Veterinary Services Laboratories (Ames, IA, USA). N, negative; WP, weakly positive; SP, strongly positive; NS, not sampled; P, positive.
†H, hemagglutinin; N, neuraminidase.
‡Uninoculated raccoons housed in adjacent cages to infected raccoons.
§These raccoons had preexisting antibodies (262:H10N7, 1:10; 263:H4N6 1:40).
¶Raccoon humanely killed 8 days postinoculation.

by staining with lectins specific for each type of receptor (Figure). Raccoons have both receptor types in their respiratory systems, similar to swine but with uneven distribution among tissues. In the upper trachea epithelium, the overwhelmingly predominant receptor is the human type SAα2,6 (Figure, panel A). As one examines tissues from deeper in the respiratory tract, increasing amounts of the avian SA α2,3 receptor are found until the 2 types of receptors are in roughly equal amounts in the lungs (Figure, panels B, C, and D).

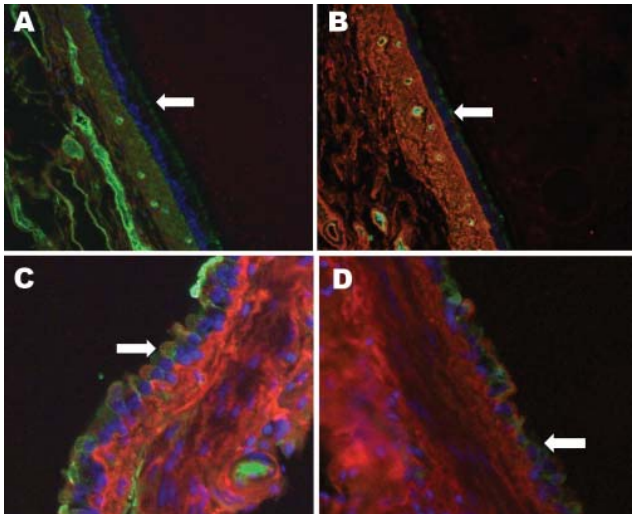

Figure. Raccoon respiratory tissues stained with lectins specific for sialic acids (SAs) with α2,6- and α2,3-linkages. A) Upper trachea; B) lower trachea; C) bronchus; D) bronchiole. Arrows indicate endothelial lining of the tissues indicated. Green staining shows a reaction with fluorescein isothiocyanate–labeled *Sambucus nigra* lectin, which indicates SAs linked to galactose by an α2,6-linkage (SAα2,6Gal). Red staining shows a reaction with biotinylated *Maackia amurensis* lectin (detected with Alexa Fluor 594–conjugated streptavidin), which indicates an SAα2,3Gal linkage. Tissues were counterstained with 4,6,-diamidino-2-phenylindole dihydrochloride. Original magnification ×40 in panels A, B, and D and ×100 in panel C.

Experimental Infection of Raccoons with Human Influenza Virus

The presence and distribution of human type receptors in raccoons led us to infect a new cohort of raccoons with human influenza virus (H3N2). Daily monitoring showed that inoculated animals shed virus nasally for up to 8 dpi (Table 4, Figure). The amounts of virus shed were larger than in the AI experimental infection study but no transmission to either co-housed, virus-free raccoon was detected. All 4 inoculated animals subsequently developed antibodies against this virus by 14 dpi (data not shown). One raccoon (272) shed small amounts of virus rectally (0.25 EID_{50} equivalents) on 5 dpi, but no other rectal shedding of virus was detected. As with AI virus infection, no obvious clinical signs of disease were observed in these animals. Infected raccoons were also capable of shedding moderate amounts of human influenza virus, although no transmission to virus-free animals was observed.

Discussion

The ecology of AI is complicated. Knowledge of the roles of wild birds and mammals in the epidemiology of the disease and how viral reassortants and variants arise are critical for the planning and preparation of future pandemics, vaccine development, and meaningful human health and agricultural risk assessments (*9*,*10*). However, other than a survey of small rodents in Pennsylvania, New Jersey, Maryland, and Virginia after an outbreak of influenza caused by virus subtype H5N2 in 1983–84 (*11*), no systematic investigation of wild mammals in influenza disease ecology has been performed.

Raccoons can carry a variety of etiologic agents. In Florida, raccoons are known to harbor 132 parasites, disease agents, and environmental contaminants, more than any other species of wild mammal (*12*). Viral diseases include rabies, canine distemper, pseudorabies, and poxvirus disease. To this list we can add West Nile virus (*13*,*14*) and now, from this study, avian and human influenza viruses.

The serologic survey of raccoons for AI virus exposure showed geographic variation in prevalence. AI in wild birds is relatively common; as much as 30% of the local waterfowl population can be infected (*15*). Raccoons often reside in these areas and can contact AI virus from their food and environment. However, the premise that areas of high waterfowl concentrations promote high antibody

Table 4. Nasal shedding of human influenza virus by experimentally inoculated raccoons*

	Day postinoculation														
Raccoon ID	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
265	–		0.2		–		–		–		–		–		–
267	–		3.2		2.4		0.3		0.3		–		–		–
269†	–		–		–		–		–		–		–		–
268†	–	–		–		–		–		–		–		–	–
271	–	–		2.0		0.5		0.2		–		–		–	–
272	–	0.2		0.2		2.0		1.2		–		–		–	–

*Shedding was determined by real-time reverse transcription–PCR of nasal swabs compared with standard curves generated from avian influenza virus stocks of known concentrations and expressed as 50% log_{10} egg infectious dose equivalents. –, no viral RNA detected.
†Uninoculated raccoons housed in cages adjacent to infected raccoons.

prevalence in raccoon populations was not always supported by these data. Raccoons in Georgia were sampled from the northwestern corner of the state, where wild fowl populations are small, and the prevalence of antibodies was 0%. In Maryland, which has one of the highest populations of overwintering and migrating waterfowl on its east coast (*16*), the prevalence of antibodies was 2.4%. Thus, data from these 2 states were logical on the basis of the waterfowl population size. However, Texas and California, with large seasonal populations of waterfowl, showed no evidence of AI virus exposure in raccoons. Wyoming and Colorado, with relatively small waterfowl populations, had the highest exposure rates of any states examined (25% and 12.8%, respectively). The reasons for higher prevalences in Wyoming and Colorado are unclear but may be related to concentrations of raccoons and waterfowl in riparian corridors in these semi-arid areas.

Wild waterfowl are the primary natural reservoir of AI virus, and different subtypes to which these raccoons were exposed are relatively common in avian populations (*17–22*). Clearly, raccoons are exposed to AI virus in the wild, and experimental studies confirm they can become infected with this virus and shed virus capable of infecting healthy animals. Also, we showed that raccoons can become infected with human influenza virus and shed moderate amounts of virus. The higher amounts of human influenza virus shed by raccoons than AI virus may indicate that human influenza virus is better adapted to mammalian physiology. The fact that we detected measurable levels of viral shedding with avian and human influenza viruses in infected raccoons is important. If one considers that only 2 uninfected raccoons were available to detect transmission of human influenza virus in this study, the fact that we did not detect transmission does not rule out the possibility that human influenza virus is also capable of being transmitted by raccoons and warrants additional research.

The abundance and distribution of avian and human influenza receptors found in raccoon tissues are similar to those in human respiratory tracts (*23*,*24*). The presence of human and AI virus receptors in raccoon respiratory systems creates the possibility of co-infection with multiple types of influenza virus and, as in swine, genetic reassortment and creation of new, possibly highly virulent strains are distinct risks.

Risks associated with wild raccoons and influenza are compounded by several factors. Raccoons are highly mobile with relatively large home ranges that include a variety of ecologic landscapes (*5*). They routinely travel between wetlands, forests, agricultural operations, and urban and suburban settings. Consequently, a raccoon that acquired AI virus in a marsh from scavenging a diseased bird could easily transport and transmit the virus to poultry and swine operations and to residential areas.

Raccoons apparently are not adversely affected by low pathogenic AI or human influenza viruses and thus remain active and potentially able to transmit virus over large areas. Because of their nocturnal habits, raccoons can be largely invisible to humans but can achieve large population densities. In fact, in some areas more raccoons can inhabit suburban areas than rural areas, reaching >90 raccoons/km^2 (*25*,*26*).

In summary, the raccoon, a common, peridomestic, wild mammal is capable of becoming infected, transporting, and potentially transmitting avian and human influenza viruses. The risks associated with raccoons and influenza to agriculture and human health are unknown but clearly warrant further research. These results underscore the importance of investigating the roles of other peridomestic species in the disease ecology of influenza.

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Enzootic Rabies Elimination from Dogs and Reemergence in Wild Terrestrial Carnivores, United States

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To provide molecular and virologic evidence that domestic dog rabies is no longer enzootic to the United States and to identify putative relatives of dog-related rabies viruses (RVs) circulating in other carnivores, we studied RVs associated with recent and historic dog rabies enzootics worldwide. Molecular, phylogenetic, and epizootiologic evidence shows that domestic dog rabies is no longer enzootic to the United States. Nonetheless, our data suggest that independent rabies enzootics are now established in wild terrestrial carnivores (skunks in California and north-central United States, gray foxes in Texas and Arizona, and mongooses in Puerto Rico), as a consequence of different spillover events from long-term rabies enzootics associated with dogs. These preliminary results highlight the key role of dog RVs and human–dog demographics as operative factors for host shifts and disease reemergence into other important carnivore populations and highlight the need for the elimination of dog-related RVs worldwide.

Rabies virus (RV) is the prototype member of the genus *Lyssavirus*, which causes acute encephalomyelitis in mammals, including humans, throughout the world. The disease is independently maintained by several species of mammals within the orders Carnivora and Chiroptera (*1*). Intraspecies RV transmission maintains the disease within geographically discrete (*2*,*3*) areas by distinctive virus variants and lineages, which can be identified either by panels of monoclonal antibodies or by genetic analysis (*4*,*5*). Interspecies transmission of rabies often results in ecological bottlenecks and epidemiologic dead ends (*1*,*4*,*6*). However, during rabies epizootics or long-term enzootics, novel rabies reservoirs may emerge and produce outbreaks involving closely related RV variants (*7*,*8*).

Dog rabies has been recognized since the first human civilizations (*9*). However, the possible origins of disease in this species likely pre-date its direct associations with humans. Over time, RVs have had the opportunity to evolve within the context of the human–dog bond and to generate a suite of genetically distinguishable and geographically circumscribed variants and lineages (*10*–*12*). Dog RVs cause >55,000 human deaths annually, mostly in Asia and Africa (*13*). In the Americas, despite a 90% reduction of cases during past decade, the domestic dog still poses the greatest public health hazard with regard to rabies (*14*).

During the first half of the 20th century, canine rabies was enzootic throughout the United States (*15*). By the 1970s, the primary dog RV variant had been eliminated by extensive mass vaccination programs and effective control of stray animal populations (*1*,*15*). During the late 1980s, the domestic dog–coyote RV variant became enzootic in the coyote (*Canis latrans*) population along the United States–Mexico border (*16*). In 1994, this variant expanded by translocation into Alabama and Florida, causing a local outbreak in domestic dogs (*17*). In 1988, a second canine RV variant, the Texas gray fox variant, was found associated with enzootic rabies in gray foxes (*Urocyon cinereoargenteus*) in west-central Texas (*16*,*17*). Aggressive oral vaccination programs in the region eliminated the domestic

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dog–coyote RV variant by 2004, while circulation of the Texas gray fox variant was substantially constrained (*18*). Thus, the United States became the most recent country to eliminate enzootic dog rabies (*19*).

To provide molecular and virologic evidence that domestic dog rabies is no longer enzootic to the United States and to identify putative relatives of dog-related RVs circulating in other carnivores, we studied RVs associated with recent and historic dog rabies enzootics worldwide. We compared historical surrogates that circulated in the United States with RV isolates suspected of being dog related, collected over the past 15 years.

Materials and Methods

Viruses

A total of 228 samples were sequenced, generating both full and partial RV nucleoprotein gene data. The 152 samples from the United States were obtained from 136 dogs and coyotes from Texas during 1991–2007; 1 dog, 1 bat, and 1 skunk from New Mexico, 2006–2007; 1 gray fox from Arizona, 1986; 3 dogs from Florida, 1994; 1 dog from Alabama, 1994; 2 mongooses and 1 dog from Puerto Rico, 2004–2006; 1 dog from New York, 1949; 1 person from California, 1954; 1 coyote from San Diego, California, 1990 (*20*); 1 dog from California, 2007; and 1 dog from Alaska, 2007. Most samples were from Texas, where canine-associated RVs have been found, although believed to be independent of dog rabies, after enzootic dog rabies was eliminated from the United States during the 1970s (*1*,*15*). The 76 samples from other countries came from 58 dogs from Mexico, representing all dog-related lineages described to date during 1961–2003 (*20*,*21*); 2 gray foxes from Sonora and Chihuahua, 1994–2002 (*21*); 3 skunks from Baja California Sur, 1998–2007 (*20*,*21*); 2 skunks from Durango and Sonora, 2001–2003 (*20*,*21*); 1 dog from Honduras, 2001; 1 person from El Salvador, 2002; and 9 vampire bats from Mexico, 2002–2004. To entail a global perspective, we compared these sequences with historical and recent sequences associated with major dog and terrestrial wildlife rabies foci in the United States and elsewhere (*11*,*12*,*20*–*24*). Vaccine strains with known American origin were included as surrogates of enzootic dog RVs in the United States. They were represented by Flury strains low egg passage and high egg passage, originally isolated from a person in Georgia in 1939 (*25*) and by Street-Alabama-Dufferin B19 and Evelyn Rokitniki Abelseth derivatives, obtained from a rabid dog in Alabama in 1935 (*26*). Surrogates of enzootic dog RVs from the Old World were also incorporated in the global analysis. Pasteur's derivatives, originally obtained from a rabid cow in Paris, France, in 1882, such as Pasteur virus, Pitman-Moore, an avirulent mutant derived from challenge virus standard AvO1, and challenge virus standard, were also considered in this regard (*27*).

Sequence Analyses

We performed multiple alignments of the partial and entire nucleoprotein RV sequences by using ClustalW (www.ebi.ac.uk/clustalw/index.html). To verify the robustness of the phylogenetic inferences, we conducted the analysis by using phenetic and cladistic methods with MEGA 2.1 (*28*) and MrBayes (www.mrbayes.net) programs. Corrected nucleotide substitutions were calculated by using the Kimura 2-parameter and general time reversible model for neighbor joining and MrBayes, respectively. Confidence limits were estimated by a bootstrap algorithm applying 1,000 iterations (*29*). To contrast RV associated with terrestrial carnivore species, we also used consensus sequences associated with bat rabies in Mexico and the United States to construct the phylogenies. We included other lyssavirus species as outgroups: European bat lyssavirus 1, European bat lyssavirus 2, and Australian bat lyssavirus (*11*,*23*).

Results

Elimination of Enzootic Dog RVs from the United States

The domestic dog–coyote RV variant lineage was found in a total of 122 samples from either dogs or coyotes. Most (118) of these were found in Texas during 1991–2004 (Figures 1, 2; online Appendix Figure, available from www.cdc.gov/EID/content/14/12/1849-appG.htm). The overall genetic identity among members of this lineage in the period analyzed was 98.9%. This lineage was monophyletic with an extinct enzootic dog rabies lineage distributed in central Mexico and west-central Mexico until the year 2000. An average identity value of 97.6% was noticed between these 2 lineages (Figure 2). A consensus amino acid change at position 134 (V replaced by I) within the nucleoprotein gene was observed in both lineages. However, a unique amino acid change at position 426 (S replaced by T) was characteristic for the domestic dog–coyote RVs. Similarly, the Sonora dog lineage, distributed along the western United States–Mexico border until 1994, was monophyletic with extant lineages circumscribed southward in west-central Mexico, southeastern Mexico (Chiapas), and Central America (Figures 1, 2). The overall genetic distance among samples within this lineage was 0.017 (98.3% identity). When compared with its closest relatives in southeastern Mexico and Central America, the identity value was 97.7% (Figures 1, 2). An amino acid change at position 40 within the nucleoprotein gene (C replaced by S), common to dog-related rabies enzootics in Africa and Asia (Africa 2 and 3, arctic fox and Arctic-like, plus all dog-related lineages in Asia and India), was found in this lineage. Within

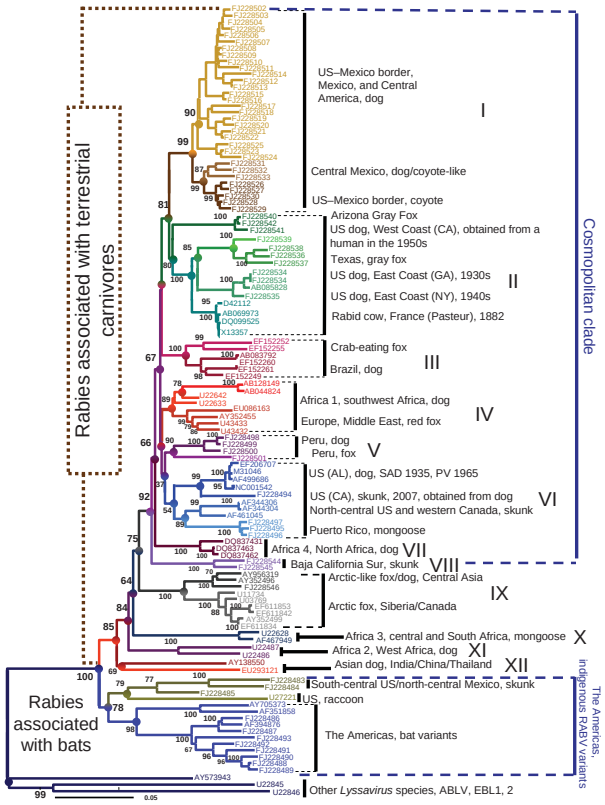

Figure 1. Neighbor-joining phyogenetic tree reconstructed by using entire nucleoprotein sequences that show the consensus topology observed with maximum-likehood and Bayes methods (www.mrbayes.net). The hierarchy encompassing phylogroups, clades, groups, lineages, and taxa of rabies viruses throughout the world is shown. SAD, Street-Alabama-Dufferin; RV, rabies virus; ABLV, Australian bat lyssavirus; EBL, European bat lyssavirus. Scale bar indicates number of nucleotide substitutions per site.

the United States, the Sonora dog lineage was detected in 2 dogs, 2 coyotes, and 1 bobcat in counties bordering northeast Mexico in 1993, 1995, and 1996, respectively, and in 1 dog in San Diego, California, in 1990.

The domestic dog–coyote RV variant and Sonora dog lineage enzootics were expected to be related because of their geographic proximity (Figure 2). However, the phylogenetic data demonstrated that the Sonora dog lineage originated from independent dog rabies enzootics translocated from Central America, Honduras, Nicaragua, and Michoacán in west-central Mexico (Figures 1, 2).

The Texas gray fox RV variant was detected in Texas only, from 8 dogs and 3 coyotes during 1991–1995, and in 20 coyotes in the western part of Texas during 2007 (online Appendix Figure). Members of the Texas gray fox variant or lineage presented a consensus amino acid change within the nucleoprotein gene at position 247 (K replaced by R) and an overall identity value of 99.1%. In 1986, a rabid gray fox from Arizona was found with the Arizona gray fox RV variant. This variant was also found circulating in northwestern Mexico (Figures 1,2); 3 consensus amino acid changes within the nucleoprotein gene at positions 9 (K replaced by R), 13 (Q replaced by H), and 421 (I replaced by V) characterized Arizona gray fox RV variant samples among all other extinct and extant enzootic canine-related variants and lineages circulating throughout the world. The average identity value for members of the Arizona gray fox RV variant lineage was 98.8%.

The Texas gray fox RV variants are enzootic to Texas, and the Arizona gray fox RV variants are enzootic to Arizona and northwestern Mexico, respectively; average identity value between variants is 92.9% (Figure 2). Distinctive consensus amino acid and nucleotide changes at the nucleoprotein gene have become fixed and have remained stable over time in these RVs circulating in gray fox populations in the southern United States. All lineages were monophyletic with historic RV (Flury high egg passage and low egg passage, CA human 1954, and NY dog 1949) obtained from virus repositories or vaccine strains that were originally recovered from canine rabies cases that occurred in the United States before dog rabies elimination (Figure 1). The topology in group II (Figure 1) details the circulation of at least 2 canine rabies lineages representing different dog rabies enzootics (before dog rabies elimination in the United States): one closely related to the Texas gray fox variant circumscribed in California (CA human 1954) and the other widely circulating over the East Coast, related to the Flury and NY dog sequences. This group also encompassed surrogates of enzootic dog rabies in Europe, which indicated an epidemiologic link between dog rabies epizootics on both continents (Figure 1).

The Puerto Rico mongoose RV variant was found in 2 mongooses and 1 dog. This lineage presented a homogeneous identity value of 99.1% and had common amino acid changes with Africa 1b and Africa 3 at position 254 (R replaced by K) and with the Arctic-like and Street-Alabama-Dufferin B19 RV at position 135 (S replaced by P), consistent with an Old World origin of these viruses. A characteristic amino acid change for this lineage was found at position 181 (I replaced by V). This variant was monophyletic with 2 North American lineages: the north-central skunk and the west Canada skunk RV. These skunk lineages, and the Puerto Rico mongoose lineage, were also monophyletic with Street-Alabama-Dufferin B19 derivatives and with a California skunk lineage, integrating group VI, which presented a low bootstrap value (Figure 1). Nonetheless, group VI was consistent when different methods for phylogenetic reconstruction were used (maximum likelihood and Bayes data are available on request). Skunk RVs in the north-central United States, southwestern Canada, and California were monophyletic with

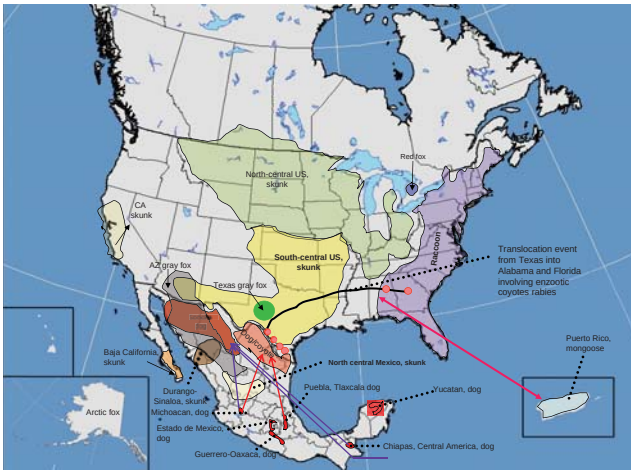

Figure 2. Current distribution of major rabies virus (RV) lineages associated with terrestrial carnivores and dogs in the United States and Mexico. Translocation movements proposed on the basis of the phylogenetic analysis (bidirectional arrows in colors) and confirmed translocations events on the basis of descriptive and epizootiologic investigations are shown. **Boldface** indicates RV lineages associated with rabies enzootics autochthonous for the New World (not associated with dogs).

Street-Alabama-Dufferin B19 and Pasteur virus strains, both surrogates for enzootic dog rabies before its elimination in the United States and Europe, respectively. These results clearly illustrate the association of enzootic skunk rabies with historic dog rabies enzootics (Figure 1).

To underscore the importance of transcontinental translocation of dog-related rabies into the United States and also to illustrate that such phenomena still occur in the 21st century, during 2007 we studied a rabid dog imported from India into Washington state and then transported into Alaska. The infecting strain was determined to be the arctic fox–like variant, which is mainly enzootic in dog populations in northern India and central Asia (Figure 1).

Conversely, interspecies transmission of rabies into domestic dogs and wildlife was also observed in this study. Two dogs, 1 from Texas and the other from New Mexico, were typed as the *Tadarida brasiliensis* RV variant, whereas 1 coyote from Texas was identified with the south-central skunk RV variant. These findings illustrate that the interspecies transmission of rabies is a process that also may occur with regularity.

Canine and Wildlife Rabies in the United States within a Historical and Global Context

To better understand dog rabies epizootiology in the United States, we also conducted a global analysis. Samples were divided into 2 main phylogroups, 1 associated with terrestrial carnivores and the other associated with bat rabies in the Americas. The terrestrial phylogroup was integrated by the cosmopolitan clade (CosC) that comprised at least 8 monophyletic groups (I to VIII, Figure 1). CosC encompassed 2–4 lineages representing independent enzootics associated with not only different species of Canidae (domestic dogs, jackals, wolves, gray foxes, red foxes, arctic foxes, raccoon dogs, Corsac foxes, crab-eating foxes, coyotes) but also with other species of terrestrial carnivores (mainly skunks, such as the Baja California skunk in Mexico and California skunks in the United States). Each of these groups consistently presented at least 1 lineage associated with rabies enzootics in domestic dogs (either extinct or extant). The remaining lineages were associated with rabies enzootics in terrestrial carnivores. The exceptions were groups VII and VIII, associated with either domestic dogs (Africa 4) or the Baja California spotted skunk *Spilogale putorious* (Figure 1). These phylogenetic patterns were consistent throughout Europe, Africa, and the Americas, where enzootic dog rabies was introduced (Figure 1). In addition, several other independent dog-related RV lineages were geographically circumscribed in different countries of the Americas, such as Argentina, Colombia, Bolivia, Peru, Ecuador, Nicaragua, and the Dominican Republic, and belong to the CosC as well (only partial nucleoprotein sequences available; tree available from the authors on request).

The average genetic distance among all members within CosC was 0.071%, equivalent to 93% average identity. The highest genetic distance observed between groups pertaining to this clade was 0.104 (89.6% average identity) and occurred between group III and group VIII (Figure 1). Meanwhile, the lowest genetic distance observed was between group I and group II 0.073 (92.7% average identity), followed by the same genetic distance of 0.074 (92.6%) between groups IV–V, IV–VI, and V–VI (Figure 1). The terrestrial phylogroup also encompassed independent groups and lineages associated with enzootic dog rabies in Asia (this group was the most divergent with an average genetic distance of 0.126, 87.4%, encompassing lineages from China, Thailand, Vietnam, and India), Africa (Africa 2, group IX with an average genetic distance of 0.079, 92.1%), the arctic fox and the arctic fox–like lineages (Arctic/Arctic-like group IX, with an average genetic distance of 0.044, 95.6%), and the African mongoose lineage (Africa 3, with an average genetic distance of 0.021, 97.9%).

Conversely, the bat phylogroup comprised RV lineages and variants associated with independent rabies enzootics maintained by different species of bats and 2 independent enzootics established in raccoons (raccoon variant) and skunks (south-central US skunk and Mexican north-central skunk), which may represent autochthonous RV lineages in the New World (Figures 1, 2). These lineages have an average genetic distance of 0.155 (84.5%) to 0.158 (84.7%) when compared with RV lineages associated with the terrestrial carnivore clade.

Discussion

On the basis of phylogenetic analysis in conjunction with historic and recent epizootiologic data on rabies, we found no evidence of enzootic dog rabies in the United States for the past 13 years, (*1*,*15*,*16*,*18*,*19*,*30*). Our findings suggest that independent rabies enzootics are now established in wild terrestrial carnivores (skunks in California and the north-central United States, gray foxes in Texas and Arizona, and mongooses in Puerto Rico), as a consequence of different spillover events from the long-term rabies enzootics associated with dogs (*31*).

The concept of dog-related lineages spilling into wildlife species with further host shifts was consistent for all phylogentic groups shown as part of CosC (I to VI) (*8*,*10*,*21–24*, *31–34*). This tendency was also consistent for other groups outside the CosC, such as group IX (*35*,*36*). Dog rabies epizootics in the Americas seem to be characterized by the circulation of multiple geographic or temporal lineages that have either become extinct or merged over time (*2*,*3*,*8*,*10*,*21*,*22*,*32–34*). Such a tendency was clearly observed in groups I, II, and VI and was implicit in groups III, IV, V, and VII (*2*,*3*,*5*,*8*,*10*,*12*,*21–23*,*31–33*, *35*,*36*). After European colonization in the 15th century, multiple dog-related RV lineages have been introduced and evolved over time in the United States and elsewhere in the Americas (*5*,*8*,*10–12*).

The historical background in Europe and the Middle East supports the suggestion that enzootic red fox rabies was derived from a dog rabies epizootic in Europe (*5*,*8*,*10–12*). However, none of the surrogates representing European enzootic dog rabies early in the 19th century (Pasteur virus, Pitman-Moore, AV01, or challenge virus standard) segregated with enzootic RVs circulating in European wild terrestrial carnivores. These observations suggest that host-switching events may have occurred from other likely extinct dog RV lineages or, alternatively, from extinct lineages contemporary to Pasteur virus yet pertaining to independent dog rabies enzootics (*8*,*10–12*).

There is no cultural, historical, or epidemiologic evidence of enzootic dog rabies in the New World before European colonization (*8*,*10*). One of the oldest rabies enzootics associated with wild canines in North America occurred in arctic and red foxes (*37*,*38*). This fox rabies enzootic was found to be associated with the expansion of arctic fox rabies from northern Asia (*35*). We did not find any direct phylogenetic linkage between CosC and arctic fox rabies, as exists between arctic fox–like rabies epizootics in Asia and arctic fox rabies prevailing in the circumpolar Arctic region (*36*).

The global origin of enzootic rabies in dogs and in other terrestrial carnivores remains unknown but likely predates the origin of dogs 15,000–100,000 years ago (*39*). A higher mitochondrial DNA haplotype diversity found in dog breeds from eastern Asia correlates with a higher genetic diversity of dog-related RV lineages in the same region (*8*,*10*,*35*,*36*,*39*). The latter observations raise questions about the possible coevolution of wild canines and RVs, the role of dog domestication in the dissemination of rabies, and the emergence of rabies enzootics in other wild terrestrial carnivores elsewhere. Nonetheless, independent rabies enzootics that persist in raccoons in the eastern United States and among skunks in the south-central United States and north-central Mexico seem to have their origin in RVs associated with indigenous bats species (*8*,*31*).

Global migration, social factors, and trading activities continue to promote and enhance long-distance movements of rabies-infected animals, increasing the potential risk of reintroducing the disease to regions where the problem has been eliminated. This trend poses new challenges for the regulation of animal movements to attain sustainable elimination.

Clearly, canine rabies elimination is needed on a global level. Vaccination of dogs should be maintained until all dog-related lineages and biotypes currently circulating in wildlife have been eliminated. The canine origin of these viruses makes them prone to return to dogs, where the disease may easily become enzootic again without proper attention related to laboratory-based surveillance, prevention, and control.

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Genetic Characterization of Toggenburg Orbivirus, a New Bluetongue Virus, from Goats, Switzerland

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A novel bluetongue virus (BTV) termed Toggenburg orbivirus (TOV) was detected in goats from Switzerland by using real-time reverse transcription–PCR. cDNA corresponding to the complete sequence of 7 of 10 double-stranded RNA segments of the viral genome was amplified by PCR and cloned into a plasmid vector. Five clones for each genome segment were sequenced to determine a consensus sequence. BLAST analysis and dendrogram construction showed that TOV is closely related to BTV, although some genome segments are distinct from the 24 known BTV serotypes. Maximal sequence identity to any BTV ranged from 63% (segment 2) to 79% (segments 7 and 10). Because the gene encoding outer capsid protein 2 (VP2), which determines the serotype of BTV, is placed within the BTV serogroup, we propose that TOV represents an unknown 25th serotype of BTV.

Bluetongue is a vectorborne disease affecting all ruminant species but causing clinical disease mostly in sheep; cattle and goats are usually considered asymptomatic reservoir hosts (*1*). The disease occurs worldwide and is caused by bluetongue virus (BTV), which belongs to the genus *Orbivirus* within the family *Reoviridae*. Other species of the orbiviruses are African horse sickness virus, epizootic hemorrhagic disease virus (EHDV), and some lesser-known viruses such as Peruvian horse sickness virus, Chuzan virus, Saint Croix River virus, and Yunnan orbivirus (*2*). The virus has a segmented double-stranded RNA genome consisting of 10 segments that code for 11 viral proteins. BTV is transmitted between its ruminant host mainly by blood-feeding midges of the *Culicoides* spp. Twenty-four serotypes of BTV can be distinguished on the basis of the antigenic profile of its major outer capsid protein VP2 (*3*).

In Europe, several serotypes of BTV were detected sporadically in the 1990s (*4*). From 1998 through 2006, a total of 5 serotypes (1, 2, 4, 9, and 16) became established in southern Europe (*5*), but they were kept confined to this area, most likely because their main insect vector, *C. imicola*, has never been detected north of the Alps in Europe (*6*). A BTV-monitoring program in cattle herds has been in effect in Switzerland since 2003 in Ticino Canton, south of the Alps (*7*–*9*). However, no BTV has been detected until now in this region of the country. Furthermore, northern Europe had never been affected by bluetongue before 2006, although BTV can also be transmitted by other *Culicoides* species, such as *C. obsoletus* and *C. pulicaris* (*6*), which are native to this area.

In 2006, BTV serotype 8 (BTV-8) of likely African origin was introduced into Belgium by an as yet unknown mechanism (*8*). This virus spread throughout many northern European countries. By mid-2008, BTV-8 had caused numerous clinical bluetongue cases in sheep and cattle in the Netherlands, Belgium, Germany, Luxembourg, France, Denmark, Czech Republic, United Kingdom, Italy, Spain, and Switzerland (*5*).

The first clinical case of bluetongue caused by BTV-8 in Switzerland was detected in October 2007 on a dairy cattle farm (*10*). Because Switzerland was already included in a surveillance zone and, after detection of the first BTV-8 infection, a protection zone, all animals were tested

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serologically and virologically before trade. Four additional cases of BTV-8 infection were detected from October through November 2007 (*10*,*11*).

Early in 2008, several BTV-positive, clinically healthy animals were detected in a goat flock in northeastern Switzerland (V. Chaignat et al., unpub. data) by BTV-specific antibody ELISAs and viral RNA detection, by using a BTV-specific real-time reverse transcription–PCR (rRT-PCR) (*10*,*12*). However, levels of antibody and viral RNA were unexpectedly low. Furthermore, presence of BTV RNA could not be confirmed by use of other BTV-specific rRT-PCR protocols (*13*,*14*), and amplification curves obtained in the screening rRT-PCR suggested that the target sequence on RNA segment 10 of this virus might be different from all known BTV strains (data not shown). For this reason, we determined the nucleotide sequence of the rRT-PCR amplification product.

Experimental infection of goats and sheep by subcutaneous and intravenous injection of blood from virus-positive animals from the herd in which the infection was first discovered demonstrated that the virus is transmissible to and replicates in goats, albeit without causing bluetongue-specific clinical symptoms. In contrast, the virus could only be sporadically detected in inoculated sheep (V. Chaignat et al., unpub. data). On the basis of the sequence of the rRT-PCR product, this newly detected virus, termed Toggenburg orbivirus (TOV), could represent an unknown orbivirus of low pathogenicity, or a new serotype of BTV. Thus, we performed a detailed genetic characterization of the TOV genome.

The complete coding sequence for the 7 RNA segments (2, 5, 6, 7, 8, 9, and 10) was determined. Segment 2 was chosen because it encodes the viral structural protein VP2 that contains the serotype-specific determinants, whereas the genome segments 5 through 10 are short enough to enable sequencing of the cloned cDNA without the need for internal sequencing primers. We present evidence that TOV is genetically related to BTV but cannot be assigned to any of the 24 known serotypes; instead, it likely represents a 25th BTV serotype.

Materials and Methods

Source of Viral RNA and Extraction of RNA

BTV-like orbivirus RNA and antibodies were detected in several adult and newborn goats in a herd in the northeastern part of Switzerland (St. Gallen Canton) (V. Chaignat et al., unpub. data). We used erythrocytes from blood collected into tubes containing EDTA from 1 adult and 1 newborn goat, which had cycle threshold (C_T) values of 22 and 32, respectively, in the rRT-PCR (*10*). Total RNA was extracted from 250 μL of erythrocyte suspension by using Trizol (Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. Precipitated purified RNA was dissolved in 20 μL of RNase-free water.

RT-PCR for Full-Length cDNA Amplification, Cloning, and Sequencing

Primers corresponding to inter-BTV serotype-conserved 5′ and 3′ terminal sequences were designed for each of the viral RNA segments to be analyzed (Table 1). Before RT-PCR, 8 μL of extracted viral RNA was mixed with 50 pM of 2 RNA segment-specific forward and reverse primers and heat-denatured for 5 min at 95°C. Reverse transcription of both RNA strands into double-stranded cDNA was performed by using SuperScript III reverse transcriptase (Invitrogen) in a final volume of 25 μL. A total of 8 μL of cDNA was then added to a PCR mixture containing Platinum Taq High Fidelity Polymerase (Invitrogen) and amplified by 40 cycles at 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min (for segments 5–10) or 72°C for 8 min (for segment 2).

PCR-amplified cDNA of the expected length was purified by agarose gel electrophoresis and ligated into plasmid vector pCR4-TOPO (Invitrogen). Clones of transformed *Escherichia coli* harboring the TOV insert-containing vector were identified by PCR preps using insert-spanning M13 primers. Miniprep DNA of ≥5 cDNA clones from each viral genome segment was sequenced by cycle sequencing using IRD800 and IRD700 infrared dye–labeled M13 (Eurofins MWG Operon, Ebersberg, Germany) and TOV segment 2–specific internal primers. Sequencing reactions were subjected to electrophoresis in a 4300L DNA sequencer (LI-COR, Lincoln, NE, USA) and analyzed by using e-Seq V3.0 and AlignIR V2.0 software (LI-COR). The coding region of each TOV genome segment analyzed in this study was compared with published orbivirus sequences by using online BLAST analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi). For phylogenetic analysis, TOV-specific sequences were aligned to a selection of available corresponding sequences from GenBank that represented all orbivirus species by using MEGA version 4.0 software (*15*) with default parameters. The open reading frame (ORF) sequences of the 7 analyzed genome segments of TOV were submitted to GenBank under accession nos. EU839840 (S2), EU839841 (S5), EU839842 (S6), EU839843 (S7), EU839844 (S8), EU839845 (S9), and EU839846 (S10).

Results

TOV was initially detected by using an RNA segment 10–specific rRT-PCR (*10*). Nucleotide sequencing of this rRT-PCR product indicated that TOV is an orbivirus closely related to, but not identical with, any known BTV serotype. The analyzed sequence showed a 1-base mismatch in the probe region of the rRT-PCR target sequence (Figure 1, panel A), which could explain the unusual amplification

Table 1. Terminal primers used to amplify complete Toggenburg orbivirus RNA segments

RNA segment	Name*	Sequence (5′ → 3′)
	Forward primer	
2	BTV4&10_S2_-21_F	GGGTTAAAAGAGTGTTCYAC
5	BTV_S5_F	GTTAAAAAAGTTCTCTAGTTGGCA
6	BTV4&10&11_S6_-30_F	GTTAAAAAGTRTTCTCCTACTC
7	BTV_S7_-17_F	GTTAAAAATCTATAGAGATGGAC
8	BTV_S8_-19_F	GTTAAAAAAWCCTTGAGTCATG
9	BTV_S9_-15_F	GTTAAAAAATCGCATATGTCAG
10	BTV_S10_F	GTTAAAAAGTGTCGCTGCCAT
	Reverse primer	
2	BTV4&20_S2_R	GTAAGTGTAAGAAGGCCACAG
5	BTV_S5_R	GTAAGTTGAAAAGTTCTAGTAGAG
6	BTV4&10_S6_1618_R	GTAAGTGTAATCTTCTCCCTC
7	BTV_S7_1142_R	GTAAGTGTAATCTAAGAGACGT
8	BTV_S8_1106_R	GTAAGTGTAAAATCCCCCCC
9	BTV_S9_1038_R	GTAAGTRTGAAATCGCCCTAC
10	BTV_S10_R	ACCTYGGGGCGCCACTC

*BTV, bluetongue virus.

curves (low delta Rn value, the magnitude of the signal generated by the given set of PCR conditions). Various additional rRT-PCR protocols all yielded negative results (data not shown). When the primer and probe sequences of these published rRT-PCR protocols for segment 1 (*13*,*14*) or segment 5 (*14*), respectively, were aligned to the corresponding TOV sequences determined in this study, numerous mismatches were found (Figure 1). These mismatches were the likely reason for the failure of these assays to detect TOV. Because sequence amplified by the rRT-PCR (Figure 1, panel A) was most closely related to several BTV-4 and BTV-10 serotypes, RNA segment–specific primers binding to the conserved 3′ and 5′ untranslated regions were designed on the basis of these serotypes whenever no consensus sequence matching with all published sequences (representing all 24 serotypes) could be found, i.e., for segments 2 and 6.

Although the amount of viral RNA in blood from infected but clinically healthy goats was low (C_T values >30 in the rRT-PCR) we could amplify the full-length cDNA sequence for all 7 RNA segments analyzed, including the 2.9-kb fragment of segment 2. PCR products all showed the expected length as predicted from the corresponding BTV genome segment. After cloning into the pCR4-TOPO vector, both DNA strands of 5 clones were sequenced for each genome segment. A consensus sequence could be unambiguously determined because the number of point mutations in the clones was low, and never more than 1 of the 5 clones showed a difference from the consensus sequence (data not shown).

BLAST analysis of the 7 TOV genome segments showed in all cases the highest sequence similarity to BTV, although the search algorithm had to be changed from megablast (highly similar sequences) to blastn (somewhat

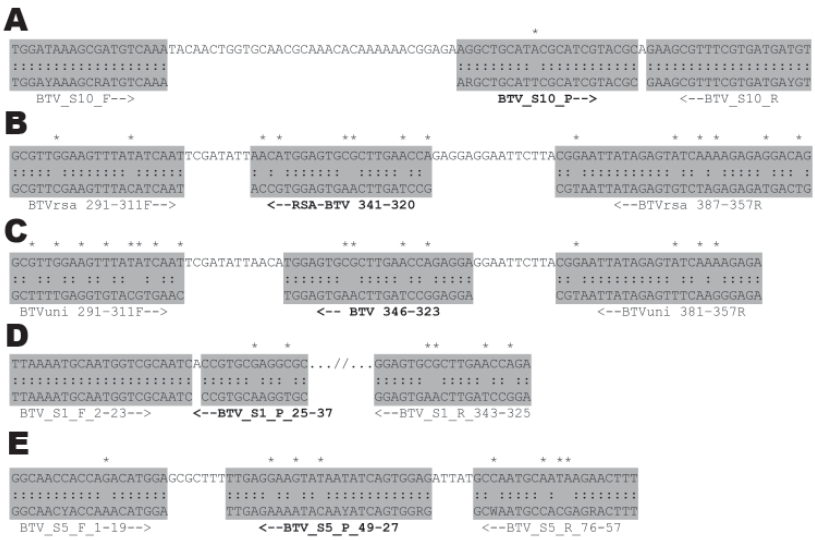

Figure 1. Nucleotide sequence alignment of target regions of published bluetongue virus (BTV) real-time reverse transcription–PCR primers and probes (in **boldface**), which were used for detection of Toggenburg orbivirus (TOV). A) Segment 10 (*10*); B) segment 1, eastern serotype specific (*13*); C) segment 1, western serotype specific (*13*); D) segment 1 (*14*); E), segment 5 (*14*). Shaded areas indicate primer and probe sequences (in sense orientation), colons indicate sequence identity, arrows indicate orientations of probes and primers, and asterisks indicate mismatches between primers/probes and TOV genome sequence.

similar sequences) to detect any sequence homology. However, results varied widely in terms of percentage of identity to their closest BTV sequence and to BTV serotype showing the highest similarity (Table 2). No BTV strain was distinctly more closely related to TOV than all other known sequences, although for several genome segments BTV serotype 4 isolates were found to be the closest relatives to TOV. When identity levels between analyzed TOV genome segments to their genetically closest BTV strain were compared with respective values of the 2 most distantly related BTV serotypes, TOV sequences were in some cases (segments 5, 8, and 10) clearly more different than the BTV sequences among themselves. However, for the remaining segments, TOV sequences were less different from their closest BTV sequence than the 2 most distantly related BTV serotypes (segments 2, 6, 7, and 9).

Results of phylogenetic analysis using ClustalW alignment (*16*) and neighbor-joining tree construction confirmed the results of BLAST analyses by locating some TOV genome segments within the BTV serogroup (segments 2, 6, 7, and 9). In contrast, for segments 5, 8, and 10, TOV sequences were outside the BTV subtree (Figures 2, 3). Furthermore, the S2 gene could not be assigned to any of the 9 proposed nucleotypes (*17*) (data not shown). Despite the different placement of the 7 TOV genome segments, they were all more closely related to BTV than to any of the other known orbivirus species, in particular to EHDV, which is the closest relative to BTV (Figures 2, 3).

Discussion

In the context of trade investigations implemented in Switzerland shortly after the first BTV-8 outbreak was detected (*11*), BTV-specific RNA was detected in clinically healthy goats in St. Gallen Canton in northeastern Switzerland by segment 10–specific rRT-PCR. However, none of the additional rRT-PCR protocols for other BTV genome segments, which were performed for confirmatory purposes, yielded positive results. Furthermore, the short (58-nt) segment 10–specific sequence amplified by the initial rRT-PCR was similar but not identical to any known BTV. Therefore, to characterize TOV as a potentially novel BTV, we performed a detailed genome analysis by cloning and sequencing the entire coding region of most viral genome segments.

cDNA of smaller genome segments that encode the structural proteins VP5 (segment 6), VP6 (segment 9), VP7 (segment 7), and nonstructural (NS) proteins NS1 (segment 5), NS2 (segment 8), and NS3/NS3A (segment 10) could be amplified and sequenced without the need for internal, TOV-specific primers. The 2.9-kb region spanning segment 2 was also included; however, a primer-walking approach had to be used for design of TOV-specific sequencing primers (data not shown). VP2, which is encoded by segment 2, is exposed at the virion surface, together with VP5 (*3*), and carries the epitopes inducing serotype-specific neutralizing antibodies (*18*). Thus, segment 2 was expected to contain the genetic information enabling assignment of TOV to 1 of the 24 BTV serotypes.

Although initial attempts to isolate TOV in several mammalian cell lines failed, there is preliminary evidence that the virus replicates in the highly BTV-susceptible KC insect cell line derived from *Culicoides* spp. (*19*). However, to avoid introduction of mutations in the viral genome caused by cell culture adaptation, we used blood as the source for cloning and nucleotide sequencing of the TOV genome.

For accurate identification of a consensus sequence for each cloned TOV genome segment, 5 clones were analyzed for each segment. When sequences of individual clones were compared, mismatches were found in <1% of the nucleotide positions (data not shown), and always in only 1 of the 5 clones. If one takes into account the error rate of the reverse transcriptase and Taq DNA polymerase used to transform the viral RNA into PCR-amplified cDNA, this finding supports reported genetic stability of the double-stranded RNA genome of orbiviruses (*20*).

BLAST analysis of the ORF sequence of the 7 TOV genome segments studied suggested that TOV most likely belongs to the BTV serogroup because although the degree of nucleotide sequence identity between TOV and the closest BTV relative was low, each of the TOV genome segments was closely related to BTV and not to another orbivirus. The BLAST results were confirmed by individual phylogenetic analysis of the 7 TOV genome

Table 2. Sequence comparisons of Toggenburg orbivirus genes by BLAST analyses*

	BLAST best hit					
RNA segment	Serotype	Isolate	Accession no.	No. identities/total	Identity level, %	Query coverage, %
2	4	Turkey 1999	DQ825670	1,855/2,919	63	100
5	16	TUR2000/10	AM773696	1,242/1,662	75	100
6	4	ARG2002/01	AJ586682	1,163/1,585	73	100
7	23	Dehradoon	AJ277802	832/1,050	79	100
8	4	Corsica	AY857499	778/1,072	72	100
9	10	10O90H	BVU55782	742/1,005	73	100
10	8	8438/vaccine	AF512919	550/694	79	100

*Only coding regions of Toggenburg orbivirus genes (including stop codons) were used (http://blast.ncbi.nlm.nih.gov/blast.cgi).

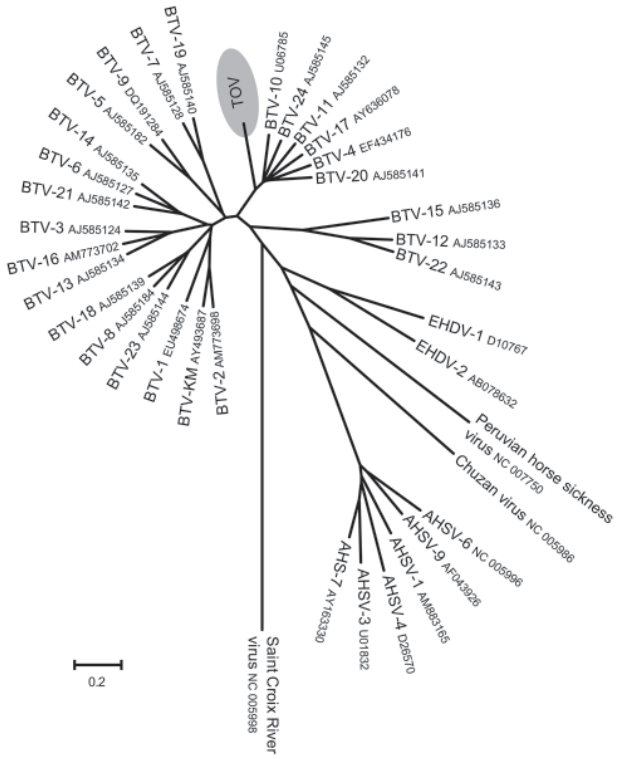

Figure 2. Phylogenetic analysis of Toggenburg orbivirus (TOV) (shaded region) genome segment 2 by ClustalW alignment (*16*) and subsequent neighbor-joining tree construction by MEGA version 4 software (*15*). GenBank accession numbers are indicated for all orbivirus sequences used to construct dendrogram. BTV, bluetongue virus; EHDV, epizootic hemorrhagic disease virus; AHSV, African horse sickness virus. Scale bar indicates number of nucleotide substitutions per site.

segments. Depending on the segment, TOV was placed in a different neighborhood among BTV and EHDV (Figures 2, 3). Although segments 2, 6, 7, and 9 encoding viral structural proteins were placed within the BTV subtree in the dendrograms, albeit being distinct from all 24 BTV serotypes, the remaining segments 5, 8, and 10 coding for NS proteins were located outside the branching area of the BTV subtree toward the EHDV subtree. These findings suggest that TOV might have diverged from BTV and evolved independently or could represent a reassortant between BTV and an unknown orbivirus closely related to EHDV. This assumption is supported by results of BLAST analysis that showed a greater evolutionary distance between TOV and BTV than among the various BTV serotypes (data not shown), which is evident in the case of the segment 10 encoding NS3/NS3A. It will be useful to determine whether TOV genome segments 1, 3, and 4, which encode viral structural proteins, are also more closely related to BTV than the NS genes.

Definitive characterization of TOV as a new BTV serotype will require a comprehensive serologic typing with BTV serotype–specific antisera and TOV-specific antisera. It is likely that TOV represents a new BTV serotype because agreement of segment 2–based genotypic and serology-based phenotypic serotype differentiation, involving a large collection of BTV strains representing all 24 known serotypes, has been reported (*17*).

We propose that TOV more likely represents an unknown 25th serotype of BTV rather than a new orbivirus species, on the basis of grouping of structural protein genes within BTV and because sera from goats from which the virus was isolated showed reactivity in several BTV-specific antibody ELISAs. These tests did not show reactivity with antibodies against EHDV, which represents the closest relative to BTV among the orbiviruses (data not shown).

TOV did not cause bluetongue-specific clinical signs in experimentally infected goats (V. Chaignat et al., unpub. data). In addition, virus load was low. These findings are consistent with the fact that goats generally show no clinical disease after BTV infection but can serve as reservoir hosts (*1*). However, TOV was only sporadically detected by rRT-PCR in inoculated sheep, and these animals did not show any pronounced clinical signs of bluetongue. If TOV also shows an apathogenic phenotype in other ruminants, this finding would further support the possibility that TOV may have evolved differently than classic BTV strains. Identification of insect vector(s) for TOV would be useful because these vector(s) might influence transmission and phenotypic properties of TOV.

An apathogenic BTV termed the KM strain has been reported in goats from Taiwan (*21*). This virus was characterized as a typical BTV and was added to BTV reference sequences for the present phylogenetic analysis (Figures 2, 3). On the basis of its high segment 2 sequence similarity with BTV-2 (Figure 2), it is likely that KM belongs to this BTV serotype. Comparison of TOV with KM shows that TOV is less related to classic BTV than KM, which rules out TOV and the KM strain having a common ancestor, although the 2 viruses share a high sequence homology in their segment 9 ORF (Figure 3). Therefore, TOV might represent a reassortant BTV that acquired its segment 9 from a BTV closely related to KM.

To determine the genetic relationship of TOV with the BTV-8 strain currently circulating in northern Europe, the segment 10 coding region of the Swiss BTV-8 isolates from 2 of the late 2007 outbreaks was sequenced and submitted to GenBank (accession nos. EU450660 and EU450661). This BTV-8 was different from a BTV-8 reference strain obtained from the Office International des Epizooties Reference Laboratory for bluetongue (Institute of Animal Health, Pirbright, UK) (accession no. EU450663). Although TOV was initially detected in Switzerland at the same time as the

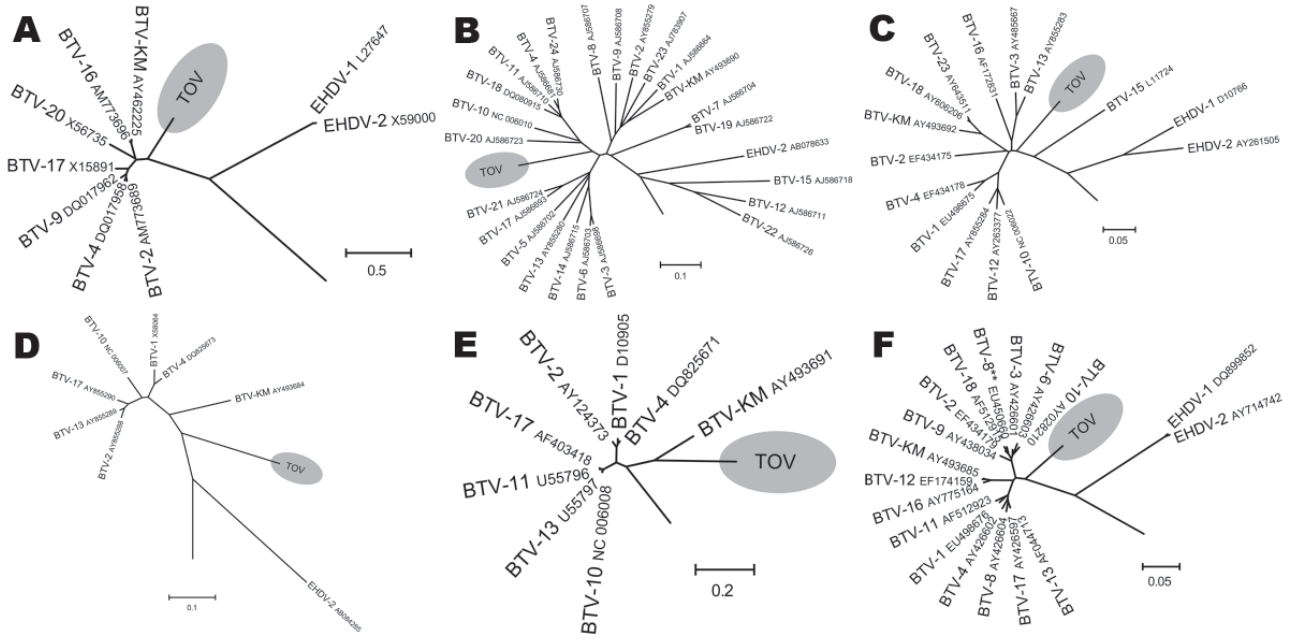

Figure 3. Phylogenetic analysis of Toggenburg orbivirus (TOV) (shaded regions) genome segments by ClustalW alignment (*16*) and subsequent neighbor-joining tree construction by MEGA version 4 software (*15*). GenBank accession numbers are indicated for all orbivirus sequences used to construct dendrograms. A) Segment 5; B) segment 6; C) segment 7; D) segment 8; E) segment 9; F) segment 10. BTV, bluetongue virus; EHDV, epizootic hemorrhagic disease virus. Segments show only relevant parts of dendrograms. **Segment 10 sequence of BTV-8 currently circulating in northern Europe. Scale bars indicate number of nucleotide substitutions per site.

first BTV-8 infections, these 2 BTV strains are genetically diverse, as shown in the dendrogram of segment 10 (Figure 3). None of the 7 genome segments analyzed or any of the 3 remaining genome segments (1, 3, and 4) (data not shown), showed sequence homologies >79% with any other BTV, and none of the BLAST analyses showed a maximal sequence identity level with the corresponding genome segment of the BTV-8 currently circulating in Europe (*22*). These findings indicate that TOV is not a reassortant between BTV-8 and another BTV.

Distinct sequence differences on RNA segments 1 and 5 may also explain why TOV was not detectable by rRT-PCR protocols designed to detect a wide range of BTV serotypes (Figure 1). Only the segment 10–specific rRT-PCR showed a positive result, whereas all segment 5– and segment 1–specific assays did not detect TOV. These findings demonstrate the importance of selecting an appropriate rRT-PCR protocol for detection of TOV and other unusual BTV-like viruses. The fact that most diagnostic laboratories use segment 1– or segment 5–specific rRT-PCRs for routine detection of BTV might also explain why TOV has never been detected in other regions.

TOV and other BTV-25 strains, as potential apathogenic viruses, will have major implications in control of bluetongue. A similar situation is found in the United States, where several BTV serotypes circulate without causing any clinical signs in domestic ruminants; nevertheless, national and international animal trade is heavily affected (*23*).

Additional animal experiments in various ruminant species and adaptation of TOV to cell culture for extended phenotypic characterization (e.g., replication kinetics) are currently under way. TOV-specific rRT-PCR protocols will be developed to determine TOV prevalence and to clarify its role as an animal pathogen. Furthermore, TOV-specific serologic tools that use immunologically dominant epitopes of VP2 and VP5 as recombinant ELISA antigens for detection of antibodies to TOV should be developed. These tools would facilitate epidemiologic studies to determine actual and retrospective seroprevalences of TOV in goats and other domestic and wild ruminants in Switzerland and throughout the world, in particular in countries that have long claimed to be free of BTV infections. Furthermore, TOV-specific diagnostic tools will enable identification of natural reservoir(s) and address how and by which insect vectors this orbivirus is transmitted.

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Sentinel-based Surveillance of Coyotes to Detect Bovine Tuberculosis, Michigan

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Bovine tuberculosis (TB) is endemic in white-tailed deer (*Odocoileus virginianus*) in the northeastern portion of Michigan's Lower Peninsula. Bovine TB in deer and cattle has created immense financial consequences for the livestock industry and hunting public. Surveillance identified coyotes (*Canis latrans*) as potential bio-accumulators of *Mycobacterium bovis*, a finding that generated interest in their potential to serve as sentinels for monitoring disease risk. We sampled 175 coyotes in the bovine TB–endemic area. Fifty-eight tested positive, and infection prevalence by county ranged from 19% to 52% (statistical mean 33%, SE 0.07). By contrast, prevalence in deer (n = 3,817) was lower (i.e., 1.49%; Mann-Whitney $U_{4,4}$ = 14, p<0.001). By focusing on coyotes rather than deer, we sampled 97% fewer individuals and increased the likelihood of detecting *M. bovis* by 40%. As a result of reduced sampling intensity, sentinel coyote surveys have the potential to be practical indicators of *M. bovis* presence in wildlife and livestock.

The emergence and reemergence of zoonotic diseases are becoming increasingly important issues for numerous reasons, including deforestation and habitat fragmentation, increased globalization of travel and trade, urbanization, and bioterrorism concerns. Diseases such as severe acute respiratory syndrome (SARS), avian influenza, transmissible spongiform encephalopathies, Rift Valley fever, West Nile disease, anthrax, and *Escherichia coli* O157 infections recently have resulted in major public health and economic concerns, as well as public anxiety. Over 60% of the 1,415 known human pathogens and 75% of the 175 emerging pathogens are zoonotic (*1*). Many emerging diseases have spilled over from wildlife directly (e.g., West Nile virus infection, hantavirus infection, and Lyme disease) or indirectly through domestic or peridomestic species (e.g., avian influenza, SARS, and Nipah virus infections, plague) (*2*). Early detection of new disease outbreaks in domestic and wild animals is an essential prerequisite of disease control and eradication. Development of methods for early detection of diseases in free-ranging wildlife is problematic.

Development of practical strategies for conducting surveillance in free-ranging wildlife to detect and monitor disease and evaluate control efforts is a necessary component of predicting and managing emerging zoonoses. A case in point is bovine tuberculosis (TB). *Mycobacterium bovis,* the bacterial pathogen that causes bovine TB, has been identified in wildlife, domestic animals, and humans (*3*–*6*). Transmission of *M. bovis* may occur through ingestion of infected tissues or, less likely, through inhalation of aerosolized bacilli (*7*); typically, granulomatous lesions develop in the thoracic lymph nodes and lung after aerosol exposure, and granulomatous lesions develop in the abdominal lymph nodes after oral exposure. Bovine TB often progresses slowly, and clinical symptoms may not appear until advanced stages are reached (*8*,*9*). In 1995, *M. bovis* was found in free-ranging white-tailed deer (*Odocoileus virginianus*) in a localized area in the northeastern Lower Peninsula of Michigan (*10*). In subsequent years, a reemergence of *M. bovis* in Michigan cattle was detected; deer

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were postulated to be the source of infection. Because the socioeconomic impact of this discovery has been immense (*11*), a strategy was developed and implemented to monitor and eradicate *M. bovis* from wildlife and cattle. Although the strategy successfully reduced the apparent prevalence of *M. bovis* in deer, the disease still persists at low levels (e.g., 2001–2006 statistical mean 2.3%) because of high deer densities (statistical mean 13/km^2) and spatiotemporal crowding resulting from supplemental feeding (*12*). As prevalence of *M. bovis* in deer decreases, the sample size required to detect positive deer increases, making monitoring of the disease in deer more difficult and costly. Eventually, prevalence in deer may become too low to accurately estimate through current methods because of the difficulty and expense of obtaining a sufficient sample size, and consequent difficulty of verifying disease eradication. We hypothesized that the presence of *M. bovis* in wild deer at low prevalence could be more accurately determined through an indirect estimator (i.e., a sentinel species).

Use of sentinel animals has been suggested as a cost-effective way to infer prevalence in host populations when direct estimation in such populations is difficult (*13*). As facultative scavengers, coyotes (*Canis latrans*) may act as biological sensors and bio-accumulators of *M. bovis*. by consuming infected host material, resulting in high rates of infection. Furthermore, social foraging by coyote populations (*14*,*15*) should increase the likelihood of multiple coyotes ingesting infected tissue from the same *M. bovis*–positive deer. As a logical corollary, the increased numeric exposure of coyotes to *M. bovis* should mediate an increased detection probability relative to sampling effort. Support for this hypothesis was provided by research (*5*), which reported an apparent prevalence of *M. bovis*. in opportunistically sampled coyotes as 4% in the general area where apparent prevalence in deer averaged 2.3% from 1995 through 2001 (*16*). Finally, coyote home-range sizes (statistical mean 14.25 km^2, 95% confidence interval [CI] 9.54–18.96 km^2) in Michigan allow for reasonable estimates of where infection was acquired (*17*).

We report on a sentinel-based surveillance program designed to detect *M. bovis* in coyotes. Specifically, we sought to determine whether 1) *M. bovis* occurrence in coyotes was detectable, given reduced sampling intensity relative to white-tailed deer, and 2) prevalence of *M. bovis* was greater in coyotes than deer for a given area. If so, coyotes should be effectual sentinels of *M. bovis* occurrence in free-ranging white-tailed deer.

Methods

We worked within the 4-county bovine TB–endemic area in Michigan's Lower Peninsula, where cattle herds continue to be infected and intensive sampling of hunter-killed deer is ongoing (Deer Management Unit [DMU] 452; Figure 1). DMU 452 is the historic core bovine TB–endemic area and remains a focal site of intensive sampling of hunter-killed deer (*18*). Prior carnivore surveillance conducted by the Michigan Department of Natural Resources (MDNR) had detected *M. bovis* in 18 of 249 sampled coyotes. Given the history of intensive surveillance and elevated *M. bovis* prevalence in the area, it was the logical choice to implement and evaluate a sentinel-based surveillance program. Habitat associations within DMU 452 were diverse; moraine uplands were dominated by forests of jack pine (*Pinus banksiana*), white pine (*P. alba*), oak (*Quercus* spp.), and maple (*Acer* spp.). Dominant lowland vegetation included tag alder (*Alnus rugosa*) and white cedar (*Thuja occidentalis*), and wetland ephemera were common. Annual precipitation typically ranged from 71 cm to 91 cm; most occurred as snowfall (*19*). Mean yearly summer and winter temperatures were 21°C and –10°C, respectively (*19*).

We trapped coyotes from December 2003 through September 2005 using padded foot-hold traps and scent lures in 15 townships within the 4-county area. We trapped coyotes in 6 townships in Alcona County, 5 in Oscoda County, 2 in Montmorency County, and 2 in Alpena County. Because a large proportion of land in the study area was privately owned (e.g., commercial hunting clubs, agricultural operations, residential development), landowner permission

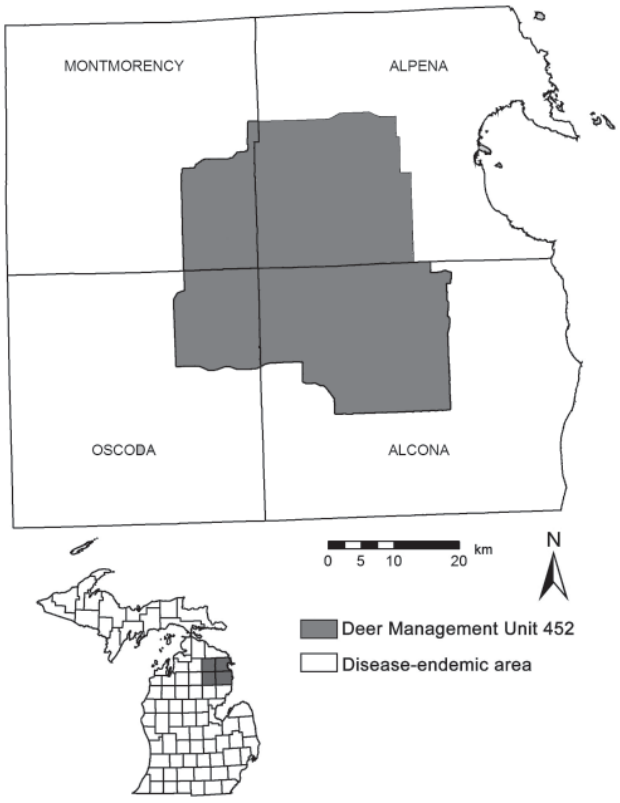

Figure 1. Coyote study area in Montmorency, Alpena, Alcona, and Oscoda Counties in the northeastern Lower Peninsula of Michigan, United States.

to access property dictated trap placement. Thus, we were unable to randomize trapping locations or distribute traps proportionally among counties. Within each township, traps were checked daily, and trapping was terminated when 10 coyotes were collected. Because multiple captures could occur on the final day of trapping, we occasionally collected >10 coyotes/township. We killed trapped coyotes with a 0.22-caliber gunshot to the brain, determined their age on the basis of tooth wear and eruption (*20*), and performed necropsy examinations on them within an hour of death to minimize autolysis. Tissues containing visible lesions as well as the parotid, mandibular, retropharyngeal, bronchial, mediastinal, and mesenteric lymph nodes were collected and submitted in formalin for histologic examination and fresh for mycobacterial culture.

Coyote samples were processed by following protocols used for histologic examination and mycobacterial culture of white-tailed deer samples (*6*,*10*). Fresh tissues for bacterial culture were digested and decontaminated with a sodium-hypochlorite-sodium hydroxide method (*21*). We then spun tissue suspensions in a refrigerated centrifuge at 6,000 × *g* for 20 minutes (*21*). Half of the supernatant was discarded, and the pellet was resuspended and swabbed on the following solid media: Middlebrook 7H10 agar containing sodium pyruvate (National Veterinary Services Laboratory [NVSL], Ames, IA, USA), Middlebrook 7H11 agar containing sodium pyruvate (NVSL), BBL Mycobactosel L-J medium slant (Becton Dickinson, Sparks, NJ, USA) and Middlebrook 7H11 with aspartic acid and pyruvate (Becton Dickinson) (*22*). We then injected the suspension (0.5 mL) into BACTEC 12 B liquid culture vials (Becton Dickinson) and BACTEC MGIT liquid culture tubes (Becton Dickinson) (*21*). The solid media tubes were incubated at 37 ± 2°C in a 10% CO_2 incubator and examined weekly until colonies were observed or until an incubation period of 8 weeks was complete, at which time tubes with no growth were discarded (*21*). We incubated the BACTEC 12 B vials at 37 ± 2°C and monitored them in the BACTEC 460 instrument for 6 weeks (*21*,*22*). We incubated MGIT 960 tubes at 37 ± 2°C and monitored them in the BACTEC MGIT 960 instrument for 6 weeks (*21*–*23*). Colonies from solid media and liquid culture bottles that showed positive signals were confirmed as *M. tuberculosis* complex identification by a combination of Ziehl-Neelsen acid-fast staining and the AccuProbe *M. tuberculosis* complex nucleic acid probes (Gen-Probe, San Diego, CA, USA) (*21*,*23*). We then used niacin and nitrate biochemical tests to distinguish *M. bovis* from *M. tuberculosis* isolates (*21*,*23*).

Formalin-fixed tissues were processed and stained with hematoxylin and eosin. Any granulomatous lesions were then stained with a modified Ziehl-Neelson procedure and an auramine orange and acridine orange procedure (*24*,*25*). When tissues were identified as having granulomatous lesions and acid-fast bacilli, they were further evaluated by PCR. The PCR was performed on the formalin-fixed, paraffin-embedded tissue by using primers for IS*6110* to identify *M. tuberculosis* complex species, which include *M. bovis*, and 16S rRNA to identify *M. avium* complex species. The PCR procedures were similar to those described previously (*22*). Animals were considered positive if bacterial cultures isolated *M. bovis* from fresh tissues and/or fixed tissues had granulomatous lesions with acid-fast bacilli that were PCR positive for IS*6110*. All histologic screenings and PCRs were conducted at NVSL.

We used a log-linear model (*26*) to determine whether the count of *M. bovis*–positive coyotes was independent among age classes and sexes. We used adjusted residuals for describing and making inferences about the true association structure among the response variables. We used a Mann-Whitney test (*27*) to compare prevalence of *M. bovis* in coyotes to white-tailed deer sampled by MDNR during the same period.

Results

We captured and collected tissues from 175 coyotes (91 males, 84 females) in 15 townships (statistical mean 11 coyotes/township, SE = 0.63) within DMU 452 and 14 control coyotes (8 males, 6 females) from Michigan's Upper Peninsula. For coyotes sampled from DMU 452, we were able to classify 101 (51 males, 50 females) as juveniles (<2 years old) and 67 (34 males, 33 females) as adults. Age data were not collected from control coyotes. We identified 58 *M. bovis*–positive coyotes from DMU 452; 16 (28%) positive coyotes were trapped within the boundaries of property owned by 7 private hunt clubs distributed throughout DMU 452. Seven coyotes (5 males, 2 females) whose age could not be determined were negative for *M. bovis*. All control coyotes were negative for *M. bovis*, and they were not included in subsequent analyses or summary statistics. Unweaned pups were not sampled.

Apparent prevalence of *M. bovis* infection did not differ by age ($\chi^2 = 3.16$, degrees of freedom [df] = 1, p = 0.07) or sex ($\chi^2 = 0.05$, df = 1, p = 0.83) class (log linear model; *26*). Percent prevalence of *M. bovis* was highest for coyotes sampled from Alpena County, followed by Alcona, Oscoda, and Montmorency Counties, respectively (Figure 2). Mean prevalence for the 4-county area was estimated at 33% (SE = 0.07; bovine TB–positive coyotes: $n_{Alcona} = 23$, $n_{Oscoda} = 18$, $n_{Alpena} = 10$, $n_{Montmorency} = 7$; Table). During the same period, MDNR identified 57 (1.49%) *M. bovis*–positive deer from a sample of 3,817 killed by hunters within DMU 452, and apparent prevalence was highest in Oscoda County, followed by Alcona, Alpena, and Montmorency Counties (Figure 2) (*18*). Mean apparent prevalence was significantly greater in coyotes than in deer (Mann-Whitney $U_{4,4} = 14$, p<0.001); this overall trend was consistent

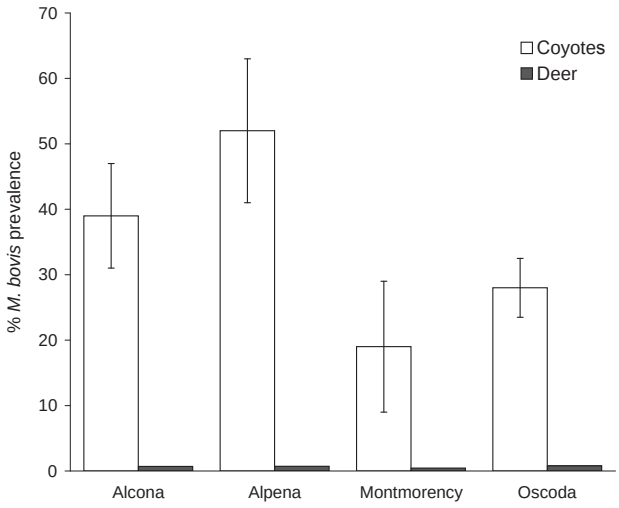

Figure 2. Percent prevalence of *Mycobacterium bovis*–positive coyotes (*Canis latrans*) and white-tailed deer (*Odocoileus virginianus*) in Montmorency, Alpena, Alcona, and Oscoda Counties, Michigan, 2003–2005. Prevalence estimates for white-tailed deer are expressed as a mean calculated from discrete sampling periods conducted in 2003, 2004, and 2005. Error bars for coyote estimates represent the standard error of the mean calculated across townships for each county. Estimates of *M. bovis* prevalence for white-tailed deer were not available for individual townships; standard errors were not calculated for counties.

for all 4 counties. The proportion of *M. bovis*–positive deer sampled from DMU 452 during 2004–2005 fell within 95% confidence limits generated by calculating the proportion of positive deer from 1996 through 2003.

M. bovis (n = 58) was the most common mycobacterium isolated, but *M. avium* complex species (n = 12), *M. intracellulare* (n = 1), and *M. kansasii* (n = 1) were also identified by culture. *M. bovis* was the most common mycobacterial isolate found within the mesenteric lymph nodes. In 31 positive cases in which anatomic location of lymph nodes was identified, 14 animals were positive only in mesenteric lymph nodes, 14 were positive in both mesenteric and combined head and thoracic lymph nodes, and 3 animals were positive only in combined head and thoracic lymph nodes. No coyotes were detected concurrently infected with multiple *Mycobacterium* types.

Lymph node lesions caused by *M. bovis* varied from focal to multifocal and ranged in size from 1 to 15 mm. Frequently, an affected lymph node contained several 1- to 5-mm granulomas. A single animal was found with multiple, large, 1- to 1.5-cm granulomas within the liver, lungs, pleura, and mesenteric lymph nodes. Microscopically, both lesions and the number of acid-fast bacilli within lesions were variable. Most lesions contained occasional acid-fast bacilli with fewer lesions containing numerous acid-fast bacilli. The most common microscopic lesion was a granuloma in the cortex of lymph nodes with large central areas of acellular, eosinophilic debris, with or without basophilic mineralized debris, and numerous cholesterol clefts. Necrotic debris was surrounded by a thin rim of macrophages, epithelioid macrophages, fibrous connective tissue, lymphocytes, only a few neutrophils, and plasma cells. Multinucleated giant cells were infrequent or absent (Figure 3). Less commonly, in some granulomas the central area of necrotic debris was almost entirely mineralized. A second type of lesion found in the cortex of the lymph nodes consisted only of small, poorly delineated aggregates of macrophages and epithelioid macrophages intermixed with low numbers of lymphocytes. In some animals, these small aggregates of macrophages were the only lesions identified (Figure 4).

Discussion

We demonstrated the potential of using coyotes as sentinels to detect *M. bovis* occurrence in an area containing endemically infected white-tailed deer with a prevalence of <2%. By focusing on coyotes rather than deer, we sampled 97% fewer animals and detected a similar number of *M. bovis*–positive animals (i.e., 58 *M. bovis*–positive coyotes; 57 *M. bovis*–positive deer), which increased detection of *M. bovis* by 40%. Smaller samples mean less expense associated with laboratory testing. Moreover, smaller samples can result in shorter times between end of sampling and disease confirmation and therefore can increase opportunities for rapid disease management response.

Early in the study, we discovered the importance of collecting diagnostic samples as soon as possible after death. Rapid autolysis of the gastrointestinal tract and associated mesenteric nodes quickly minimizes the utility of these tissues for histologic and microbiologic examination. Delays between time of euthanasia and tissue collection reduced the ability to identify lesions and associated acid-fast organisms as well as to propagate the organism in culture and consequently lower the apparent incidence of disease. Related to this, because MDNR only submitted diagnostic samples from deer with visible lesions and because samples collected from deer were not taken as quickly after death as those from coyotes, the prevalence rates in deer may have been underestimated.

Table. Number of coyotes sampled and determined to be *Mycobacterium bovis* positive,* 4 counties, Michigan, USA, 2003–2005

	No. sampled (no. positive)			
County	Adult M	Adult F	Juvenile M	Juvenile F
Montmorency	6 (1)	8 (2)	6 (3)	5 (1)
Alpena	5 (2)	4 (2)	7 (5)	6 (1)
Alcona	11 (5)	12 (5)	16 (5)	20 (8)
Oscoda	12 (7)	9 (4)	23 (6)	18 (1)

*Determined by histologic examination and mycobacterial culture, followed by PCR for strain identification.

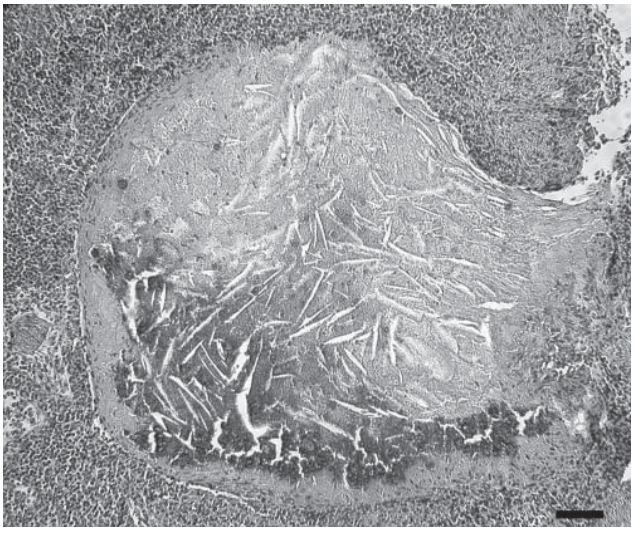

Figure 3. Granulomatous lymphadenitis caused by *Mycobacterium bovis* in a coyote (*Canis latrans*). The granulomas consist of a large central necrotic area with mineralization and cholesterol clefts surrounded by a thin rim of primarily macrophages and fibrous connective tissue. Scale bar = 55 μm.

Also, infection of coyotes was independent of age groups and sex, which suggested that our sampling design did not bias detection of *M. bovis* occurrence relative to coyote demographic characteristics. This finding is critically important as to whether focal species are considered effectual disease sentinels (*28*) because age- or sex-biased dispersal can severely confound attempts to correlate the spatial distribution of disease occurrence between the sentinel and host. Capture biases in wildlife studies can be a legitimate concern, particularly where complex social behavior, such as agonism, can differentially influence the vulnerability of animals to various methods of capture. Our decision to collect coyotes exclusively by means of foot-hold traps, rather than hunting with dogs or with predator calls (the methods preferred by sport hunters), should have minimized sampling bias: socially dominant individual animals are potentially more susceptible to predator calls (*29*). Furthermore, standardizing sampling effort to a single trapping period with a goal of 10 animals/transect should have ensured that the animals that were captured, and their disease status, were representative of the at-large population (*30*).

Additional bias could accrue if infirmity influenced the probability of capture, thereby resulting in over- or underestimates of apparent prevalence (*31*–*33*). However, TB is a chronic infection, and animals usually survive in relatively good condition until severe clinical symptoms, such as extreme malaise (*8*), appear at the penultimate stage of disease (*9*). Because of this, there is a relatively short temporal frame (≈2 weeks) between the onset of moribund condition and death (*9*) when capture probabilities may be biased by disease status. We found no evidence of physical debilitation positively or negatively influencing capture probability. Of 58 *M. bovis*–positive coyotes captured, none showed symptoms of severe emaciation or lethargy suggestive of advanced disease, and only 1 coyote bore widely disseminated lesions visible on gross inspection during necropsy. Thus, we believe our trap-transect method of sampling coyotes was robust to potential bias associated with coyote disease status. Because the animals were euthanized upon capture, our work was not replicable. Therefore, we could not use a design based on mark-recapture to determine if, in fact, our sampling protocol produced stable, increasing, or diminishing prevalence estimates over successive trapping sessions.

It appears that for coyotes infected with *M. bovis*, lesions predominantly localized to the lymphoid tissue of the gastrointestinal tract, although lesions concurrently developed in lymph nodes of the head in 16 coyotes. Lesions ranged from acute to chronic; marked fibrosis and few acid-fast organisms were noted in the chronic lesions. Only 1 animal had evidence of advanced disease, as evidenced by lesions in the lung and liver, which may have been caused by a large infectious dose, a compromised immune system, or long-term infection. The spectrum and locations of lesions led us to postulate that coyotes may acquire *M. bovis* orally and have the immunologic ability to minimize and possibly eliminate the bacteria. Our study was not designed to determine route of transmission or whether coyotes were a maintenance reservoir for *M. bovis*. However, preliminary results of current research indicate that excretion of *M. bovis* by coyotes experimentally inoculated with oral doses (ranging from 10 to 10^5 CFU) is probably unlikely or undetectable (M. Dunbar, National Wildlife Research Center, pers. comm.). If excretion of *M.*

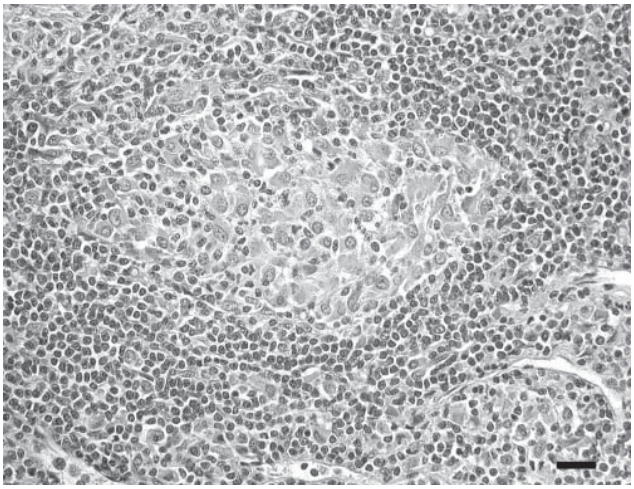

Figure 4. Focal histiocytic lymphadenitis caused by *Mycobacterium bovis* in a coyote (*Canis latrans*). Note the small, poorly delineated, aggregates of primarily macrophages within the lymph node cortex. Scale bar = 25 μm.

bovis is not likely in orally inoculated coyotes, then it is not likely to result in widespread infection among coyotes that would have become infected by ingesting infected tissue. Moreover, the absence of *M. bovis* in control coyotes sampled from the Upper Peninsula, where bovine TB has not been detected in white-tailed deer or cattle (*12*), lends further credence to the belief that coyotes are spillover rather than maintenance hosts.

For agrarian areas where livestock operations predominate, regular testing of domestic animals and slaughter of reactors can effectively prevent the long-term maintenance of *M. bovis* within localized livestock (*34*). However, in areas where livestock densities are low, *M. bovis* prevalence in wildlife must be surveyed directly (*13*). The disparity in prevalence relative to sampling effort between coyotes and deer is strong evidence that coyotes could be useful for monitoring *M. bovis* occurrence in Michigan (*4*). Coyotes in Michigan generally have larger home ranges than deer (coyotes, statistical mean 14.25 km^2; white-tailed deer, statistical mean 2.11 km^2; *17*,*35*) and appear to have a much higher per capita probability of developing detectable infection. However, because of discrepant home-range sizes, attempts to spatially correlate sources of infection for coyotes, sympatric wildlife, and domestic livestock will be confounded by spatial scale. Thus, some question about the source of infection in coyotes will always remain; the presence of an infected coyote can only provide a broad indication of the location of the original source of infection. Although we noted that 44% of all *M. bovis*–positive coyotes were trapped within the boundaries of private hunt clubs, we cannot infer that coyotes acquired the pathogen within club boundaries. The only way to circumvent this inferential deficit is to gather spatial information on a large sample of animals before killing them to determine their infection status (*13*) and then to develop probabilistic resource selection models (*36*).

As with other tools (e.g., radio transmitters, global positioning systems) and techniques (e.g., telemetry, population estimation), the sentinel species concept may not be applicable in some instances. For example, others have followed our lead to investigate the feasibility of using coyotes as sentinels for *M. bovis* in Manitoba, Canada, without documenting *M. bovis* in coyotes (*37*). Their results could have occurred because prevalence rates in cervids were so low that they were not detected, given the number of coyotes sampled; coyotes are not the appropriate sentinel species; or both. Just as it is useful to determine why coyotes can function well as sentinels in Michigan, it is valuable to point out why the same does not appear so in Manitoba. We concur with the authors of the Manitoba study (*37*) that their negative results could be due to 1) the fact that it was unknown if trapped coyote ranges overlapped cervid ranges (much less if they overlapped the ranges of potentially infected cervids), 2) too low coyote sample size relative to prevalence rate in cervids, and 3) coyotes not being likely to prey on elk (*Cervus elaphus*); if they scavenge kills of other predators (wolves [*Canis lupus*], black bears [*Ursus americanus*]; which may be appropriate sentinels in Manitoba), infected tissues are likely no longer present (*38*). Other reasons for their negative results could include the following: 1) ranges and diets of coyotes in the area were unknown, 2) the prevalence rate for cervids during the life of most coyotes collected was unknown and likely very low (<0.1%), and 3) if sample quality from carcasses salvaged from trappers or collected opportunistically was compromised, it could negatively affect the ability to detect *M. bovis*.

The potential benefits of using coyotes as sentinels for *M. bovis* occurrence ultimately relate to increased sampling efficiency and disease detection. Our work shows that coyotes are sensitive indicators of disease presence in Michigan. The collection protocol we designed to sample coyotes ensured the likelihood that sampled individuals were representative of the population and estimates of disease prevalence were relatively bias-free. Sentinel coyote surveys appear to be effectual cost- and labor-sensitive indicators of *M. bovis* presence in sympatric wildlife and domestic livestock. We concur with others (*1*,*28*) who endorse the use of sentinel-based surveillance programs, particularly when project goals include monitoring spatiotemporal changes in disease risk. In addition, we believe sentinel-based programs could facilitate adaptive monitoring of disease occurrence where the likelihood of horizontal transmission is great and/or spatial epidemiology is uncertain. From another perspective (*39*), we also believe that wildlife can serve as effective biologic sensors and satellites of some infectious disease epidemics and bioterrorism that threaten human health and safety.

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African Swine Fever Virus Isolate, Georgia, 2007

Rebecca J. Rowlands, Vincent Michaud, Livio Heath, Geoff Hutchings, Chris Oura, Wilna Vosloo, Rahana Dwarka, Tinatin Onashvili, Emmanuel Albina, and Linda K. Dixon

African swine fever (ASF) is widespread in Africa but is rarely introduced to other continents. In June 2007, ASF was confirmed in the Caucasus region of Georgia, and it has since spread to neighboring countries. DNA fragments amplified from the genome of the isolates from domestic pigs in Georgia in 2007 were sequenced and compared with other ASF virus (ASFV) isolates to establish the genotype of the virus. Sequences were obtained from 4 genome regions, including part of the gene *B646L* that encodes the p72 capsid protein, the complete *E183L* and *CP204L* genes, which encode the p54 and p30 proteins and the variable region of the *B602L* gene. Analysis of these sequences indicated that the Georgia 2007 isolate is closely related to isolates belonging to genotype II, which is circulating in Mozambique, Madagascar, and Zambia. One possibility for the spread of disease to Georgia is that pigs were fed ASFV-contaminated pork brought in on ships and, subsequently, the disease was disseminated throughout the region.

African swine fever (ASF), classified as a notifiable disease by the World Organisation for Animal Health (OIE), causes an acute hemorrhagic fever in domestic pigs. It often results in major economic losses because of the high rates of illness and death associated with the disease. ASF has the potential to spread rapidly and since a vaccine is currently not available, control options are limited to rapid diagnosis of the disease and culling of infected animals and animals in contact with them.

ASF virus (ASFV) infects wildlife hosts and ticks of *Ornithodoros* spp., and these can provide a reservoir of virus that is not possible to eliminate. In Africa, where ASF is widespread, the virus causes long-term, persistent infections—but no clinical manifestation of disease—in warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus porcus*) (*1*). In contrast, ASFV causes clinical disease and high numbers of deaths in wild boars (*Sus scrofa*). The disease has been reported in pigs from most sub-Saharan countries and continues to spread to previously uninfected countries within the region. In 1998, ASF was reported in Madagascar for the first time; it is now considered to be endemic. At the end of 2007, ASF was introduced on a second Indian Ocean island, Mauritius (*2*).

Once introduced into countries, ASF is difficult to eradicate for several reasons, including the presence of wildlife reservoirs, lack of a vaccine, insufficient laboratory support for rapid and accurate diagnosis, and inadequate funding for veterinary services to enforce the appropriate control measures. This situation was amply demonstrated in Portugal and Spain, where the disease remained endemic until the 1990s, after its introduction into Portugal in 1957 and again in 1960. Other European countries, the Caribbean, and Brazil have had outbreaks of ASF, but extensive control programs have led to successful eradication, with the exception of Sardinia, where ASF has remained endemic since 1982 (*1*).

The genetic characterization of the viral strain associated with disease outbreaks is important for tracing possible sources of infection and ensuring that appropriate diagnostic reagents are used. ASFV isolates have previously been characterized by restriction enzyme site mapping or sequencing of different genome regions. Partial sequencing

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of the *B646L* gene encoding the major capsid protein p72 has so far led to the identification of 22 ASFV genotypes. Twenty-one of these genotypes were identified in isolates from domestic pigs or from wildlife hosts in eastern and southern Africa. The level of diversity between isolates from these regions is attributed to the long-term evolution of virus within wildlife hosts. In contrast to the other genotypes, genotype I predominantly comprises isolates from domestic pigs in West and Central Africa, Europe, the Caribbean, and Brazil obtained during a 40-year period since 1957. Isolates belonging to genotype I share considerably higher sequence identity across the p72 gene compared to isolates from the sylvatic cycle, which suggests that this genotype probably evolved from a single source introduction (*3*–*5*). PCR amplification and sequencing of more variable genome regions have been used to distinguish between closely related isolates and identify virus subgroups within several of the 22 genotypes (*4*). The additional genome regions that have been described thus far include the *E183L* and *CP204L* gene regions, encoding the p54 and p30 proteins, respectively, as well as the central variable region within the open reading frame (ORF) *B602L*.

ASFV can infect pigs by a variety of mechanisms, including direct contact between pigs, bites from infected ticks, indirect transmission by means of fomites, and ingestion of infected meat. The main route by which ASF infections spread over long distances is thought to be infected meat products. The transcontinental spread of ASF has been a relatively rare event, and it was unexpected when, in June 2007, cases of ASF affecting domestic pigs in the Caucasus region of the former Soviet republic of Georgia were confirmed by the OIE ASF reference laboratory (*6*). It has been suggested that the outbreak started in April 2007 near the Black Sea Port of Poti. Catering waste, including infected pig meat from ships in the port, is considered to be the most likely source of the infection. As of July 9, 2007, the outbreak had spread to 56 of 61 districts in Georgia. Reports to OIE indicated that >80,000 pigs had died or been destroyed in Georgia. Outbreaks of ASF were also reported in neighboring regions, including the autonomous republic of Abkhazia (*7*). On August 29, 2007, ASF was confirmed in Armenia and on November 4, 2007 (*8*), in Nagorno-Karabakh (*9*), a de facto independent republic that is officially part of Azerbaijan and near its border with Armenia. On November 5, 2007, infection of a wild boar was confirmed in the Russian Republic of Chechnya near the border with Georgia. To control the spread of disease, wild boars were killed in 17 different regions in Chechnya, and the slaughter of the entire pig population was ordered (*10*). Further outbreaks of ASF were reported in Nagorno-Karabakh in April 2008, where it is believed that ≈8,500 pigs have died as a result of disease since the beginning of the outbreaks.

Here we describe the genetic characterization of the ASFV isolates implicated in the 2007 outbreak of the disease in Georgia. Results of the analysis showed that the Georgia isolates group within genotype II, which suggests that the virus is closely related to ASFV isolates typically found in Mozambique, Madagascar, and Zambia (*4*,*11*,*12*).

Materials and Methods

Virus Isolates

In June 2007, samples were collected from 2 pigs that were showing clinical signs of ASF. The first pig originated from the Imereti Province in western Georgia; the second was sampled in the Kakheti Province in eastern Georgia. Five tissues samples were collected from each pig, including serum and samples from the kidney, spleen, lung, and lymph nodes. The samples were subsequently submitted to the OIE reference laboratory, Institute for Animal Health, Pirbright, United Kingdom. The presence of ASFV in these samples was confirmed by pathogen isolation on primary leukocyte cultures, real-time PCR, and ELISA (*13*,*14*).

Viral DNA Extraction, PCR Amplification, and Sequencing

Viral DNA was extracted directly from cell culture isolates or from suspensions of clinical samples by using the High Pure Viral Nucleic Acid Kit (Roche, Indianapolis, IN, USA) following the manufacturer's guidelines. The extracted DNA was used as template for the amplification of the respective gene regions. Details of isolates studied are shown in the online Appendix Table (available from www.cdc.gov/EID/content/14/12/1870-appT.htm).

PCRs were performed with the Accuprime *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Reactions contained 22.5 μL Accuprime *Pfx* Supermix, 100 ng DNA, and a final concentration of 200 nmol/L of each primer in a total reaction volume of 25 μL. Thermocycling condition included a 2-min denaturation step of 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 68°C with a 10-min elongation step at 68°C. Part of the gene encoding the p72 gene was amplified by using the primers P72-D and P72-U (*3*), which amplify a 478-bp fragment from the 3′ end of the *B646L* gene. The primer pair ORF9L-F (5′-AATGCGCTCAGGATCTGTTAAATCGG-3′) and ORF9L-R (5′-TCTTCATGCTCAAAGTGCGTATACCT-3′) was used to amplify a region from the central variable genome within the ORF B602L (*16*); E183L-F (5′-TCACCGAAGTGCATGTAATAAACG-3′) and E183L-R (5′-TCTGTAATTTCATTGCGGCCACAACATT-3′) were used to amplify a 681-bp fragment of the *E183L* gene. Primer pairs p30-F (5′-ATGAAAATGG AGGTCATCTTCAAAAC-3′) and p30-R (5-AAGTT

TAATGACCATGAGTCTTACC-3′) were used to amplify 521 bp of the *CP204L* gene.

Primers used for the amplification of p72, p54, p30, and B602L gene regions, as described above, were used in the respective sequencing reactions. Sequencing of PCR products was performed by using the Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). Thermocycling consisted of 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 3 min. Completed reactions were processed following the manufacturer's instructions. Data was processed by using the default sequence analysis parameters and analyzed with Beckman Coulter CEQ 8000 software.

Sequence Analysis

Analysis of sequence data was performed with Beckman Coulter CEQ8000 software, Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and ClustalX version 1.83 (www.clustal.org). A summary of the sequences is shown in the online Appendix Table.

Phylogenetic analysis was conducted by means of the "criterion of neighborhood based on the principle of parsimony" (www.megasoftware.net/index.html; *17*,*18*), selecting the correction of Kimura (*19*). Bootstrap confidence values were calculated on 1,000 replicates according to the maximum likelihood approach of Felsenstein (*20*).

Results

Partial Sequence of *B646L* Gene Encoding the p72 Capsid Protein

Sequence analysis of the *B646L* gene has been used extensively for phylogenetic analysis of ASFV isolates (*3*,*5*,*15*) by focusing on a 478-bp fragment corresponding to the C-terminal end of the *B646L* gene that broadly defines the virus genotypes. Twenty-two genotypes (*4*) have thus far been identified by analyzing this region of the viral genome.

The *B646L* partial sequences from each of the 5 tissue samples from the east and west Georgian samples showed that they were identical at the nucleotide level (results not shown). Comparison of these sequences to other isolates of known genotypes identified the Georgia 2007 sequence as falling within *B646L* genotype II (Figure), together with 1 isolate from Zambia (Lus 1/93), isolated from a domestic pig after an outbreak of ASF in 1991 (*10*); 9 from Mozambique (Moz 60–98, Moz 61–98, Moz 63–98, Moz 70–98, Moz 77–98, Moz 1/02, Moz 2/02, Moz 1/03, and Moz 1/05), obtained from outbreaks in 1998–2005 (*5*,*11*,*12*); and a pig isolate from Madagascar (Mad 1/98), obtained after the first introduction in 1998 (*3*,*21*).

Sequence Analysis of *B602L* Region

The central variable region of the ORF *B602L* is characterized by tetrameric repeats, the number and composition which can be used to distinguish between closely related isolates (*16*). Sequence analysis of this region from the *B602L* gene (also designated central variable region ORF9L, 9RL) of >100 ASFV isolates has shown that the number of tandem repeat tetramers in individual genomes may vary from 7 to 34. Twenty-two sequence variants of the 4-aa repeats have also been identified (*15*).

Amplification of the *B602L* variable fragment from each of the east and western Georgian isolates yielded PCR products of ≈200 bp, which corresponded in size and sequence to the other genotype II isolates with 10-aa tetramers. The sequences of this region differed from that of all other genotypes (online Appendix Figure 1, available from www.cdc.gov/EID/content/14/12/1870-appF1.htm). Despite also containing 10 copies of amino acid tetramers, the *B602L* sequence of 2 South African isolates from genotype XXI differed from Georgia 2007 and the other genotype II isolates.

Sequence Analysis of *E183L* Gene Encoding Protein p54

Amplification of the fragment containing the complete *E183L* gene from all the Georgian isolates produced PCR products of ≈550 bp, which were identical in sequence

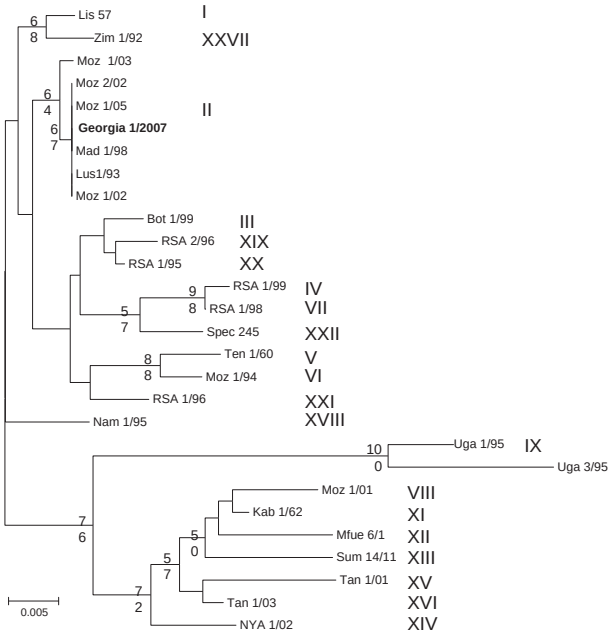

Figure. Phylogram depicting the *B646L* gene relationships of selected isolates representative of the 22 African swine fever virus genotypes. Because all the Georgian isolates had identical nucleotide sequences, only 1 isolate is presented in the tree (in **boldface**). The consensus tree was generated from 1,000 replicates; only bootstraps >50% are shown. Genotypes are indicated in roman numerals. Moz, Mozambique. Scale bar indicates number of nucleotide substitutions per site.

(results not shown). Amplification of this fragment from other isolates from Africa, Europe, and Madagascar produced fragments that ranged from 528 bp to 600 bp. The discrepancy in the size of the respective fragments is due to sequences encoding 2 arrays of amino acid repeats, which vary in number and sequence. The nucleotide sequence of the *E183L* gene from the Georgia 2007 isolate was identical to sequences from 5 Madagascar isolates obtained from outbreaks that in 1998–2003 (Mad 1/98, Ampani/99, Tolagna/99, Chrome/01, and Antani/03) and 2 Mozambican isolates (Moz 1/02, Moz 2/02) (online Appendix Figure 2, available from www.cdc.gov/EID/content/14/12/1870-appF2.htm). However, the p54 nt and protein sequences of isolates Moz 1/03, Moz 1/05, and Lus 1/93 differed from that of the Georgia 2007 isolates; the latter contained a single deletion between positions 341 and 355, resulting in a 5-aa deletion within the central portion of the protein. The nucleotide sequence of the 2 other isolates from Mozambique obtained in 2003 and 2005 (Moz 1/03 and Moz 1/05) were identical to each other but differed from that of the Georgia 2007 isolates at several positions throughout the gene region (online Appendix Figure 2).

Sequence Analysis of *CP204L* Gene Encoding p30 Protein

Amplification of a fragment containing the *CP204L* gene from each of 2 Georgian isolates produced a PCR product of ≈550 bp. As was the case in all the other gene regions, the sequence of the 2 Georgian isolates was identical across the length of the gene (results not shown). The nucleotide sequences of the Georgia isolates were unique within genotype II but shared a high degree of similarity with the isolates from Madagascar, Zambia, and Moz 2/02 (nucleotide identity >99%). In contrast, isolates implicated in the most recent outbreaks of the disease in Mozambique, isolates Moz 1/03 and Moz 1/05, differed from the Georgian isolates by >2.5% at the nucleotide level (online Appendix Figure 3, available from www.cdc.gov/EID/content/14/12/1870-appF3.htm).

Discussion

We analyzed the sequence of 4 genomic fragments of the ASFV genome to characterize the viruses responsible for the outbreak of ASF in Georgia in 2007. The 4 regions of the genome—*B646L, E183L, CP204L,* and the variable region within the ORF *B602L*—were amplified by PCR. The nucleotide and amino acid sequences of these ORFs from samples collected at 2 different geographic locations in Georgia were then compared with ASFV isolates from other regions of the world. Because all DNA and amino acid sequences for each genome region from all tissue samples obtained from Georgia that we tested were identical, we concluded that the ASF outbreaks in Georgia and the surrounding regions were probably due to a single introduction of the virus. Sequence analysis of the p72 gene region placed the Georgian isolate within genotype II together with isolates from Madagascar, Mozambique, and Zambia (*3*–*5*). Genotype II occurred in Mozambique in outbreaks in 1998–2005 (*12*) and affected the northeastern provinces of Cabo Delgado and Nampula (which were most recently affected in 2004), the northwestern province of Tete, and the southern province of Maputo (most recently in 2005) (*12*). Three other genotypes of ASFV have also been identified as having occurred in Mozambique—genotypes II, V, and VI (*12*).

The genotype II Madagascar isolate, MAD 1/98, was obtained from a domestic pig in 1998 during the first outbreak of ASF that affected the island country. The more recent Madagascar pig isolates obtained in 1999–2003 are presumed to have derived from this first introduction because they belong to the same genotype. Mozambique has been speculated to be the most likely source of infection for the 1998 ASFV outbreaks occurring in Madagascar because the isolates from Mozambique were genotype II and identical across the *B602L* region (*22*). Before 1998, the island of Madagascar was free of the disease (*21*,*22*). The genotype II isolate from Zambia (Lus 1/93) was isolated from an infected domestic pig in 1991, whereas the viruses from Mozambique were isolated from domestic pigs during outbreaks of the disease along the eastern coast of the country in 2002–2005.

Further sequence analysis was performed on 2 other conserved regions of the ASFV genome; the ORFs *E183L* and *CP204L*, which encode the structural proteins p54 and p30, respectively. Analysis of the *E183L* gene showed that the Georgia 2007 isolates were most closely related to 4 isolates from Madagascar, which were in circulation in 1999–2003, and 2 isolates from Mozambique but distinguishable from the group II isolate Lus 1/93 and the Mozambique isolates Moz 1/03 and Moz 1/05. Similarly, analysis of the *CP204L* gene encoding p30 showed the Georgia 2007 isolates were distinguishable from all other isolates, although they were most closely related to the 4 isolates from Madagascar in circulation in 1999–2003, the Zambian Lus 1/93 isolate, and one of the Mozambique isolates (2/02).

Fragment size analysis has identified *B602L* as the most variable genome region (*15*). The variable region of *B602L* contains amino acid tetramers that vary in number and type. Sequence analysis of the *B602L* gene from the Georgian isolates identified 5 different amino acid tetramer sequences encoded in this genome region. One of these tetramer sequences was CTST, which is one of the less common tetramer sequences (*15*). The sequence of the *B602L* variable region from the Georgian isolate grouped it with isolates in circulation in Madagascar (1999–2003) as well as isolates from Mozambique from outbreaks in 1960, 1961, 1963, 1970, and 1998.

The first case of ASF in Georgia was observed in the Samegrelo region on the west coast, which suggests a possible connection to the port of Poti on the Black Sea. One possibility is that that the virus entered Georgia through meat products since ASFV may remain viable for long periods in infected pig tissues, meat, and processed pig products. Most pigs in Georgia are kept on a free-ranging, scavenging system, and so access to or swill feeding of dumped port waste is possible. However, several events would be required to cause an outbreak, making this a relatively rare event and providing an explanation for the relatively few incidents of transcontinental spread of ASFV. Our analysis showed that the Georgia strain is most similar to isolates from Madagascar. However, since few ASFV samples are submitted for genotyping, it is possible that viruses belonging to genotype II may be more widespread. However, it seems likely that the source of infection of the Georgia 2007 outbreak is from the eastern side of southern Africa or Madagascar rather than west or central Africa or Sardinia.

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Dr Rowlands studied for her PhD at the Institute for Animal Health Pirbright Laboratory on the interaction of ASFV with the tick vector *Ornithodoros erraticus* and on the molecular epidemiology of ASFV. She is currently conducting postdoctoral work on the function of ASFV genes involved in inhibiting interferon responses.

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Clinical Characteristics and Molecular Subtyping of *Vibrio vulnificus* Illnesses, Israel

Ronit Zaidenstein, Chantal Sadik, Larisa Lerner, Lea Valinsky, June Kopelowitz, Ruth Yishai, Vered Agmon, Michele Parsons, Cheryl Bopp, and Miriam Weinberger

CME ACTIVITY

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Learning Objectives

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

- List the predisposing factors for infections caused by *Vibrio vulnificus* biotypes 1 and 2.
- Identify the differences between infections caused by *V. vulnificus* biotypes 1 and 2 and biotype 3.
- Describe the types of fish associated with *V. vulnificus* biotype 3 infection.
- Describe the mortality associated with *V. vulnificus* biotype 3 infection.
- List the predictors of mortality in *V. vulnificus* biotype 3 infection.

Editor

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During 1996–1997, a new *Vibrio vulnificus* biotype 3, which caused severe soft tissue infection after fishbone injury, emerged in Israel. We conducted a follow-up study from 1998 through 2005 to assess changing trends, outcomes, and molecular relatedness of the implicated strains. A total of 132 cases (71% confirmed and 29% suspected) of *V. vulnificus* biotype 3 infection were found. Most infections (95%) were related to percutaneous fish exposure, mainly tilapia (83%) or common carp (13%). Bacteremia, altered immune status, and history of ischemic heart disease were identified as independent risk factors for death, which reached a prevalence of 7.6%. Pulsed-field gel electrophoresis patterns of strains from 1998 through 2005 and from 1996 through 1997 showed a high degree of homogeneity and were distinct from those of *V. vulnificus* biotype 1. Infections caused by *V. vulnificus* biotype 3 continue to affect the public's health in Israel.

Vibrio vulnificus, a gram-negative bacterium of the family *Vibrionaceae*, is a worldwide inhabitant of salt water (*1*–*3*). *V. vulnificus* biotypes 1 and 2 are capable of causing severe human infection, including necrotizing fasciitis and septicemia; the death rate is substantial (*4*–*6*). Persons with chronic liver disease, particularly liver cirrhosis, are more prone to developing infection and at greatest risk for an adverse outcome (*7*,*8*). Other predisposing factors are iron overload and hemochromatosis and immunosuppression caused by steroid treatment, malignancy, HIV infection, renal failure, and organ transplantation (*1*,*9*,*10*).

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During 1996–1997, a new biotype, *V. vulnificus* biotype 3, emerged as a cause of severe soft tissue infection and bacteremia in Israel (*11*,*12*). Several important features differentiate the illness caused by the new *V. vulnificus* biotype from previously described *V. vulnificus* infections. First, a new vector, a pond fish (tilapia) grown in fresh water, has been associated with *V. vulnificus* infection. Second, infection is caused by direct injury from the fish backbone while purchasing, cleaning, or handling the live fish, as opposed to contamination of a prior injury by immersion in seawater or ingestion of contaminated seafood. Bacteriologically, the new *V. vulnificus* biotype differs from other *V. vulnificus* strains by its biochemical features (salicin-, cellobiose-, citrate-, and lactose-negative, plus delayed reaction for o-nitrophenyl-β-D-galactopyranoside [ONPG]). These biochemical differences initially prevented correct identification of the strain by routine laboratory methods (*11*,*12*). Furthermore, molecular analyses using several methods have shown that *V. vulnificus* biotype 3 is genetically distinct from biotypes 1 and 2 (*11*–*15*). Bisharat et al. suggested that biotype 3 is a recombinant clone that may have emerged as a result of hybridization of 2 *V. vulnificus* populations (*14*). Currently, biotype 3 is geographically restricted to Israel; biotypes 1 and 2 have a worldwide distribution (*16*).

The 1996–1997 Israeli cluster involved 62 persons, with a slight male predominance (58%) and a median age of 56 years. Although no deaths were reported, 41 persons (66%) had conditions that required surgical debridement, 1 had total limb amputation, and 7 had finger amputations (*11*). A new, aggressive, live-fish marketing initiative in the northern part of Israel was implicated in the outbreak, and the outbreak was followed by new instructions from the Israeli Ministry of Health that recommended selling only precleaned, ice-chilled tilapia (*17*).

We studied the epidemiology and the trends in illnesses associated with the new *V. vulnificus* biotype 3 during a 7-year period (1998–2005) following the initial 1996–1997 cluster. Our study assesses the effects of infection, risk factors for death, possible spread to other fish species, and molecular relatedness of the *V. vulnificus* biotype 3 strain.

Methods

Clinical Data

Clinical data were obtained from the records of the Infectious Diseases Department, Epidemiology Unit, Israel Ministry of Health, Jerusalem. Subdistrict health offices reported data collected from persons with *V. vulnificus* infections; a standardized questionnaire was used. Investigation was initiated after passive reporting from primary physicians who treated patients with suspected *V. vulnificus* infection or when clinical isolates were positively identified as *V. vulnificus* biotype 3 by the Vibrio Reference Laboratory in the Government Central Laboratories, Israel Ministry of Health, Jerusalem. The data collected were patient's age, sex, and place of residence; underlying diseases; circumstances of exposure and type of fish involved; time lapse from exposure to seeking treatment in the emergency department; site of infection; length of hospitalization; clinical symptoms; source of isolation; antimicrobial drug treatment; and outcome.

Cases were classified as laboratory-confirmed when a patient with suggestive history had *V. vulnificus* or *V. vulnificus* biotype 3 isolated from blood or soft tissue or as suspected when a patient had suggestive history without positive cultures. Suggestive history was considered to be the development of soft tissue infection or septicemia after recent (within 7 days) fish exposure or immersion in a water pool.

Source of Isolates and Laboratory Diagnosis

Initial identification of *V. vulnificus* was performed in the microbiology laboratories of hospitals where infected patients had been admitted, using the API 20E strip (bioMérieux, Marcy-l'Etoile, France). The laboratories submitted the isolates for confirmation and further identification to the Vibrio Reference Laboratory, Government Central Laboratories in Jerusalem. Identification of *V. vulnificus* biotype 3 was performed solely in this laboratory by using biochemical tests (failure to ferment citrate, lactose, salicin, cellobiose, and a negative test result for ONPG).

Random isolates were also submitted for molecular analysis of the cytotoxin-hemolysin gene. This test was developed in the Vibrio Reference Laboratory and can differentiate between *V. vulnificus* biotype 3 and biotypes 1 and 2 by demonstrating the unique restriction fragment length polymorphism (RFLP) patterns of biotype 3 (Figure 1). Briefly, the *V. vulnificus* cytotoxin-hemolysin gene (*vvh*A) is amplified from crude bacteria lysate (boiling a loop full of bacteria suspended in 100 μL Tris-EDTA buffer for 10 min) using primers with the sequences RRCTH 5′-CAGCTCCAGCCGTTAACCGAACCACCCGC-3′ and LCTH 5′-TTCCAACTTCAAACCGAACTATGAC-3′. This step is followed by RFLP analysis. For the enzymatic restriction reaction, 2 μL of the PCR-amplified DNA was added to a reaction mixture to give a final volume of 20 μL, according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA), in 2 separate reactions. The restricted DNA was separated by electrophoresis in a 2% gel that was stained with ethidium bromide and visualized for the specific biotype 3 restriction patterns. Restriction sites for 2 enzymes, *Kpn*I and *Pst*I, existed in the sequence of *vvh*A of *V. vulnificus* biotype 3 but not in the corresponding *vvh*A gene of biotypes 1 and 2 (Figure 1).

Molecular Subtyping

Twenty randomly selected laboratory-confirmed isolates of *V. vulnificus* biotype 3 from study years (1998–2003) plus 4 retrospective isolates from 1997 were sent to Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, for molecular subtyping. The isolates were recovered from the blood or wound sites of patients with a history of exposure to various fish. An additional isolate was recovered from the remnants of a tilapia fish found in the refrigerator of 1 of the infected patients (the fish remnants were diced and inoculated onto *Vibrio*-selective agar with and without enrichment). These 25 isolates were available for further molecular studies. Eight submitted human *V. vulnificus* biotype 1 isolates stored at CDC were used for comparison.

V. vulnificus isolates were subtyped by pulsed-field gel electrophoresis (PFGE) in accordance with the PulseNet protocol for *V. cholerae* (*18*) using *Sfi*I for primary enzyme digestion with 1 modification: thiourea (Sigma-Aldrich, St. Louis, MO, USA) was routinely added to the electrophoresis running buffer at a final concentration of 50 μmol/L (*19*) to prevent DNA degradation, which was commonly found during the initial runs. All DNA fingerprints were captured using a Gel Doc EQ system (Bio-Rad, Hercules, CA, USA). The PFGE fingerprints were analyzed in BioNumerics, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Gels were normalized by aligning the bands of the PulseNet universal standard *Salmonella enterica* serotype Braenderup (H9812) placed in every fifth lane on the gels (*20*). Dendrograms were made of the similarities of the DNA fingerprints by using the Dice similarity coefficient and unweighted pair group method with averages (unweighted pair group method with arithmetic mean) clustering. An optimization of 1.5% and tolerance window of 1.5% were used. The 25 isolates sent to CDC as described above were submitted for susceptibility tests using the E-test method according to Clinical Laboratory Standards Institute standards for *Enterobacteriaceae* (*21*).

Statistical Methods

Proportions were compared by using the Fisher exact test or the χ^2 test, and continuous variables were compared by using the Kruskal-Wallis test. Variables associated with death at the 0.05 significance level were entered into a stepwise forward logistic regression model for mortality rate. Analyses were performed by using SPSS version 15 software (SPSS, Inc., Chicago, IL, USA).

Results

A total of 134 cases of *V. vulnificus* infection were identified during the 8-year study period from 1998 through 2005 (Figure 2). Most cases (96, 71.6%) were laboratory-confirmed; 70 (52%) were submitted to the Vibrio Reference Laboratory and identified as *V. vulnificus* biotype 3. PCR-RFLP analysis performed on 34 of these 70 isolates (49%) showed the unique *Kpn*I, *Pst*I pattern. Two patients with laboratory-confirmed *V. vulnificus* biotype 3 infection were excluded from further analyses because clinical data were missing. The median age of the remaining 132 patients was 66 years (mean 58.9 years, range 10–93 years) (Figure 3). Overall, infection rates for women were only slightly higher than those for men (1.1:1); this predominance increased in those ≥70 years of age (1.7:1). The sites of *V. vulnificus* isolation included wounds in 61 patients (65% of 94 patients with laboratory-confirmed infection), blood in 24 (26%), or both in 7 (7%). The source was not recorded in 2 patients.

Clinical characteristics of the patients and outcomes are summarized in Table 1. Compared to patients with laboratory-confirmed infection, patients with suspected infection were more likely to be men, with a longer incubation period after exposure, less severe clinical symptoms, a shorter hospitalization time, and a more favorable outcome. Information regarding the type of exposure was available for 93 patients. Most of these patients (75, 81%) were injured while purchasing or preparing fish for cooking. Fish were purchased from fish stores (52, 56%), mobile selling units (9, 10%), or stands near fishponds (14, 15%). Other types of exposures included involvement in fish marketing (14, 15%), either in selling (11/14) or cleaning the fishponds (3/14). One person was infected after fishing at a fishpond. Four persons had no connection to the pond fish industry. Three persons with suspected infection became ill after fishing in the Sea of Galilee, and 1 with laboratory-confirmed *V.*

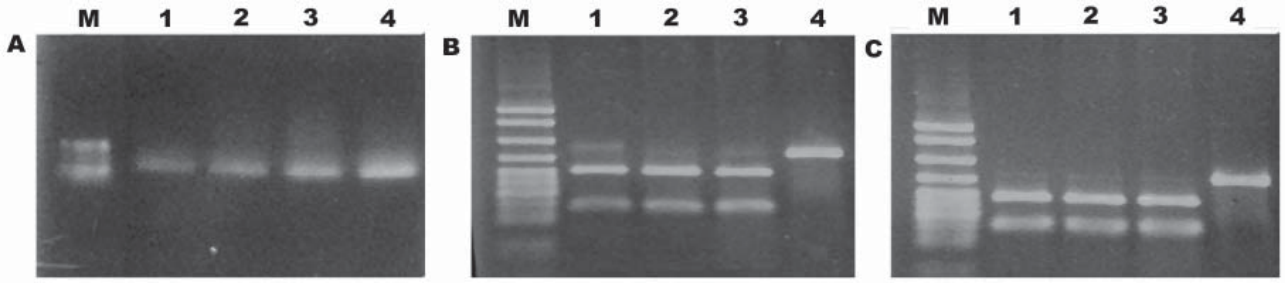

Figure 1. PCR restriction fragment length polymorphism of *Vibrio vulnificus* cytotoxin gene *vvhA*. A) PCR amplicon of *vvhA* gene restriction digested with B) *Pst*I or C) *Kpn*I. Gel shows molecular size standards (M) and *V. vulnificus* biotype 3 (lanes 1–3) and biotype 1 (lane 4).

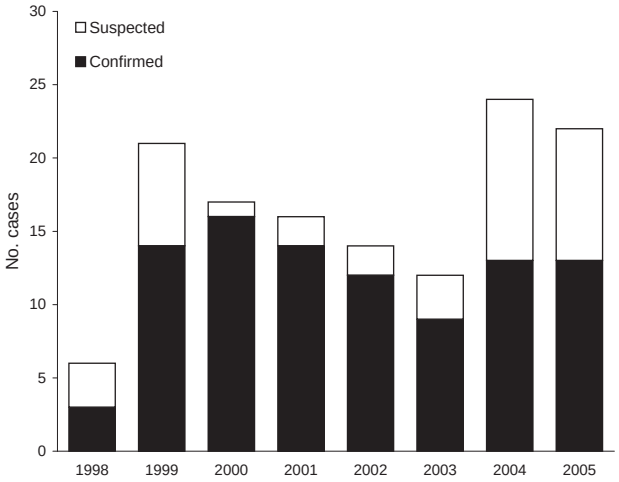

Figure 2. Annual distribution of laboratory-confirmed and suspected *Vibrio vulnificus* biotype 3 infections.

vulnificus biotype 3 infection reported immersion in a natural spring near Jerusalem with an open wound. Most of the injuries affected the hands; only 9 affected the legs.

Most of the infections (74%) occurred in the warm months of the year between June and November (Figure 4). Most patients resided in northern Israel, where freshwater fish aquaculture takes place. Only 6 patients lived in other areas of Israel (3 in the central part, 2 in the southern part, and 1 near Jerusalem). A total of 104 patients provided details regarding fish exposure. Tilapia (St. Peter's fish) was the primary fish involved in 86 of these cases (83%), common carp (*Cyprinius carpio*) in 14 cases (13%), and both in 2 cases (2%). Two other patients reported exposure to saltwater fish: gilt-head sea brim (*Sparus aurata*) that was purchased in a fish store (laboratory-confirmed infection) and common gray mullet (*Mugil cephalous*) that was purchased at a fish stand near a fishpond (suspected infection).

Course and Outcome

A total of 124 patients (94%) were hospitalized for a mean duration of 10.9 days (median 9 days, range 2–50 days). Information regarding treatment with antimicrobial drugs was available for 112 patients and included a variety of regimens. The most frequent regimens among these patients included only a single drug, usually ampicillin-clavulanate (47 patients, 42%), doxycycline (27, 24%), and ceftriaxone (6, 5%). Combinations of drugs were also used and included third-generation cephalosporins (ceftriaxone or ceftazidime) plus doxycycline (18, 16%), third-generation cephalosporins plus ampicillin-clavulanate (3, 3%), or doxycycline plus ampicillin-clavulanate (8, 7%).

Ten patients (7.5%) died and 9 (6.8%) underwent amputation of fingers or part of the arm (7 patients) or a leg (2). Two of the patients who underwent amputation subsequently died. Seven of the patients who died (70%) were ≥70 years of age, 2 were in their 50s, and 1 was in his 60s. All patients who died or underwent amputation had laboratory-confirmed infection.

Ninety patients with laboratory-confirmed infections were included in the analyses of risk factors associated with death. Factors that were significantly associated with death by a univariate analysis are outlined in Table 2. We could not show that any of the antimicrobial drug regimens influenced outcomes. Variables associated with death were entered into a stepwise forward logistic regression model. All initially entered variables remained in the model as independent predictors of a fatal outcome: presence of bacteremia (odds ratio [OR] 6.03, 95% confidence interval [CI] 1.2–30.8, p = 0.031), altered immune status (OR 6.7, 95% CI 1.1–40.8, p = 0.038), and history of ischemic heart disease (OR 15, 95% CI 2.5–91.1, p = 0.003) (p value for the model <0.001). When septic shock was added, only septic shock (OR 24.4, 95% CI 4.3–140.2, p<0.001) and history of ischemic heart disease (OR 9.7, 95% CI 1.3–72.7, p = 0.027) remained in the model (p value for the model <0.001). The model did not change when adjusted for age.

Antimicrobial Drug Susceptibility Tests

V. vulnificus isolates were susceptible to all antimicrobial agents tested by criteria for *Enterobacteriaceae*. The MIC ranges for ampicillin were 0.5–1.0 μg/mL; cephalothin, 4–8 μg/mL; chloramphenicol, 0.5–0.75 μg/mL; ciprofloxacin, 0.012–0.023 μg/mL; kanamycin, 4–16 μg/mL; nalidixic acid, 0.25–1 μg/mL; streptomycin, 8–16 μg/mL; tetracycline, 0.5–7.5 μg/mL; and trimethoprim-sulfamethoxazole, 0.064–0.094 μg/mL.

Molecular Subtyping

Analysis of the PFGE subtyping results from 25 isolates (21 isolates from 1998 through 2003, 4 isolates from

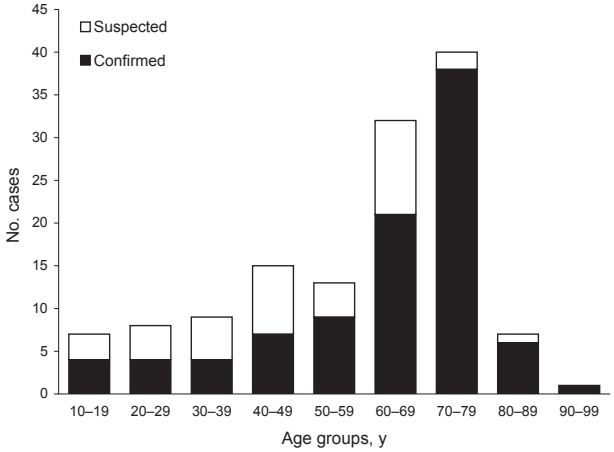

Figure 3. Age distribution of patients with laboratory-confirmed and suspected *Vibrio vulnificus* biotype 3 infections.

Table 1. Clinical characteristics of patients with *Vibrio vulnificus* biotype 3 infections, Israel, 1998–2005*

Clinical characteristics	All patients	Patients with laboratory-confirmed infection	Patients with suspected infection	p value†
No. patients studied (%)	132 (100)	94 (100)	38 (100)	
Males, no. (%)	64 (48.5)	40 (42.6)	24 (63.2)	0.036
M:F ratio	0.94:1	0.7:1	1.7:1	
Mean age, y; median (range)	58.9; 66 (10–93)	63.1; 68 (10–93)	48.7; 47.5 (11–88)	NS
Clinical presentation				
Mean incubation time, h; median (range)	17.7; 12 (1–96)	13.9; 12 (0.5–48)	28; 20 (1–96)	<0.001
Bacteremia, no. (%)	31 (23.5)	31 (33.0)	NA	
Septic shock, no. (%)	11 (8.7)‡	10 (11.0)§	1 (2.9)¶	NS
Necrotizing fasciitis, no. (%)	16 (12.7)‡	16 (17.6)§	0	0.006
Uncomplicated cellulitis, no. (%)	92 (69.7)‡	62 (68.1)§	30 (85.7)¶	0.072
Abscess formation, no. (%)	12 (9.8)‡	7 (7.7)§	5 (14.3)¶	NS
Peritonitis, no. (%)	1 (0.8)‡	1 (1.1)§	0	NS
Underlying diseases#				
None, no. (%)	59 (44.7)	49 (52.1)	10 (26.3)	0.007
Liver disease, no. (%)	18(13.6)	18 (19.1)	0	0.002
Diabetes mellitus, no. (%)	18 (13.6)	13 (13.8)	5 (13.2)	NS
Ischemic heart disease, no. (%)	9 (6.8)	8 (8.5)	1 (2.6)	NS
Altered immune status,** no. (%)	13 (9.8)	9 (9.6)	4 (10.5)	NS
Hemolytic anemia, no. (%)	3 (2.3)	2 (2.1)	1 (2.6)	NS
Outcome				
Mean hospitalization time, d; median (range)	10.9; 9 (2–50)	12.7; 11 (2–50)	6.2; 5 (2–20)	0.003
Amputation, no. (%)	9 (6.8)	9 (9.6)	0	0.059
Death, no. (%)	10 (7.6)	10 (10.6)	0	0.062
No. patients with known fish exposure††	104	72	32	
Tilapia, no. (%)	86 (82.7)	60 (83.3)	26 (81.3)	NS
Carp, no. (%)	14 (13.5)	10 (13.9)	4 (12.5)	NS
Tilapia plus carp, no. (%)	2 (1.9)	1 (1.4)	1 (3.1)	NS
Other, no. (%)	2 (1.9)	1 (1.4)	1 (3.1)	NS

*NA, not available; NS, not significant.
†For comparison between patients with laboratory-confirmed and suspected infections.
‡n = 126.
§n = 91.
¶n = 35.
#Patients may have ≥1 underlying diseases.
**Due to malignancy or immunosuppressive state.
††Percentages of exposure to each fish type based on total no. patients with known fish exposure in each category.

1997) showed that the selected isolates represented 18 unique but similar (≥88% pattern similarity; 1–3 fragment differences) PFGE fingerprint patterns regardless of the type of fish exposure (tilapia vs. carp) and year of isolation (Figure 5). A tilapia isolate from 2003 generated an *Sfi*I PFGE pattern, which was indistinguishable from the PFGE patterns of 3 patient isolates (1 from 2002, 2 from 2003) with reported exposure to tilapia from wound sites. When compared with biotype 1 strains reported from the United States, the biotypes separated into 2 distinct clusters with ≈70% pattern similarity (Figure 5).

Discussion

Our study showed that infections caused by *V. vulnificus* biotype 3 continued to occur after the initial cluster during 1996–1997, with an average of ≈16 cases annually. Although the annual rate of infection during 1998–2005 was half the rate of infection during 1996–1997 (62 cases) (*11*), outcomes were more grave. Our study found that 10 persons (7.6%) died; no deaths were reported during the 1996–1997 outbreak (*11*). Possible explanations for this disparity could be that patients in our study were older (median age 66 vs. 56 years, respectively) with a higher proportion of laboratory-confirmed infections (70.1% vs. 53%, respectively). We have shown that patients without laboratory confirmation had a much milder form of the disease and a more favorable outcome.

Notably, our findings show a high proportion of infected women (52%), including 8 (80%) of the 10 patients who died. Previous studies have stressed a male predominance (*7*,*9*,*10*,*22*–*25*), and have even argued that female sex hormones protect against contracting the disease (*26*). With *V. vulnificus* biotype 3 infections in Israel, it was more likely that women purchased and prepared the fish before cooking, and thus were more likely to be exposed to fishbone injuries.

There are 3 major clinical syndromes of *V. vulnificus* illnesses, including primary bacteremia (mostly related to

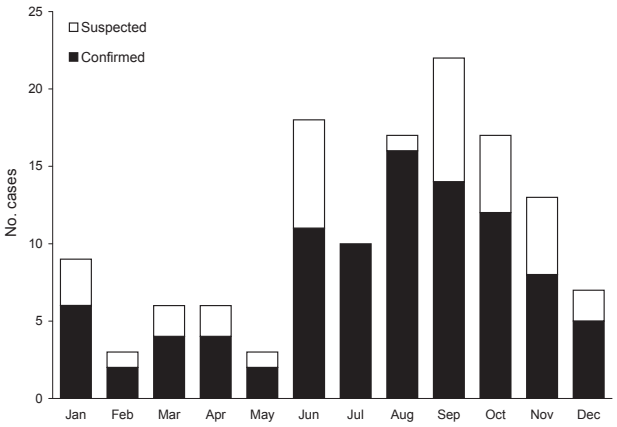

Figure 4. Seasonality of *Vibrio vulnificus* biotype 3 illnesses, Israel, 1998–2005.

raw seafood consumption), wound infection (mostly related to immersion in contaminated water or to injury by seafood preparation), and gastroenteritis (after consumption of seafood or swallowing contaminated water) (*9*,*23*,*27*). Patients with primary bacteremia caused by non–biotype 3 strains are more likely to have predisposing conditions, particularly liver diseases in ≥80% of patients (*7–9*), whereas patients with wound infection are more likely to be previously healthy and have a more favorable outcome (*9*,*10*,*23*,*24*,*28–30*). The death rate may exceed 50% in the most seriously ill patients (*7–9*,*23*).

Our study describes a large, uniform group of patients who acquired infection through percutaneous exposure. All sought treatment for a wound infection, which in 18% was complicated by secondary bacteremia. In 38 patients (29%), laboratory confirmation of infection was lacking. These patients tended to delay seeking treatment and to have a milder form of infection, compared with patients who had laboratory-confirmed infection. Some of the patients who lacked laboratory confirmation may not have had *V. vulnificus* infection. However, patients with milder forms of *V. vulnificus* infection may have been less likely to undergo extensive microbiologic workup and the yield of cultures, if taken, would have been lower.

Only 45% of the patients in the group had underlying diseases. Liver disease occurred in 14% of patients overall, and in 19% of patients with confirmed cases, but was not a statistically significant risk factor for death. The case-fatality rate was 7.6% for the entire study population and 10.6 % for patients with laboratory-confirmed infection. These characteristics concur with prior reports of wound infection syndrome caused by non–biotype 3 *V. vulnificus* (*9*,*10*,*23*,*24*,*27–30*).

Of concern is our finding that *V. vulnificus* infection was not limited to tilapia exposure. Fourteen persons (13%) reported exposure to the common carp, or to both tilapia and common carp (2 persons, 2%). Tilapia and common carp are co-cultivated in several fishponds in northern Israel, which may have resulted in cross-contamination. For those exposed to salt water fish (gilt-head sea brim and common gray mullet, 1 person each), contamination may have occurred at the place of purchase. *V. vulnificus* infection after exposure to the common carp caused the death of 1 patient and the amputation of a finger in another. One person infected after exposure to the gilt-head sea brim also died. Notably, orthopedic surgeons from hospitals in northern Israel have also pointed out severe soft tissue infections, including those caused by *V. vulnificus*, after fishbone injury from the common carp (*31*).

The *V. vulnificus* biotype 3 strains studied were uniformly susceptible to all tested antimicrobial agents. These results concur with those of similar studies of clinical and environmental *V. vulnificus* biotype 1 isolates from the United States (*32*,*33*) and Taiwan (*22*). Only a few isolates from Taiwan showed resistance to ceftazidime and moxalactam.

Molecular subtyping of the *V. vulnificus* biotype 3 strains by PFGE showed no specific association between fish species and PFGE pattern. The results indicate that the biotype 3 strains are homogeneous with limited heterogeneity between the isolates but cluster distinct from biotype 1 strains. PFGE has been shown (*34*) to offer sufficient discrimination when subtyping biotype 1 strains, but published findings that evaluate the utility of PFGE to sufficiently discriminate between biotype 3 strains are limited. Modifications in restriction sites may alter the number and size of DNA fragments, which define the PFGE pattern and result in observable true differences.

Table 2. Variables associated with death in laboratory-confirmed *Vibrio vulnificus* infection, by univariable analysis, Israel, 1998–2005*

Clinical characteristics	Alive,† n = 84	Dead,† n = 10	p value
Mean age ± SD, y	62.3 ± 18.2	69.2 ± 15.8	NS
Female, no. (%)	60 (49.2)	8 (80)	NS
Septic shock, no. (%)	41 (4.9)†	6 (60)	<0.001
Bacteremia, no. (%)	24 (28.6)	7 (70)	0.013
Ischemic heart disease, no. (%)	4 (4.8)	4 (40)	0.004
Altered immune status,‡ no. (%)	6 (7.1)	3 (30)	0.052

*NS, not significant.
†n = 81.
‡Due to malignancy or immunosuppressive state.

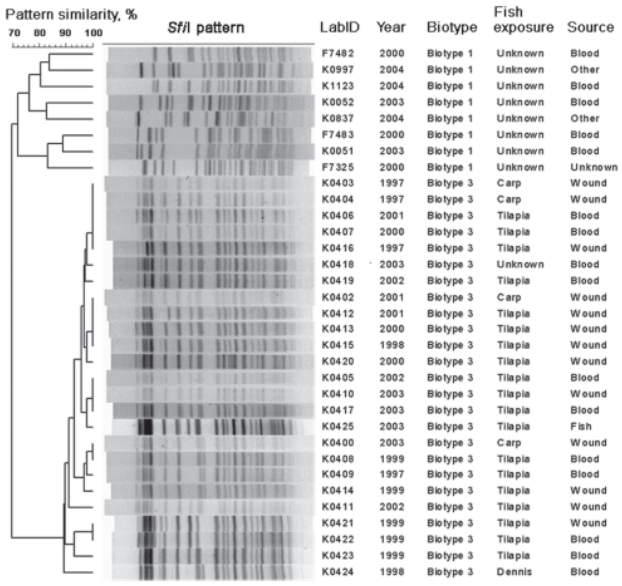

Figure 5. Dendogram comparing pulsed-field gel electrophoresis patterns of 25 *Vibrio vulnificus* biotype 3 isolates and a reference set of biotype 1 isolates when restricted with *Sfi*I enzyme.

A high degree of homogeneity among the *V. vulnificus* biotype 3 strains and distinction from the biotypes 1 and 2 has been also observed by other authors applying various methods, including random amplified polymorphic DNA, (*13*), multilocus sequence typing (*14*,*16*), and analysis of variations in simple sequence repeat loci (*35*). Notably, the latter method was able to demonstrate small-scale variations among the biotype 3 strains (*35*). The PFGE results also support the conclusion that this biotype 3 is distinct from the other *V. vulnificus* biotypes. The high degree of homogeneity is another indicator that the emergence of biotype 3 is a recent evolutionary event (*14*,*16*,*36*).

We identified independent risk factors for death in our group, including bacteremia, altered immune status, and history of ischemic heart disease. Septic shock was also found to be a strong predictor of death; however, septic shock may also be an outcome variable. Nonetheless, prior reports have also identified septic shock or hypotension as important risk factors for death (*8*,*23*). A recent large study from Taiwan (*29*) found that treatment with third-generation cephalosporins combined with tetracycline was an independent predictor of lower death rates in a subgroup of patients with hemorrhagic bullous necrotic cutaneous lesions. We did not demonstrate any correlation between a specific antimicrobial drug regimen and death rate in our study population. The predisposing diseases that were associated with death in previous reports were liver disease and neutropenia (*8*,*23*). Ischemic heart disease was not previously recognized as a classic predisposing factor predicting death; however, in the *Vibrio*-associated wound infections after Hurricane Katrina in Louisiana, USA, ischemic heart disease occurred in 7 of 13 patients (54%) with more severe illness (*25*).

Before the introduction of *V. vulnificus* biotype 3 into the fish aquaculture no infections were reported in Israel (*37*). Also, no reports have been made of *V. vulnificus* infection acquired through marine activities in the Mediterranean Sea. Almost the entire impact of *V. vulnificus* infections in Israel is associated with the freshwater fish industry. In response to the new threat, the Israeli Ministry of Health has issued regulations forbidding the selling of live, uncleaned tilapia (*11*,*38*). Fish stores and fishpond workers have been instructed to use protective gloves when handling fish, to keep fresh fish packed in ice, and to prevent direct contact between buyers and live fish. The public has been instructed accordingly. Apparently, compliance with these regulations is not universal.

Our findings outline the substantial effects of *V. vulnificus* illnesses in Israel and support a call for more strict regulations of fresh fish marketing as well as public education. Research efforts should focus on how *V. vulnificus* has penetrated the freshwater aquaculture in Israel and ways in which this trend can be reversed.

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Novel Borna Virus in Psittacine Birds with Proventricular Dilatation Disease

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Pyrosequencing of cDNA from brains of parrots with proventricular dilatation disease (PDD), an unexplained fatal inflammatory central, autonomic, and peripheral nervous system disease, showed 2 strains of a novel Borna virus. Real-time PCR confirmed virus presence in brain, proventriculus, and adrenal gland of 3 birds with PDD but not in 4 unaffected birds.

Borna disease virus (BDV) is the causative agent of Borna disease, a meningoencephalitis of horses and sheep in central Europe (*1*). As the prototype and only known member of the family *Bornaviridae* in the order Mononegavirales (nonsegmented, negative-strand RNA viruses), BDV is atypical in its nuclear localization of transcription, alternative splicing, and differential use of initiation and termination signals. Sequence analysis of isolates obtained from various species over several decades has shown remarkable sequence conservation; only 2 genotypes are known. The virus is highly neurotropic and infects the central, peripheral, and autonomic nervous systems. Although ungulates remain the best known natural host, the introduction of sensitive molecular and serologic assays enabled by subtractive cloning of the BDV genome facilitated surveys that indicated wider geographic and species distribution (*1*). Experimental infections are described in a wide variety of vertebrates including chickens, quails, rats, rabbits, cats, shrews, and nonhuman primates; manifestations of disease range from fatal meningoencephalitis to subtle behavioral alterations or asymptomatic persistent infection (*2*). Intestinal colic is frequently observed in infected ungulates (*2*,*3*). An outbreak of neurologic disease in farmed ostriches in Israel has been attributed to BDV (*4*). BDV nucleic acids have been reported in feces of wild mallards and jackdaws in Sweden (*5*).

Proventricular dilatation disease (PDD), also known as proventricular dilatation syndrome or macaw wasting disease, is a disorder of birds wherein inflammation of the central, peripheral, and autonomic nervous systems is associated with gastrointestinal dysfunction and neurologic signs that may include ataxia and seizures (*6*). Although a presumptive diagnosis can be achieved through imaging studies and biopsy, for most animals, definitive diagnosis is made postmortem, only after detailed histologic analysis indicates the accumulation of lymphocytes in nerves that supply the proventriculus and ventriculus, in their associated ganglia, and in brain. Features consistent with PDD have been reported in >50 avian species; however, PDD is most commonly described in exotic companion birds such as macaws and parrots. Whether this reflects better case ascertainment or factors that influence exposure or susceptibility is unclear. An infectious basis is supported by the observation that disease can be transferred to naive birds through inoculation with tissue homogenates or fecal material from affected birds (*7*). One electron microscopic study showed the presence of spherical, 83-nm particles in macaw embryo cells after inoculation with feces from a diseased macaw; other researchers have described particles in tissue consistent in appearance with adenoviruses or paramyxoviruses. Whether any of these agents can be implicated in the pathogenesis of PDD is unknown.

On a quest for the causative agent of PDD, we investigated 3 birds with a PDD diagnosis based on clinical history and histologic criteria (Table 1). RNA was extracted from brains, pooled and randomly amplified for unbiased high-throughput sequencing (*8*), yielding 96,698 reads, ranging from 40 nt to 353 nt. After implementation of algorithms for vertebrate sequence subtraction and contiguous fragment assembly, GenBank searches using BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) indicated a relation to BDV for a total of 11 contigs covering ≈1.1 kb of sequence distributed in 6 clusters throughout the N (230 nt), P (450 nt), G (250 nt), and L (120, 80, and 250 nt) genes (Figure 1). Divergent sequences between multiple overlapping contigs in these 6 regions indicated at least 2 different strains. Analysis of ≈5.5 kb of genomic sequence generated by standard PCR by using primers (sequence available upon request) based on the identified sequence fragments confirmed 2 strains, one from bird 1367, another from birds 1034 and 1322 (GenBank accession nos. FJ169440 and FJ169441, respectively) and indicated conservation of the unique genome organization that is characteristic for the family *Bornaviridae* (Figure 1). Sequence divergence between the avian strains (86% identity at nucleotide level) is

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Table 1. Real-time PCR measurement of viral sequences in birds with PPD*

Case no.	Species†	Age/sex	PDD/ control	Primer/probe set	Organ	Ct	Virus copies‡ (300 ng total RNA)
1034	Canindae macaw	30 y/M	PDD	1034–1322	Brain	17.94	8.88×10^7
					Adrenal gland	20.51	8.77×10^6
					Proventriculus and gizzard	25.26	1.22×10^5
1322	Vinaceous Amazon	1 y/M	PDD	1034–1322	Brain	17.64	1.17×10^8
					Proventriculus	28.47	6.76×10^3
1367	Canindae macaw	30 y/F	PDD	1367	Brain	27.33	8.89×10^3
					Adrenal gland	18.92	3.87×10^6
					Proventriculus	23.82	1.13×10^5
5473	Leadbetter's cockatoo	17 y/M	Control	1034–1322 and 1367	Brain	>36§	Negative
4858	Long-tailed parakeet	Adult/M	Control	1034–1322 and 1367	Brain	>36§	Negative
2020	Cockatiel	Young/M	Control	1034–1322 and 1367	Brain	>36§	Negative
3616	Eclectus parrot	0.25 y/F	Control	1034–1322 and 1367	Brain	>36§	Negative

*PPD, poventricular dilatation disease; Ct, cycle threshold.
†All birds were captive animals from California, USA.
‡Copy numbers were calculated on the basis of a standard curve generated from cloned target sequences.
§A Ct of >36 was rated as negative based on the highest dilution of standard representing 5 copies.

similar to that observed between the most divergent strains isolated from ungulates (84% identity). Pairwise comparison of the avian strains with these 2 ungulate isolates that represent the 2 previously known genotypes of BDV, strain V, NC_001607 (*9*), and No/98, AJ311524 (*10*), indicated <70% sequence conservation at the nucleotide level and <80% at the overall amino acid level (Figure 2, Table 2). These data are compatible with the avian strains representing a new species.

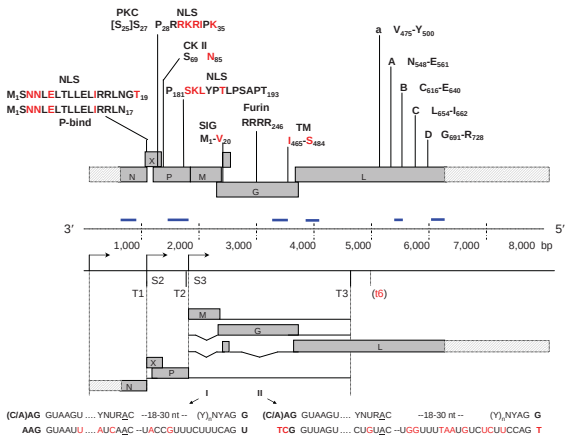

Figure 1. Conservation of genome organization, regulatory sequences, and protein domains of Borna disease virus (BDV) in novel strains from parrots 1034, 1322, and 1367. N, nucleoprotein; P, phosphoprotein; X, X protein; M, matrix protein; G, glycoprotein; L, L-polymerase protein. Genome regions not yet sequenced in the novel strains are shaded. P-bind, binding site for P on X; NLS, nuclear localization signals of X and P; PKC, protein kinase C epsilon phosphorylation sites in P; CK II, casein kinase phosphorylation sites in P; SIG, signal peptide; Furin, furin cleavage site; TM, transmembrane anchor of G; A – D, conserved RNA-dependent RNA polymerase motifs. Conserved sites/residues with respect to BDV strain V are shown in black; divergent sites/residues are indicated in red; K_{32} in P NLS-1 is divergent only in 1034/1322, K_{35} in NLS-1 and K_{183} in NLS-2 are divergent only in 1367. S2 and S3, start sites of transcription units 2 and 3, respectively, showing the conserved GAA initiation triplet; T1, T2, and T3, transcription termination sites showing the conserved TA_6 consensus sequence; (t6) indicates a nonconserved TA_6 sequence found by some BDV isolates. Blue bars indicate the 6 clusters represented by contigs obtained through pyrosequencing. Consensus splice site sequences corresponding to established introns I and II in genes M and G of BDV strain V are aligned to corresponding sequences of the novel strains.

Primers and probes for quantitative real-time PCR were selected in the amino-terminal region of the phosphoprotein (P) gene matching P sequences for strain 1367 (set 1367, forward: 5′-AGAAGACCCGCTGACAGCA-3′, reverse: 5′-AAGCTTCTCGACGGGAACAG-3′, probe: 6FAM-5′-TCGTGGGGACCTCGATCTCACTCG-3′-TMR) or strain 1034/1322 (set 1034–1322, forward: 5′-CAGACAGCACGTCGAGTGAGA-3′, reverse: 5′-AGTTAGGGCCTCCCTGGGTAT-3′, probe: 6FAM-5′-AGGTCCCCGCGAAGGAAGCGA-3′-TMR); the diagnostic assay has been made available through ProMed mail (www.promedmail.org, archive no. 20080726.2287). Real-time PCR showed levels of viral RNA exceeding 10^3 copies in all tissues tested from birds with PDD but not in control birds. In 2 birds (1034 and 1322) virus load was higher in the brain than in the proventriculus and gizzard or adrenal gland; in 1 bird (1367), the load was lower in the brain than in the adrenals or proventriculus (Table 1).

Western immunoblot and nondenaturing dot blot experiments were pursued by using brain, proventriculus, and adrenal homogenates of the 3 animals that were quantitated by real-time PCR, and 2 rabbit polyclonal antibodies raised against recombinant BDV strain V nucleoprotein (N) or P, as well as immune sera from BDV He/80–infected rats. Positive and negative controls were BDV He/80–infected rat brain homogenate and uninfected rat brain homogenate, respectively. A strong signal was obtained with positive control material in western immunoblots and dot blots that

Table 2. Percent sequence conservation between Borna disease virus strain V(No/98) and novel strains

Amino acid	Nucleotide			
	Strain V	No/98	1034/1322	1367
Strain V		84	67	66
No/98	P: 99/96*			
	X: 84/81			
	M: 100/98		66	66
	G: 95/94			
	L: 98/96			
1034/1322	P: 76/61	P: 77/63		
	X: 55/51	X: 49/45		
	M: 91/85	M: 91/85		86
	G: 75/66	G: 75/66		
	L: 81/74	L: 81/74		
1367	P: 75/60	P: 76/63	P: 98/95	
	X: 59/53	X: 53/48	X: 88/88	
	M: 91/82	M: 91/81	M: 99/95	
	G: 74/66	G: 74/66	G: 94/93	
	L: 82/76	L: 82/76	L: 97/95	

*Amino acid conservation is indicated as percent similarity/percent identity.

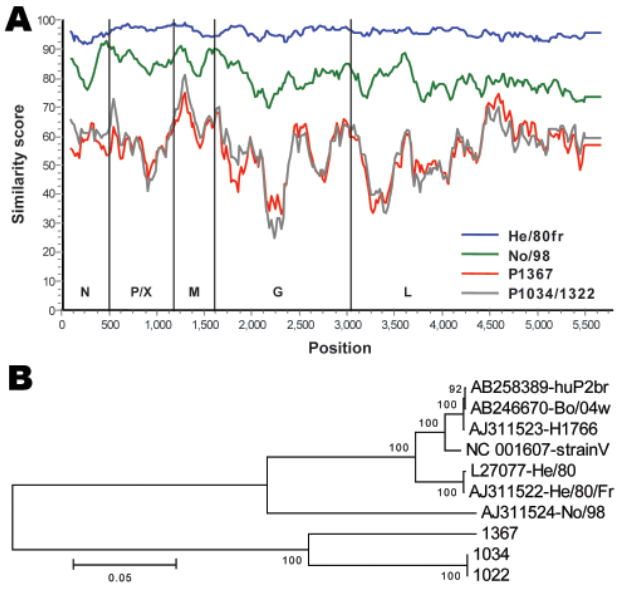

Figure 2. A) Similarity plot between Borna disease virus (BDV) prototype strain V nucleotide sequence and those of characterized BDV strains He/80fr and No/98 compared with novel strains 1367 and 1034/1322. Gene regions corresponding to the nucleoprotein (N), phosphoprotein (P), X protein (X), matrix protein (M), glycoprotein (G) and L-polymerase protein (L), and nucleotide positions are indicated. B) A tree representing the evolutionary history was inferred by using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (number of base substitutions per site; see scale bar). Evolutionary distances were computed by using a Kimura 2-parameter model; a total of 5,449 positions in the final dataset were analyzed by using MEGA4 software (www.megasoftware.net).

used either antisera to recombinant viral proteins or rat immune sera; no signal was obtained with bird homogenates or uninfected rat brain homogenate.

Conclusions

We have not yet determined whether purified virus induces PDD or an adaptive immune response occurs in association with disease. Nonetheless, given what is known about BDV pathogenesis with other strains in other hosts (*11*), the bornaviruses identified in these birds must be considered a biologically plausible candidate causative agent. Infection, lymphocyte infiltration, and dysfunction of the central, peripheral, and autonomic nervous system are common to PDD as well as to classical Borna disease in natural disease and experimental models (*2*,*12*,*13*). Proteins and antisera we have used for 2 decades for BDV diagnostics failed to detect this virus in our PCR-positive birds. Thus, it will be important to revisit epidemiologic surveys that we, and others, have undertaken to investigate the role of bornaviruses in human disease (*14*). From a personal perspective, we are intrigued that whereas molecular discovery of the first BDV in the late 1980s required an investment of 2 years in subtractive cloning (*15*), high-throughput sequencing, bioinformatics, and sequence databases enabled discovery of these 2 strains in 2 weeks.

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During review of this manuscript similar findings were independently reported by Kistler AL, Gancz A, Clubb S, Skewes-Cox P, Fischer K, Sorber K, et al. Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. Virol J. 2008;5:88.

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Possible Emergence of West Caucasian Bat Virus in Africa

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The prevalence of neutralizing antibody against West Caucasian bat virus (WCBV) in *Miniopterus* bats collected in Kenya ranged from 17% to 26%. Seropositive bats were detected in 4 of 5 locations sampled across the country. These findings provide evidence that WCBV, originally isolated in Europe, may emerge in other continents.

Bats are reservoir hosts of several emerging zoonotic RNA viruses (*1*). In particular, bats host a range of lyssaviruses, as has been reported from different continents (*2*). Presently, 7 species are recognized within the genus *Lyssavirus* (order Mononegavirales, family *Rhabdoviridae*): *Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssavirus,* types 1 and 2, and *Australian bat lyssavirus*. Another 4 lyssaviruses, all isolated from bats, have been assigned to the genus as putative species: Aravan virus, Khujand virus, Irkut virus, and West Caucasian bat virus (WCBV) (*3*).

RABV infection of bats is known in the Americas, but not in the Old World. Four lyssavirus species have been documented in Africa. Of these, RABV and MOKV have been isolated exclusively from terrestrial mammals, whereas LBV and DUVV are associated with bats and have been isolated only occasionally from other mammals (*4*). On the basis of this diversity and on the serologic cross-reactivity of MOKV with African non-lyssa rhabdoviruses, it has been hypothesized that Africa is the continent of the origin and initial evolution of members of the genus *Lyssavirus* (*5*). However, this hypothesis has been called into question by the isolation of WCBV in southeastern Europe. WCBV is the most divergent member of the genus *Lyssavirus* to date and has long genetic distances and lack of serologic cross-reactivity to other lyssaviruses (*6*,*7*). Our study objective was to enhance pathogen discovery for zoonotic agents in African bats, with particular focus on lyssavirus surveillance in Kenya.

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The Study

During 2006–2007, bats of at least 30 species were collected from 25 locations in Kenya (Figure 1, panel A; online Appendix Table, available from www.cdc.gov/EID/content/14/12/1887-appT.htm). The sample numbers and collection protocol were approved by the National Museum of Kenya and the Kenya Wildlife Service. Of the 1,221 bats collected, only 12 were sick or dead; the others appeared healthy. Captured animals were anesthetized by an intramuscular injection of ketamine hydrochloride (0.05–0.1 mg/g) and euthanized under sedation in compliance with a field protocol approved by the Animal Institute Care and Use Committee of the Centers for Disease Control and Prevention. Bat size, sex, and species were identified. When phenotypic species determination was not possible, DNA specimens were submitted for identification to the University of Guelph (Ontario, Canada), where partial sequences of the cytochrome oxidase gene were generated and compared with those available from the Barcode of Life Data Systems (www.boldsystems.org).

The brain and other organs of bats were collected into sterile plastic tubes. Oral swabs were collected and placed in tubes containing Minimum Essential Medium (MEM-10, Invitrogen, Grand Island, NY, USA) for virus isolation or in TRIzol (Invitrogen, Carlsbad, CA, USA) for RNA extraction. Serum was separated from the blood clot by centrifugation. All samples were transported on dry ice and stored at –80°C.

The brains (n = 1,182) were subjected to the direct fluorescent antibody test for lyssavirus antigen (*8*). In addition, the 277 brains that were collected in 2006 were homogenized and tested for virus isolation by the intracerebral mouse inoculation test as described elsewhere (*9*). Virus isolation was attempted for only a subset of brain samples (n = 210) from the specimens collected during 2007.

Total RNA was extracted from the oral swabs (n = 931) and subjected to nested reverse transcription–PCR, as described previously (*10*). We used primers designed for the nucleoprotein genes of LBV, MOKV, and WCBV.

The virus neutralizing antibodies in bat serum samples were determined by a modification of the rapid fluorescent focus inhibition test. We used 4-well (6-mm) Teflon-coated glass slides (Cel-Line, Erie Scientific, Portsmouth, NH, USA) as described elsewhere (*10*). Previous tests for RABV neutralizing antibodies have demonstrated that re-

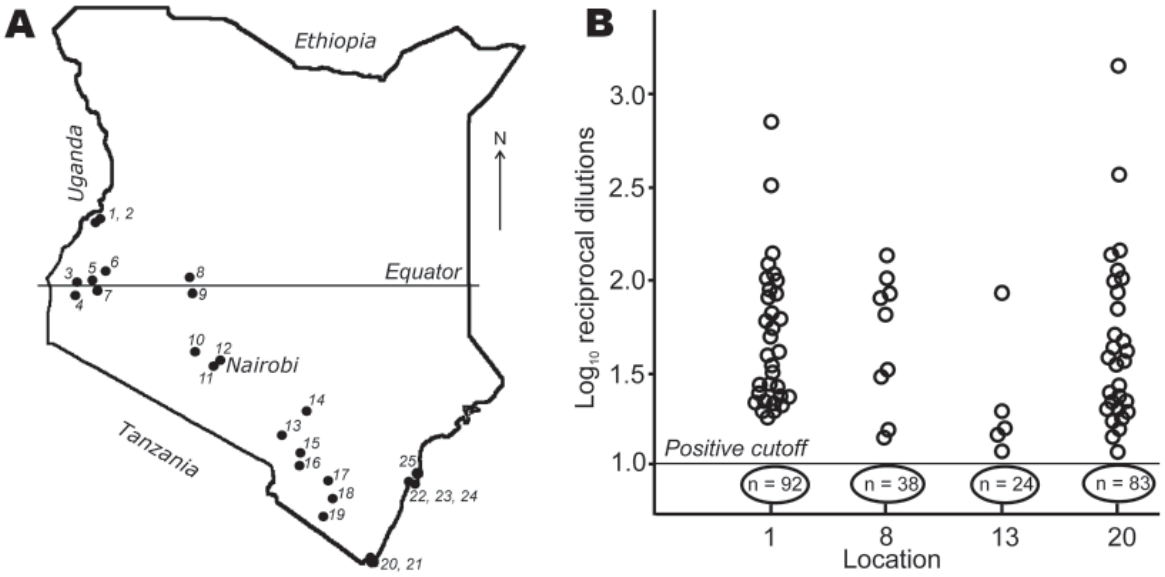

Figure 1. A) Map of Kenya showing locations of the bat collections, numbered in order of collection. B). Antibody titers to West Caucasian bat virus (WCBV) in *Miniopterus* bats from 4 of the locations. A modified rapid fluorescent focus inhibition test for WCBV-neutralizing antibodies was used. Bats with 50% end-point neutralizing titers >1 $\log_{10}$ were considered seropositive. Numbers of negative bats for each location are circled below the cutoff line.

sults of this micromethod are comparable to those of the classical test in chamber slides. The neutralizing activity of each serum sample was determined against LBV, MOKV, DUVV, RABV, and WCBV. All samples were initially screened in dilutions of 1:10 and 1:25. Samples that showed reduced fluorescence or no fluorescence were subjected to additional titration in dilutions of 1:10 to 1:1,250. The 50% end-point neutralizing titers were calculated by the method of Reed and Muench (*11*). The samples that had a 50% end-point neutralizing titer >1 $\log_{10}$ were considered positive.

Circulation of LBV was detected in fruit bats *Eidolon helvum* and *Rousettus aegyptiacus* as described previously (*10*). No other viruses were isolated during the study, and no lyssavirus RNA was identified in oral swabs. However, virus-neutralizing activity against WCBV was detected in serum of *Miniopterus* insectivorous bats (Figure 2) from 4 of the 5 locations where these species were collected (online Appendix Table). Among 76 serum samples with WCBV-neutralizing activity (Figure 1, panel B), only 1 sample additionally neutralized DUVV, but no cross-neutralization to other lyssaviruses was detected. This observation supported specificity of the reaction and reliability of the selected cutoff threshold. The seroprevalence varied by roosts, 17% to 26% (95% confidence interval 17–27). In general, seroprevalence among females (n = 201; seroprevalence 26%) was greater than that among males (n = 112; seroprevalence 19%). Although statistically insignificant ($\chi^2 = 2.38$; $p = 0.12$), this difference was consistent across locations 1, 13, and 20. Only females were available from location 8. At all locations, *Miniopterus* bats shared caves with other species of insectivorous and fruit bats. However, no serologic activity against WCBV was detected in these other species. Of note, serum from fruit bats *R. aegyptiacus* that shared caves with *Miniopterus* bats neutralized LBV but not WCBV. Conversely, serum from *Miniopterus* bats neutralized WCBV but not LBV. This finding suggests that bats of different species, even those roosting in the same caves, do not readily expose each other to lyssaviruses.

Conclusions

We found WCBV-neutralizing antibodies in bats in Africa. Because limited serologic cross-reactivity between lyssaviruses and other rhabdoviruses has been demonstrated (*12*), the WCBV seroprevalence we detected may have been caused by some other serologically related virus. However, to date no other agent that could cross-neutralize WCBV is known (*7*).

We cannot explain why 1 WCBV-neutralizing sample additionally neutralized DUVV. This finding could indicate nonspecific virus inhibition, or it could be evidence of coexposure of the bat to several lyssaviruses. Our inability to isolate viruses in this study is not surprising because lyssavirus prevalence in bat populations is usually low (<1%), even when seroprevalence is as high as 40%–70% (*10*,*13*,*14*). Indeed, the seroprevalence may reflect past exposures and peripheral virus activity rather than survival after clinical lyssavirus infection.

WCBV was first isolated in 2002 in southeastern Europe from *Miniopterus schreibersii* (*6*) bats, and only 1

Figure 2. Colony of *Miniopterus minor* bats in cave.

isolate is available to date. Seroprevalence to this virus in African *Miniopterus* bats is intriguing. Perhaps WCBV and similar viruses are specifically associated with *Miniopterus* bats and distributed quite broadly. *Miniopterus* bats are common in the tropics and subtropics of the Old World (*15*). They segregate into large colonies in caves. For example, in Kenya, *Miniopterus* colonies consisted of thousands of bats. Many of these caves are regularly visited by local residents and by tourists. Although no data exist for WCBV pathogenicity in humans, the absence of reliable vaccine protection against this virus and the ability of WCBV to cause fatal encephalitis in animal models (*7*) suggest the need for improved surveillance and public education to avoid exposure to bats.

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Detection and Phylogenetic Analysis of Group 1 Coronaviruses in South American Bats

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Bat coronaviruses (Bt-CoVs) are thought to be the precursors of severe acute respiratory syndrome coronavirus. We detected Bt-CoVs in 2 bat species from Trinidad. Phylogenetic analysis of the RNA-dependent RNA polymerase gene and helicase confirmed them as group 1 coronaviruses.

Bats are of particular interest as reservoirs for potentially emergent pathogens. Because of their abundance, wide distribution, and mobility, bats confer a greater risk for zoonotic transmission than other animals (*1*). Bats have long been known as the natural hosts for rabies virus and other lyssaviruses and were more recently identified as the reservoirs for emerging viruses such as Ebola, Hendra, and Nipah virus (reviewed in *1*). The search for the animal reservoir of the severe acute respiratory syndrome coronavirus (SARS-CoV) led to extensive surveys of coronaviruses in wild and domestic animal populations in China, resulting in the detection of a wide variety of novel bat coronaviruses (Bt-CoVs) (*2*–*5*). The data suggest that the progenitor of the SARS-CoV, and all other coronaviruses in other animal hosts, originated in bats (*4*). Recent reports by Dominguez et al. (*6*) and Gloza-Rausch et al. (*7*) confirmed the existence of Bt-CoVs outside China, in the United States and Germany, respectively. Additionally antibodies reactive with SARS-CoV have been detected in African bat species (*8*). We report the detection and characterization of CoVs in bats from Trinidad, the southernmost island of the Caribbean archipelago, located 9 km (5.5 miles) off the northeastern coast of South America.

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The Study

A total of 114 bats collected from their natural habitats from December 2006 through July 2007 (8 species from 10 locations; Table) were euthanized after being deeply anesthetized with 2% xylazine and 10% ketamine administered subcutaneously (in some cases after sedation with CO_2). The bats were then taxonomically classified on the basis of morphology, and the carcasses were stored at –70°C until used. For sampling, the carcasses were thawed at 4°C for 3–4 hours, then oropharyngeal and anal samples were taken with Dacron-tipped swabs that were then placed in RNA*later* (Ambion, Austin, TX, USA) and stored at –20°C until used.

CoV detection and sequencing were conducted as previously described (*4*). Briefly, viral RNA was extracted from swabs by using the QIAamp viral RNA minikit (QIAGEN, Westburg, the Netherlands) and used as the template for reverse transcription–PCR (RT-PCR) detection of the CoV RNA–dependent RNA polymerase (RdRp) gene (*9*). Primers based on the RdRp gene, conserved for all known coronaviruses, were then used for RT-PCR detection. The RdRp PCR products were gel purified by using the QIAquick PCR purification kit (QIAGEN) and sequenced to confirm virus species. RNA from samples positive for coronavirus was then used for cDNA synthesis by using random hexamer, gene-specific, and oligo(dT) primers. The RdRp gene and 1b open reading frames, including the helicase (HEL) domain, were then sequenced, also as previously described (*4*). Sequences derived from this study were deposited in GenBank (accession nos. EU769557 and EU769558).

Sequences were aligned with previously published CoV sequences from GenBank by using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX) then manually aligned by using the Se-Al program (http://tree.bio.ed.ac.uk/software/seal). The GenBank accession numbers of all sequences used are noted in the taxon names in Figures 1 and 2. The RdRp sequences were trimmed to equal length, which created 2 datasets of 780 bp (n = 40) and 378 bp (n = 45). The latter included Bt-CoV sequences from North America and Germany that were too short to be included in the first dataset. A third dataset comprised an alignment of the HEL domain (n = 46) trimmed to 1,797 bp. Maximum likelihood (ML) phylogenies were inferred under a General Time Reversible (GTR + Γ_4 + I) model, which was identified as the best-fit model of nucleotide substitution using MODELTEST version 3.7 (*10*). Bootstrapping was performed to assess the robustness of tree topologies by using 1,000 replicate neighbor-joining (NJ) trees under the ML substitution model. All analyses were performed with

Table. Number and location of bat species collected and tested from December 2006 through July 2007, with bat coronavirus species status

Species (family)	Location (no.)	Total no. tested (no. positive)
Carollia perspicillata (Phyllostomidae)	Arima (2), Fyzabad (2)*, Tabaquite (1)	5 (1)
Glossophaga soricina (Phyllostomidae)	Couva (12)*, Tabaquite (7)	21 (1)
Noctilio leporinus (Noctilionidae)	Couva (6)	6 (0)
Desmodus rotundus (Phyllostomidae)	Fyzabad (3), Morne Diablo (3), Rousillac (1), La Brae (7)	14 (0)
Pteronotus parnelli (Mormoopidae)	Tabaquite (29), Wallerfield (2)	31 (0)
Molossus major (Molossidae)	Talparo (25)	25 (0)
Mormoops sp. (Mormoopidae)	Tamana (1)	1 (0)
Phyllostomus hastatus (Phyllostomidae)	Wallerfield (11)	11 (0)
Total	10 locations; 8 species	114 (2)

*Indicates group from which coronavirus positive bats originated.

PAUP* version 4.0b (Sinauer Associates, Inc., Sunderland, MA, USA).

CoV RNA was detected in 1 of 21 *Glossophaga soricina* and 1 of 5 *Carollia perspicillata* bats tested**.** The latter, designated Bt-CoV/Trinidad/1FY2B, was an adult male from the area of Fyzabad, and virus was detected in anal and oropharyngeal swabs. The infected *G. soricina* specimen (Bt-CoV/Trinidad/1CO7B) was an adult female from the area of Couva, and the virus was detected in the anal swab only. Subsequent sequencing was performed on virus from the anal swabs. A total of 3,905 bp of RdRp from Bt-CoV/Tri/1CO7B and 5,160 bp from Bt-CoV/Tri/1FY2B were sequenced, in addition to 1,782 bp from the HEL domain of both samples (full sequence data not shown). Across the shared 3,798-bp region of RdRp, divergence was 28.7% at the nucleotide level and 15.5% at the amino acid level. Similarly, the HEL domains of the 2 viruses showed 27.3% divergence at the nucleotide level and 13.3% at the amino acid level.

ML phylogenies inferred from the RdRp genes of 40 viruses (780 bp) and the HEL domains of 46 viruses (1,797 bp) are shown in Figure 1. In both cases, the 5 groups proposed by Tang et al. (*4*) on the basis of NJ trees, and the lineage containing the recently reported novel CoV sequences (*11*), were strongly supported with bootstrap values >95% in all cases. In each case the Trinidadian sequences clustered with group 1 CoVs within a clade containing all other group 1 bat and human CoVs as well as porcine CoV. To determine the phylogenetic relationship between the Trinidadian Bt-CoVs and North American Bt-CoVs, for which only relatively short sequences for RdRp were available, we inferred a second RdRp ML phylogeny based on a 378-bp fragment (Figure 2). When this shorter fragment was used, 5 groups were

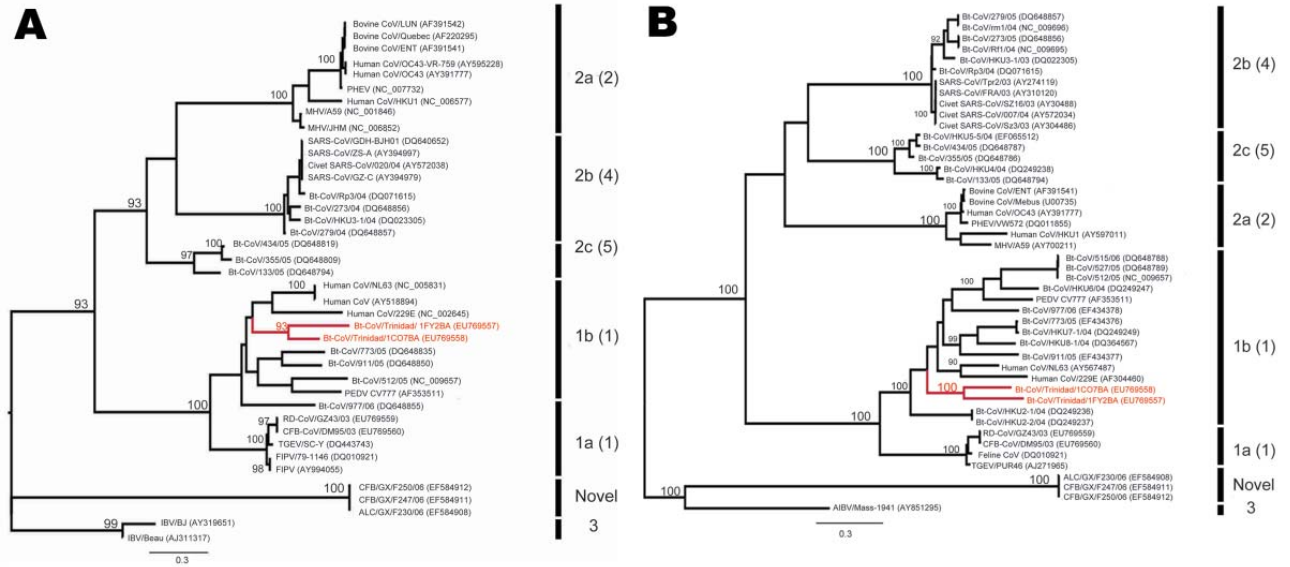

Figure 1. Maximum likelihood trees of coronaviruses based on A) 780-bp fragment of the RNA-dependent RNA polymerase gene and B) 1,797 bp of the helicase (HEL) domain of open reading frame 1b. Trees were inferred under the General Time Reversible (GTR + Γ_4 + I) model by using PAUP* version 4.0b (Sinauer Associates, Inc., Sunderland, MA, USA). Bootstrap support values >90% are indicated. Previously defined phylogenetic groups and a putative novel group (*10*) are delineated by the bars on the right. The numbering of these groups is as described in the eighth report of the International Committee on Taxonomy of Viruses with the alternative grouping proposed by Tang et al. (*4*) in brackets. Trinidadian bat coronavirus sequences are highlighted in red. GenBank accession numbers are noted in parentheses. Scale bars indicate number of nucleotide substitutions per site.

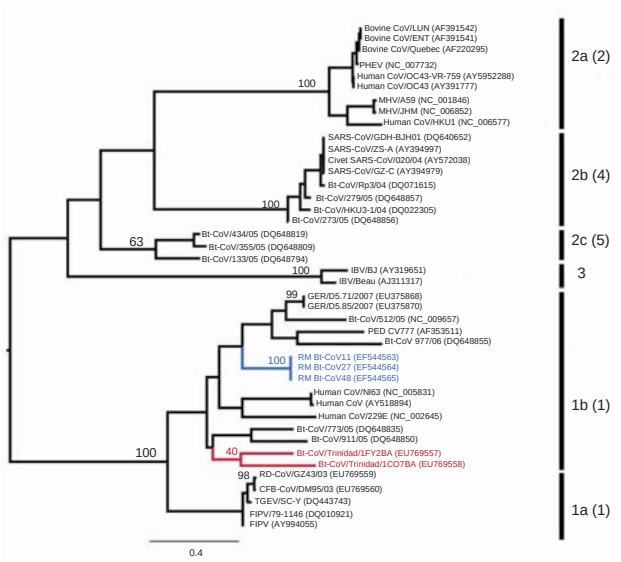

Figure 2. Maximum likelihood tree of coronaviruses based on 378-bp fragment of the RNA-dependent RNA polymerase gene. The tree was inferred under the General Time Reversible (GTR + Γ_4 + I) by using PAUP* version 4.0b (Sinauer Associates, Inc., Sunderland, MA, USA). Trinidadian bat coronavirus (Bt-CoV) sequences are highlighted in red and North American Bt-CoV in blue. Previously defined phylogenetic groups and a putative novel group (*10*) are delineated by the bars on the right. The numbering of these groups is as described in the eighth report of the International Committee on Taxonomy of Viruses with the alternative grouping proposed by Tang et al. (*4*) in brackets. Bootstrap support values for groups 1a, 1b, 2a–c, 3, and the lineage containing Trinidadian Bt-CoVs are shown. GenBank accession numbers are noted in parentheses. Scale bars indicate number of nucleotide substitutions per site.

again defined, but bootstrap support for the putative group 5 (*4*) was lower. The level of divergence between Trinidadian sequences was notably higher than among the North American (*6*) and German sequences (*7*).

Conclusions

Our detection of RNA from group 1 CoVs in Trinidadian bats shows that Bt-CoVs have a wider distribution than previously suspected and is added support for bats as the original host species for these viruses. Group 1 CoVs form 2 well-supported clades designated 1a and 1b (*12*). The Trinidadian bt-CoV clustered within the latter clade, which contains all other group 1 Bt-CoVs, including those from Germany and North America, and the 3 known group 1 human CoVs associated with respiratory illness (*13–15*). Despite the geographic proximity of the bats from which the Trinidadian Bt-CoV sequences were derived—Couva and Fyzabad are 28 km (17 miles) apart, and Trinidad has an area of only 4,769 km^2 (1,864 square miles)—they are relatively highly divergent. This divergence might reflect virus adaptations to different host species; however, more data would be needed to confirm this. Given the mobility of bats, the possibility of the viruses having different geographic origins (perhaps even from outside Trinidad) cannot be ruled out. Further work on CoV diversity in Trinidad and the rest of the Americas, as well as on the ecology and behavior of susceptible bat species, is needed to understand the origins, evolution, and dispersal of these viruses.

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Rickettsia parkeri in Argentina

Santiago Nava, Yasmin Elshenawy, Marina E. Eremeeva, John W. Sumner, Mariano Mastropaolo, and Christopher D. Paddock

Clinical reports of an eschar-associated rickettsiosis in the Paraná River Delta of Argentina prompted an evaluation of *Amblyomma triste* ticks in this region. When evaluated by PCR, 17 (7.6%) of 223 questing adult *A. triste* ticks, collected from 2 sites in the lower Paraná River Delta, contained DNA of *Rickettsia parkeri*.

Argentina is a large, ecologically diverse country, with at least 10 Neotropical *Amblyomma* tick species that bite humans, including *Amblyomma triste* (*1*,*2*; Figure 1, panels A, B). Spotted fever group rickettsiae have been identified in 3 *Amblyomma* species in Argentina: *Rickettsia amblyommii* and *R. bellii* in *A. neumanni* ticks from Córdoba Province (*3*), a novel *Rickettsia* sp. in *A. parvum* ticks from Córdoba Province (*4*), and *R. rickettsii* and *R. bellii* in *A. cajennense* ticks from Jujuy Province (*5*). Human diseases in Argentina attributable to tick-borne rickettsiae have been recognized only recently, including several fatal cases of Rocky Mountain spotted fever caused by *R. rickettsii* in Jujuy Province (*5*,*6*), and a milder, eschar-associated, spotted fever rickettsiosis in the Paraná River Delta of Buenos Aires Province (*7*) that closely resembles a newly recognized rickettsial spotted fever in the United States caused by *R. parkeri* (*8*). *R. parkeri* has been detected recently in *A. triste* ticks collected in Uruguay and Brazil (*9*,*10*). We report the occurrence of *R. parkeri* in *A. triste* ticks collected along the Paraná River close to the locations of several recently identified cases of eschar-associated spotted fever.

The Study

Tick collections occurred at 2 sites in Buenos Aires Province, Argentina, during January through December 2007: Reserva Natural Otamendi (34°15′S, 58°52′W) (Figure 1, panel C) and Estación Experimental, Instituto Nacional de Tecnología Agropecuaria (INTA), Delta del Paraná, (34°11′S, 58°50′W) (Figure 1, panel D). Both are located in the lower Paraná River Delta region (Figure 2), which is the southern extension of the Paranense Province of the Amazon Phytogeographic Dominion. The region is characterized by a system of levees that surround temporarily or permanently flooded freshwater marshes (*11*). Humboldt's willow (*Salix humboldtiana*), Cockspur coral tree (*Erythrina crista-galli*), and *Sapium hematospermum* grow on the levees, and several species of bulrush (*Scirpus giganteus, Schoenoplectus californicus*, *Scirpus americanus*, and *Typha* sp.) and espadaña (*Zizaniopsis bonariensis*) comprise the dominant vegetation in the marshes. Medium to large mammals found at the study sites include wild marsh deer (*Blastocerus dichotomus*), capybara (*Hydrochoerus hydrochaeris*), pampas fox (*Lycalopex gymnocercus*), Geoffroy's cat (*Oncifelis geoffroyi*), cattle, horses, and dogs.

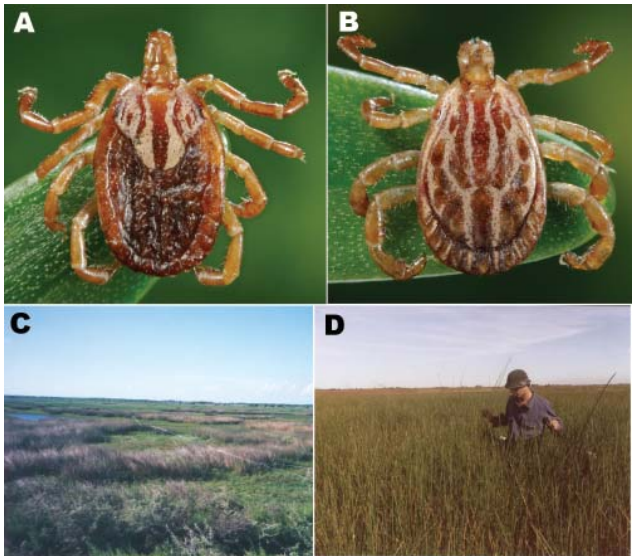

Figure 1. Adult female (A) and male (B) *Amblyomma triste* ticks and tick collection sites in the lower Paraná River Delta of Buenos Aires Province, Argentina, showing freshwater marsh habitats in the Reserva Natural Otamendi (C) and Estación Experimental, Instituto Nacional de Tecnología Agropecuaria, Delta del Paraná (D).

Questing adult ticks were collected from vegetation on the levees and in the marshes by using cloth flags and preserved in 96% ethanol. All ticks were identified by using standard taxonomic keys (*12*). *A. triste* ticks were the only ticks collected from vegetation. For molecular analyses, individual specimens were removed from the ethanol solution, air-dried, and minced with a sterile scalpel blade. DNA was extracted by using a QIAamp DNA Mini-Kit (QIAGEN, Valencia, CA, USA) and eluted in a final volume of 100 μL. DNA extracts were evaluated by using a nested PCR designed to amplify a segment of the rickettsial outer membrane protein A gene (*ompA*) as described previously (*13*). In brief, 5 μL of each DNA extract was used as template with primers 190.70 and 190.701 in the primary

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reaction. Two microliters of each completed primary reaction was used as template with primers 190-FN1 and 190-RN1 in the nested reactions. Primers were used at a final concentration of 300 nmol/L in a 50-μL reaction mixture. All amplicons were sequenced and compared to those in GenBank by using the BLAST 2.0 program (http://blast.ncbi.nlm.nih.gov/blast.cgi). Separate laboratory rooms were used for extracting tick DNA, performing primary and nested PCRs, and sequencing reactions. Water blanks were used for each primary and nested assay, and all extracts that provided amplicons of the expected size were retested to confirm the result.

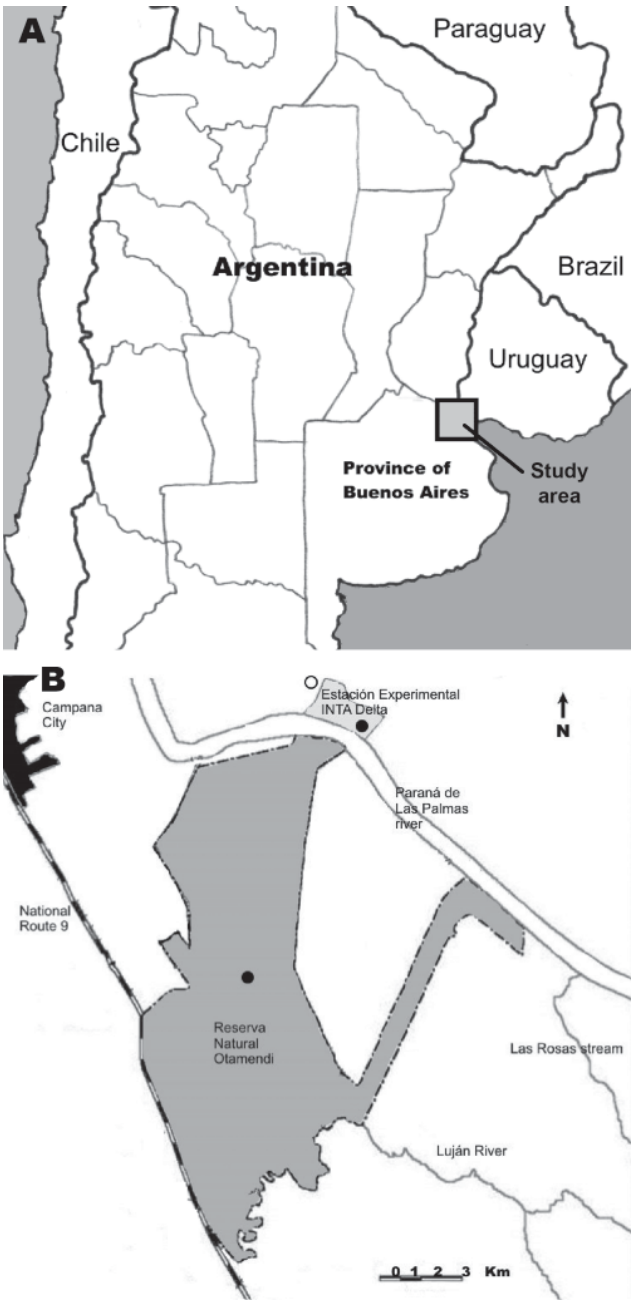

Figure 2. A) Location of study area in the lower Paraná River Delta of Argentina. B) Tick collection sites along the Paraná River (dark circles) and a recently reported case of eschar-associated rickettsiosis (open circle) identified by clinicians in Buenos Aires Province, Argentina *(7)*.

Amplicons were obtained from DNA extracts of 4 (5.8%) of 69 *A. triste* ticks collected from Reserva Natural Otamendi and 13 (8.4%) of 154 ticks collected from Estación Experimental INTA Delta del Paraná (Table). All 17 DNA samples produced amplicons of the expected sizes in primary reaction and nested reactions of the assay. Each 590-bp product (excluding primers) from the primary reaction was sequenced, and all sequences showed 100% identity with each other (GenBank accession no. FJ172358) and with the corresponding *ompA* sequence of *R. parkeri* (U43802).

Conclusions

This study provides definitive evidence of *R. parkeri* in Argentina. Our findings have relevance for public health because the infected ticks were collected from the lower Paraná River Delta near the origin of several recently identified cases of eschar-associated rickettsiosis (*7*; Alfredo Seijo, pers. comm.). In Argentina, at least 15 species of hard ticks bite humans (*1*); however, the only *Amblyomma* tick reported to bite humans in the lower Paraná River Delta is *A. triste* (*2*). Our data suggest that *A. triste* ticks are vectors of *R. parkeri* in this region of Argentina. The prevalence of *R. parkeri*–infected *A. triste* ticks identified at these 2 locations is within the range of the infection prevalence of this agent reported in questing adult ticks collected in the state of São Paulo, Brazil (9.7%) and in Canalones County in southern Uruguay (2.6%) (*9*,*10*).

In South America, *R. parkeri* has been detected only in *A. triste* ticks (*9*,*10*), and in the United States, *R. parkeri* is found almost exclusively in *A. maculatum* ticks (*8*,*13*). *A. triste* and *A. maculatum* ticks are phylogenetically and morphologically similar, and *R. parkeri* appears to be strongly associated with these closely related tick species. Another human-biting Neotropical tick, *A. tigrinum*, is closely related to *A. triste* and *A. maculatum* ticks (*12*). In this context, *A. tigrinum* ticks may also be involved in the transmission of *R. parkeri* in South America. The distribution of *A. triste* ticks extends from Argentina to Mexico, but this tick has been reported to bite humans only in a few regions of Argentina, Uruguay, and Venezuela (*1*). Because we are not aware of any records to indicate that immature stages of *A. triste* ticks will bite humans (*1*,*2*,*14*), our investigation focused on adult questing ticks for evidence of infection with *R. parkeri*. Preliminary studies indicate that peak adult *A. triste* abundance and activity in the lower Paraná River Delta occurs during August through November (S. Nava, unpub. data), similar to the seasonal distribution described

Table. Prevalence of infection with *Rickettsia parkeri* in adult *Amblyomma triste* ticks collected in the lower Paraná River Delta, Buenos Aires Province, Argentina, 2007

Collection site	Month of collection	No. females (no. positive)	No. males (no. positive)	Total no. tested (no. positive)
Reserva Natural Otamendi	Jan	20 (0)	6 (0)	26 (0)
	Mar	5 (0)		5 (0)
	Jun	1 (0)		1 (0)
	Jul	2 (1)	3 (0)	5 (1)
	Nov	10 (0)	5 (0)	15 (0)
	Dec		17 (3)	17 (3)
Estación Experimental, Instituto Nacional de Tecnología Agropecuaria, Delta del Paraná	Aug	60 (5)	64 (3)	124 (8)
	Nov	15 (4)		15 (4)
	Dec	15 (1)		15 (1)
Total		128 (11)	95 (6)	223 (17)

for *A. triste* populations in southern Uruguay (*14*); most cases of eschar-associated disease in Argentina occur during this same interval (*7*; A. Seijo, pers. comm.).

No immature *A. triste* ticks were collected by flagging during this investigation. However, larvae and nymphs were found at these study sites attached to the guinea pig (*Cavia aperea*) and several species of sigmodontine rodents, including Azara's grass mouse (*Akodon azarae*), the yellow pygmy rice rat (*Oligoryzomys flavescens*), the black-footed pygmy rice rat (*O. nigripes*), the red hocicudo (*Oxymycterus rufus*), and the Argentine swamp rat (*Scapteromys aquaticus*) (S. Nava, unpub. data). These findings suggest that one or more of these species may be involved in the natural transmission cycle of *R. parkeri* in this region.

Ecologic studies of *A. triste* ticks collected along the Paraná River in the states of São Paulo and Mato-Grosso do Sul in Brazil indicate that this tick is well-adapted to marsh habitats (*15*); the results of this investigation support this observation. The occurrence of an *R. parkeri* rickettsiosis-like disease in humans in the Paraná River Delta suggests that similar cases of human illness may occur in palustrine regions of other Central and South American countries where this tick is found. Additional studies are needed to better understand the natural history of *R. parkeri* in Argentina and in other countries of the Western Hemisphere.

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Transmission of Atypical Bovine Prions to Mice Transgenic for Human Prion Protein

Vincent Béringue, Laëtitia Herzog, Fabienne Reine, Annick Le Dur, Cristina Casalone, Jean-Luc Vilotte, and Hubert Laude

To assess risk for cattle-to-human transmission of prions that cause uncommon forms of bovine spongiform encephalopathy (BSE), we inoculated mice expressing human PrP Met^{129} with field isolates. Unlike classical BSE agent, L-type prions appeared to propagate in these mice with no obvious transmission barrier. H-type prions failed to infect the mice.

The epizootic of bovine spongiform encephalopathy (BSE) is under control in European countries >20 years after the first cases were diagnosed in the United Kingdom. Thus far, BSE is the only animal prion disease known to have been transmitted to humans, leading to a variant form of Creutzfeldt-Jakob disease (vCJD) (*1*). The large-scale testing of livestock nervous tissues for the presence of protease-resistant prion protein (PrP^{res}) has enabled assessment of BSE prevalence and exclusion of BSE-infected animals from human food (*2*). This active surveillance has led to the recognition of 2 variant PrP^{res} molecular signatures, termed H-type and L-type BSE. They differ from that of classical BSE by having protease-resistant fragments of a higher (H) or a slightly lower (L) molecular mass, respectively, and different patterns of glycosylation (*3*–*5*). Both types have been detected worldwide as rare cases in older animals, at a low prevalence consistent with the possibility of sporadic forms of prion diseases in cattle (*6*). Their experimental transmission to mice transgenic for bovine PrP demonstrated the infectious nature of such cases and the existence of distinct prion strains in cattle (*5*,*7*–*9*).

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Like the classical BSE agent, H- and L-type prions can propagate in heterologous species (*7*–*11*). Thus, both agents are transmissible to transgenic mice expressing ovine PrP (VRQ allele). Although H-type molecular properties are conserved on these mice (*9*), L-type prions acquire molecular and neuropathologic phenotypic traits undistinguishable from BSE or BSE-related agents that have followed the same transmission history (*7*). Similar findings have been reported in wild-type mice (*8*). An understanding of the transmission properties of these newly recognized prions when confronted with the human PrP sequence is needed. In a previous study, we measured kinetics of PrP^{res} deposition in the brain to show that L-type prions replicate faster than BSE prions in experimentally inoculated mice that express human PrP (*7*). In a similar mouse model, the L-type agent (alternatively named BASE) was also shown to produce overt disease with an attack rate of ≈30% (*12*). However, no strict comparison with BSE agent has been attempted. As regards the H-type agent, its potential virulence for mice that express human PrP Met^{129} remains to be assessed. We now report comparative transmission data for these atypical and classical BSE prions.

The Study

The bovine isolates used in this study have been previously described; they all exhibited high infectivity levels in bovine PrP mice (*4*,*7*,*9*). The equivalent of 2 mg of infected bovine brain tissue was injected intracerebrally into *tg650* mice. This line of mice overexpresses (≈6-fold) human PrP with methionine at codon 129 (Met^{129}) on a Zurich mouse PrP null background and has been shown to be fully susceptible to vCJD agent (*13*). The resulting transmission data available to date are summarized in the Table. The primary transmission of classical BSE isolates was inefficient as judged by the absence of clear neurologic signs and by Western blot detection of PrP^{res} in the brain of only 4/25 inoculated mice. The PrP^{res} banding pattern was essentially similar to that of vCJD (low molecular mass fragments and predominance of diglycoform species; Figure 1).

Secondary passages were performed by using PrP^{res}-negative or PrP^{res}-positive individual mouse brains. Every time brain homogenate from an aged mouse (≥630 days of age) was inoculated, transmission was observed in >80% of the mice, as determined by clinical signs and PrP^{res} accumulation. The mean survival time was ≈600 days (Table; additional data not shown). By the third passage, mean survival time approached ≈500 days, as is usually observed with vCJD cases (Table; *13*). The vCJD-like PrP^{res} profile was conserved in all 50 positive brains analyzed (Figure 1). Transmission of L-type isolates to *tg650* mice produced markedly different results.

Table. Transmission of classical and atypical BSE isolates to transgenic mice expressing human prion protein (Met^{129})*

Isolate	Origin (identification no.)	1st passage		2nd passage		3rd passage	
		Total affected†	Mean survival time‡	Total affected†	Mean survival time‡	Total affected†	Mean survival time‡
BSE	France (3)	1/6§	872	6/7	568 ± 65	8/8	523 ± 22
	France (3)	2/6	627, 842				
	Germany¶	1/4§	802	6/6	677 ± 54	8/8	555 ± 24
	Italy (128204)	0/5	606–775				
	Belgium	0/4	696–829				
L-type	Italy (1088)	9/9	607 ± 23	11/11	653 ± 13		NA
	France (7)	7/7	574 ± 35	0/8#	>450		
	France (10)	8/8	703 ± 19				
	France (11)	9/9	647 ± 26				
H-type	France (1)	0/6	376–721	0/7	350–850		NA
	France (2)	0/6	313–626	0/8	302–755		NA
	France (5)	0/10	355–838		NA		

*BSE, bovine spongiform encephalopathy; NA, not available (experiments still ongoing).
†Mice with neurologic signs and positive for protease-resistant prion protein by Western blotting.
‡Days ± SE of the mean. For mice with negative results, only the range of survival time is given.
§First passage performed on hemizygous mice. Note that the primary transmission of France (3) isolate was performed on both hemizygous and homozygous mice.
¶One passage on transgenic mice expressing bovine prion protein (*7*).
#Ongoing experiment.

First, the 4 L-type isolates induced neurologic disease in almost all mice; survival times averaged 600–700 days (Table). Second, PrP^{res} accumulated in the brain of all 33 mice analyzed. The molecular profile was distinct from BSE or vCJD; prominent monoglycosylated PrP^{res} species resembled those found in cattle (Figure 1). Third, we found neither shortening of the survival time on subpassage (1 isolate tested, 2 brains) (Table; data not shown) nor change in PrP^{res} profile (Figure 1).

In sharp contrast, BSE H-type isolates failed to transmit disease to or even infect *tg650* mice. None of the inoculated mice had a detectable level of PrP^{res} in the brain (22 analyzed). Secondary passages were performed with brains of mice that died at various time points. All inoculated mice survived, and none showed a PrP^{res} signal in the brain (15 mice analyzed).

To further compare the behavior of the 3 bovine prions in *tg650* mice, we examined the regional distribution

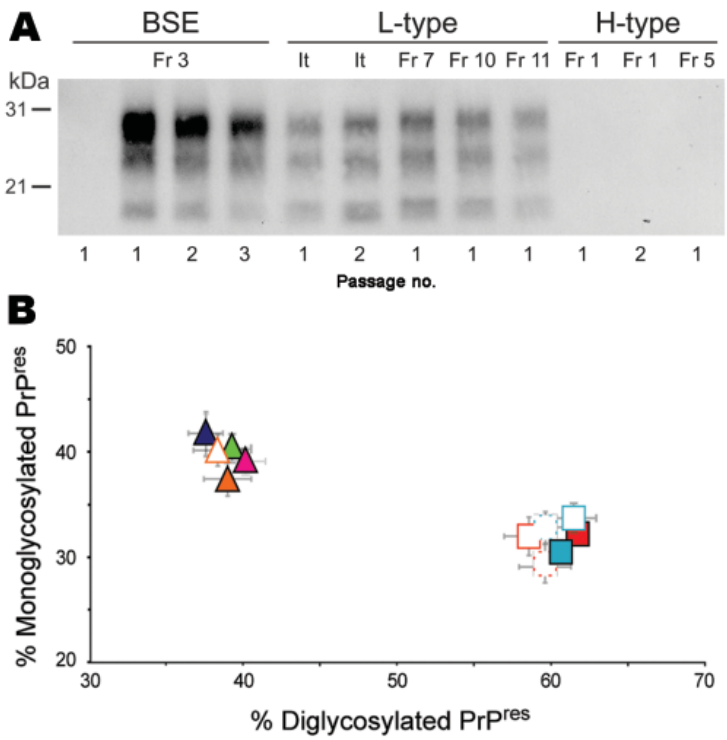

Figure 1. Protease-resistant prion protein (PrP^{res}) in the brains of human PrP transgenic mice infected with atypical or zoonotic bovine spongiform encephalopathy (BSE) agents. A) Representative Western blot analysis of PrP^{res} extracted (for detailed protocol, see *7*) from brain homogenates of mice at terminal stage of disease or at end of lifespan after serial transmission of atypical (L-type and H-type) or classical BSE isolates. The amount of equivalent brain tissue loaded onto the gels was 0.01 mg (BSE; Fr3 isolate, 2nd and 3rd passage), 0.3 mg (L-type and 1st passage of BSE), and 10 mg (PrP^{res}-negative samples). Anti-PrP monoclonal antibody Sha31 was used for PrP^{res} detection. Immunoreactivity was determined by chemiluminescence. B) Ratio of diglycosylated and monoglycosylated PrP^{res} species in the brains of mice after serial transmission of L-type or BSE isolates (data plotted as means ± standard error of the mean). Primary passage of L-type isolates are represented as triangles (orange, It; blue, Fr7; green, Fr10; pink, Fr11) and BSE as squares (light blue, Fr3; red, Ge). Passages are indicated by unfilled symbols of the same color (solid line, second passage; broken line, third passage). The ratio was determined after acquisition of PrP^{res} chemiluminescent signals with a digital imager as previously described (*7*). Note the distinct glycoform ratio between L-type and BSE groups. It, Italy; Fr, France; Ge, Germany.

and intensity of PrP^{res} deposition in the brain (*14*). Histoblot analyses (3 brains per infection) were performed on primary (L-type, H-type) and secondary passages (L-type, H-type, BSE). As shown in Figure 2, L-type and BSE agents showed clear differences according to both the aspect and localization of the PrP deposits. Granular PrP deposits were scattered throughout the brain with BSE, as has been previously observed with vCJD (*13*). The ventral nuclei of the thalamus, cerebral cortex, oriens layer of the hippocampus, and raphe and tegmentum nuclei of the brain stem were strongly stained. With BSE-L, the staining was finer and essentially confined to the habenular, geniculate, and dorsal nuclei of the thalamus; the lateral hypothalamus; the lacunosom moleculare layer of the hippocampus; the superficial gray layer of the superior colliculus; and the raphe nuclei of the brain stem. Finally, PrP^{res} could not be detected on brain sections from mice inoculated with H-type isolates (data not shown), thus confirming the Western blot data.

Conclusions

We found that atypical L-type bovine prions can propagate in human PrP transgenic mice with no significant transmission barrier. Lack of a barrier is supported by the 100% attack rate, the absence of reduction of incubation time on secondary passage, and the conservation of PrP^{res} electrophoretic profile. In comparison, transmission of classical BSE agent to the same mice showed a substantial barrier. Indeed, 3 passages were necessary to reach a degree of virulence comparable to that of vCJD agent in these mice (*13*), which likely reflects progressive adaptation of the agent to its new host. At variance with the successful transmission of classical BSE and L-type agents, H-type agent failed to in-

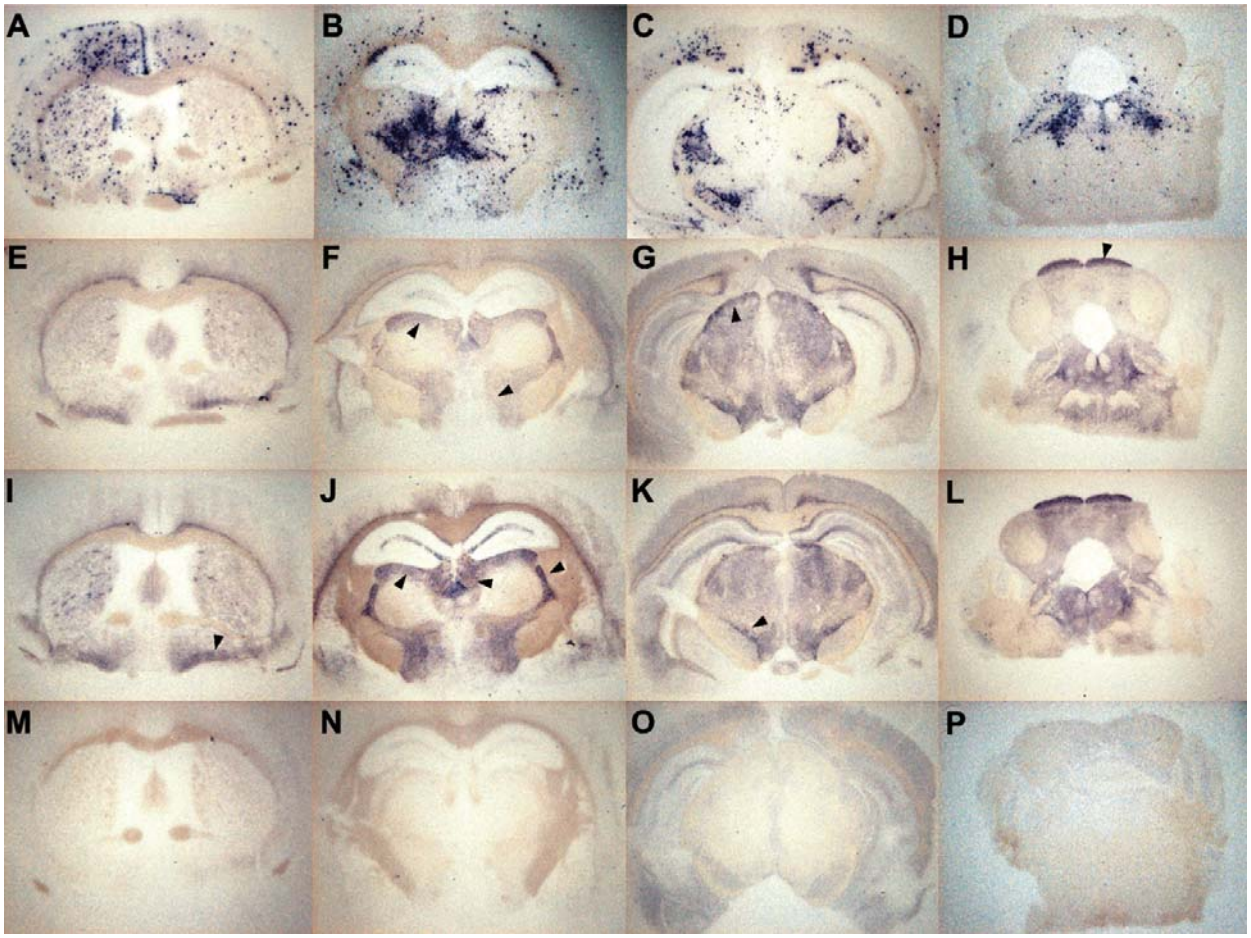

Figure 2. Representative histoblots in 4 different anteroposterior sections showing the distribution of disease-specific PrP^{res} deposits in the brains of *tg650* mice infected with bovine spongiform encephalopathy (BSE) or L-type BSE. Panels A–D show infection with BSE (second passage of France 3). Panels E–H show infection with L-type BSE (first passage of France 7). Panels I–L show infection with L-type BSE (second passage of Italy). Panels M–P show brain sections of an age-matched, mock-infected mouse, euthanized while healthy at 700 days postinfection, for comparison. Note the differing aspect and distribution of PrP^{res} deposits between brain of mice infected with BSE and BSE-L (arrowheads). Assignment of the positive brain regions has been made according to a mouse brain atlas after digital acquisition.

fect *tg650* mice. These mice overexpress human PrP and were inoculated intracranially with a low dilution inoculum (10% homogenate). Therefore, this result supports the view that the transmission barrier of BSE-H from cattle to humans might be quite robust. It also illustrates the primacy of the strain over PrP sequence matching for cross-species transmission of prions (*15*).

Extrapolation of our data raises the theoretical possibility that the zoonotic risk associated with BSE-L prions might be higher than that associated with classical BSE, at least for humans carrying the Met^{129} PrP allele. This information underlines the need for more intensive investigations, in particular regarding the tissue tropism of this agent. Its ability to colonize lymphoid tissues is a potential, key factor for a successful transmission by peripheral route. This issue is currently being explored in the *tg650* mice. Although recent data in humanized mice suggested that BSE-L agent is likely to be lymphotropic (*12*), preliminary observations in our model suggested that its ability to colonize such tissues is comparatively much lower than that of classical BSE agent.

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Human Illnesses Caused by *Opisthorchis felineus* Flukes, Italy

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We report 2 outbreaks of *Opisthorchis felineus* infection caused by the consumption of tench filets (*Tinca tinca*) from a lake in Italy. Of the 22 infected persons, 10 (45.4%) were asymptomatic. When present, symptoms (fever, nausea, abdominal pain, and myalgias) were mild. Eosinophilia occurred in all infected persons.

Opisthorchis felineus is a trematode that is transmitted to humans through the consumption of raw freshwater fish of the family Cyprinidae. Worldwide, the number of cases of human infection has been estimated to be 1.2 million (*1*). A high prevalence has been reported in Byelorussia, Russia, and the Ukraine. In the European Union, sporadic human infections have been documented in Germany, where the parasite has been detected in red foxes and cats, and in Greece (*2*–*6*).

In Italy, *O. felineus* was first described in cats and dogs in Pisa (Tuscany Region) and in cats in Turin (Piedmont Region), yet for over 100 years the infection was not detected or reported in animals and humans and no one investigated this pathogen (*7*,*8*). With regard to human infection, cases were reported in 2003 and 2005, when 2 outbreaks of opisthorchiasis occurred after persons consumed fish from Lake Trasimeno (central Italy) (*9*). Our study describes 2 recent outbreaks and provides the results of the epidemiologic investigation.

The Study

In August 2007, an outbreak in central Italy involved persons who had consumed fish at a private dinner. In October–November 2007, a second outbreak involved persons who had also eaten fish. For both outbreaks, index case-patients were interviewed to trace others who had eaten these meals. A case of opisthorchiasis was defined as *Opistorchidae* spp. eggs in a fecal sample or immunoglobulin (Ig) G antibodies to *Opisthorchis* in a serum sample from persons who had consumed raw freshwater fish.

We searched for parasites in fecal samples after formol-ether concentration by light microscopy. To investigate the presence of trematodes in fish from the lake where they had been caught, 800 specimens of 17 species were collected. Muscle tissues from these fish were digested with 1% pepsin and 1% HCl at 40°C to detect metacercariae.

Stool samples (4 g) were concentrated by a modified formalin-ethyl acetate procedure. Parasite DNA was purified from 200 μL of fecal pellets by using the QIAamp DNA stool kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The primers OP1 (5′-CGAGGGTCGGCTTATAAAC-3′) and OP2 (5′-AGCCTCAACCAAAGACAAAG-3′) were used to amplify the ITS2 region of the rDNA of the parasite eggs and metacercariae (*10*). The 250-bp fragment was sequenced and compared with the internal transcribed spacer (ITS2) sequences of *O. felineus*, *O. viverrini*, and *Clonorchis sinensis* present in the GenBank database. We used ELISA to search for IgG antibodies to *O. felineus* in blood samples by using excretory/secretory antigens, according to a standard protocol (*11*).

On August 4, 2007, 34 men from different villages in Viterbo Province attended a dinner in a private home, where they consumed marinated fish filets of tench (*Tinca tinca*) and of white fish (*Coregonus* sp.) from Lake Bolsena (Viterbo Province, central Italy). The fish had been frozen at –10°C for 3 days; they were then cut into filets ≈1-cm thick and marinated with vinegar and wine for 24 hours before consumption. On August 29, two of the men sought treatment at the hospital in Viterbo with symptoms of fever, abdominal pain, and diffuse myalgias; onset of symptoms had occurred 10 days earlier. In both men, laboratory findings showed marked leukocytosis (17.4 and 18.8 $\times 10^3$ cells/μL) with eosinophilia (10.1 and 13.9 $\times 10^3$ cells/μL) and elevated levels of alanine aminotransferase (ALT) (125 and 205 U/L). Examination of fecal samples showed *Opisthorchis* sp. eggs (Figure 1). Of the other 32 men who had attended the dinner at the private home, fecal samples of 18 were positive for *Opisthorchis* sp. eggs. Nine of these men had fever, nausea, abdominal pain, and myalgias. Specific IgG antibodies to *O. felineus* were detected only in the 20 men whose fecal samples were positive for eggs (attack rate 58.8%).

In all 11 symptomatic persons, onset of symptoms occurred ≈2 weeks after they consumed the fish. A patient with high levels of aspartate aminotransferase (AST) and

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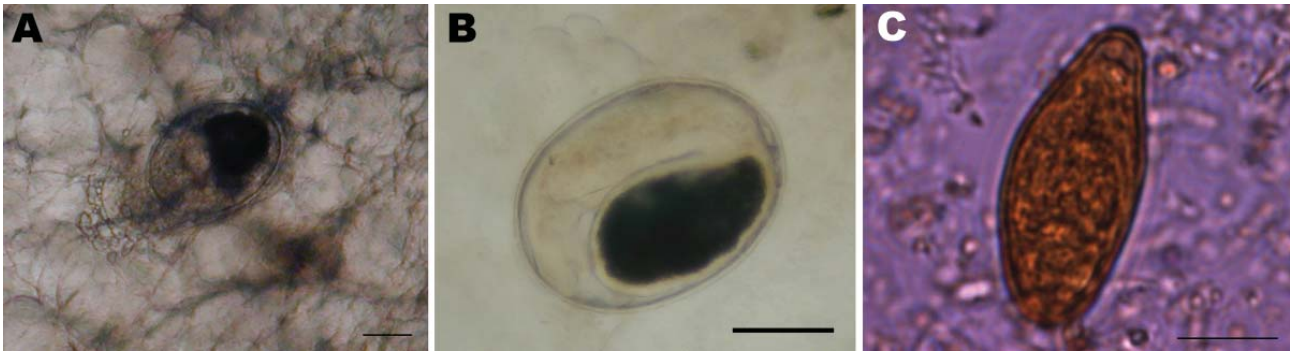

Figure 1. A) Metacercaria of *Opisthorchis felineus* in muscles of a tench (*Tinca tinca*) from Lake Bolsena (Latium region, central Italy). Scale bar = 100 μm. B) Metacercaria of *O. felineus* collected from a tench filet by digestion with 1% pepsin and 1% HCl. Scale bar = 100 μm. C) Egg detected in feces of the index patient of the August 2007 outbreak. Scale bar = 10 μm.

ALT (315 and 899 U/L, respectively) was hospitalized for 7 days and completely recovered after 4 weeks.

All 20 infected persons showed eosinophilia (mean 4 × 10^3cells/μL; range 0.27–14 × 10^3 cells/μL), and 8 of them had elevated ALT levels (mean 182.6 U/L; range 57–899 U/L). Nine and 11 persons were treated with praziquantel (25 mg/kg orally 3×/day for 1 day) or albendazole (10 mg/kg/day orally in 2 doses for 7 days), respectively. In all 20 patients, there was complete remission of symptoms, and eosinophilia and aminotransferase levels returned to normal. Eggs were detected posttreatment in the fecal sample of only 1 patient who had been treated with albendazole for 7 days, although the patient's leukocyte count was 6.9 × 10^3 cells/μL and eosinophilia had disappeared. He was treated successfully with praziquantel.

On November 22, 2007, a woman sought treatment of fever and diarrhea at the hospital in Rieti. Laboratory investigations showed eosinophilia (19.6 × 10^3 cells/μL) and elevated AST and ALT levels (118 and 364 U/L). Examination of a fecal sample showed *Opisthorchis* sp. eggs. The woman was treated with albendazole (400 mg/day orally in 2 doses for 7 days). Within 5 days, symptoms disappeared; aminotransferase levels slowly decreased, and the woman was discharged from the hospital. After treatment, no eggs were detected in the woman's feces. She reported that 33 days before seeking treatment, she had eaten marinated tench filets at a restaurant. A friend of the woman was also present at the meal but he had only tasted the marinated tench filets. No eggs were detected in the fecal sample taken from the friend, although eosinophilia was slightly increased. Nonetheless, IgG antibodies to *O. felineus* were detected in a serum sample collected 58 days after the man had consumed tench (attack rate 100%). The restaurant owner stated that the origin of the infected fish was Lake Bolsena.

Epidemiologic investigation of metacercariae in fish from Lake Bolsena showed a high level of infection in tenches (83.1% of the fish tested, range 1–146, median 4.5; Figure 1), yet metacercariae were not detected in any other species. To identify the species of *Opisthorchis*, we conducted PCR on eggs taken from patients' fecal samples and on metacercariae from tenches. Eggs and metacercariae were identified as those of *O. felineus*; we observed no difference in the amplified sequences (GenBank accession no. EU926762) between eggs and metacercariae of Italy, and between parasites from Lake Bolsena and Germany (reference DNA) (Figure 2). In addition, we observed no difference in 16 of the 17 sequences deposited in GenBank (from metacercariae collected from the fish of several Russian rivers) with the exception of 1 (GenBank accession no. EF688142), which is different from the others because of the presence of an A instead of a T at position 32 of the ITS2 sequence (data not shown).

Conclusions

In Italy, raw fish has become more popular in recent years, and the outbreaks we discuss reflect this change in eating habits. In fact, although *O. felineus* has apparently been circulating in Italy at least since the 19th century (*7*), the lack of cases of infection may be attributable to the low commercial value of the tench and the fact that it is traditional to cook fish well done in Italy.

In the 4 outbreaks in Italy, i.e., those investigated in 2003 and 2005 (*9*) and the 2 outbreaks that we investigated, the incubation period in symptomatic persons ranged from 2 to 4 weeks, which is consistent with reports in the literature (*12*). The attack rate was 100% in all but 1, the August 2007 outbreak (attack rate of 58.8%). This finding may be due to the fact that not all of the persons present at the private dinner had eaten marinated tench.

In the 4 outbreaks, 19 (59%) of the 32 infected persons were asymptomatic; no one had severe symptoms. The other 21 persons had only mild symptoms, probably because of the low number of parasites ingested and because infected persons did not regularly eat marinated tench. This epidemiologic and clinical picture differs from that observed in

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Patient 1     CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCACGCATTTG----------
Patient 2     CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCACGCATTTG----------
Metacercaria  CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCACGCATTTG----------
O. felineus   CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCACGCATTTG----------
O. viverrini  CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCGCGCAATTG----------
C. sinensis   CGAGGGTCGGCTTATAAACTATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCATGATTTCCCCACACAATTGTGTGTATGTG

Patient 1     TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTGGATGAGGGGGTGGCGGCGG
Patient 2     TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTGGATGAGGGGGTGGCGGCGG
Metarcecaria  TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTGGATGAGGGGGTGGCGGCGG
O. felineus   TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTGGATGAGGGGGTGGCGGCGG
O. viverrini  TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTAGATGAGGGGGTGGCGGCGG
C. sinensis   TGTGGGGTGCCGGATCTATGGCTTTTCCCCAATGTGCCGGACGCAACCATGTCTGGGCTGACTGCCTAGATGAGGGGGTGGCGGCGG

Patient 1     AGTCGTGGCTCAATTGTTGTTGTTATTGTTGTGAATGTGCGCGCTCCGTTGTTGGTCCTTTGTCTTTGGTTGAGGCT
Patient 2     AGTCGTGGCTCAATTGTTGTTGTTATTGTTGTGAATGTGCGCGCTCCGTTGTTGGTCCTTTGTCTTTGGTTGAGGCT
Metacercaria  AGTCGTGGCTCAATTGTTGTTGTTATTGTTGTGAATGTGCGCGCTCCGTTGTTGGTCCTTTGTCTTTGGTTGAGGCT
O. felineus   AGTCGTGGCTCAATTGTTGTTGTTATTGTTGTGAATGTGCGCGCTCCGTTGTTGGTCCTTTGTCTTTGGTTGAGGCT
O. viverrini  AGTCGTGGCTCAATTGTTGTTATTGTTGTGAATGCGCGCGCTCCGTTGTTGTTCCTTTGTCTTTGGTTGAGGCT
C. sinensis   AGTCGTGGCTCAATTGTTGTTATTGTT---GTGAATGTGCGCGCTCCGTTGTTGGTCCTTTGTCTTTGGTTGAGGCT
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Figure 2. Alignment of the internal transcribed spacer 2 region of rDNA. Patient 1, DNA from eggs of a patient of the August 2007 outbreak; patient 2, DNA from eggs of the patient of the October–November 2007 outbreak; metacercaria collected from a tench (*Tinca tinca*) from Lake Bolsena (Latium region, central Italy); *Opisthorchis felineus*, reference DNA from an adult worm of *O. felineus* from Germany; *O. viverrini*, reference DNA from an adult worm of *O. viverrini* from Thailand; *C. sinensis*, DNA from an adult worm of *Clonorchis sinensis* from China. Primer sequences are underlined, different bases are in **boldface**, and gaps are represented by dashes.

endemic regions of eastern Europe and Asia, where people frequently eat raw fish infected with *O. felineus* and where more severe symptoms have been reported (*12*,*13*).

Praziquantel and albendazole were effective treatments for all case-patients, except for 1 man, who had the greatest number of eggs in his stool sample. He was first treated unsuccessfully with albendazole and then successfully with praziquantel. Our data confirm the efficacy of praziquantel; however, albendazole is also apparently effective in eliminating worms and can be used when praziquantel is not available.

After the 2 most recent outbreaks, the local health service informed restaurant owners, fishermen, and the population in the areas of Lakes Bolsena and Trasimeno about the risks related to eating raw fish. At the same time, epidemiologic surveys on stray cats in the area showed that *O. felineus* eggs were present in their feces, with a prevalence of infection ranging from 23.5% to 40.0% (*14*,*15*). Given that most of these cats had eaten garbage from restaurants and fish carcasses discarded by fishermen, educating restaurant owners and fisherman on proper garbage disposal is important. According to the literature, metacercariae may be killed by freezing at –10°C for 5–70 days or at –28°C for 24 hours, depending on the size of the fish (*13*). In the August 2007 outbreak, the fish had been frozen at –10°C for 3 days, which did not completely kill the metacercariae. Even if fish are frozen in a home freezer, there is no way of knowing the internal temperature of the fish. Consumers should be warned about the risk of consuming raw fish regardless of where it has been frozen.

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Identification of New Rabies Virus Variant in Mexican Immigrant

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A novel rabies virus was identified after death in a man who had immigrated from Oaxaca, Mexico, to California, USA. Despite the patient's history of exposure to domestic and wild carnivores, molecular and phylogenetic characterizations suggested that the virus originated from insectivorous bats. Enhanced surveillance is needed to elucidate likely reservoirs.

Rabies is an acute, progressive, fatal encephalitis caused by viruses in the family *Rhabdoviridae*, genus *Lyssavirus*. Globally, 11 major genotypes have been identified as etiologic agents of this zoonosis (*1*). *Rabies virus* (RV), the type species, is the most widespread and epidemiologically important member of the genus and the only taxon documented in the New World. Major mammalian reservoirs reside in the orders Carnivora and Chiroptera. Several specific RV variants have been characterized from different mammalian hosts, such as dogs, foxes, mongooses, and other carnivores, and bats. Within North America, distinct RV variants have been associated with rabid wildlife, including foxes, coyotes, raccoons, skunks, and multiple species of frugivorous, insectivorous, and hematophagous bats. Antigenic and genetic characterization of RV isolates, combined with traditional epidemiologic methods, is used to infer transmission events when a history of animal exposure is lacking or inconclusive.

In March 2008, a man who had recently immigrated from Mexico went to a hospital in Santa Barbara County, California, USA, where he died. Rabies was suspected, and a history was obtained of prior dog exposure and a confirmed fox bite in Oaxaca, Mexico, 110 days before the onset of neurologic symptoms (*2*). The primary objective of this study was to identify and molecularly characterize the isolate obtained from this patient. Our aims were to determine likely transmission event(s) associated with the case and to demonstrate the need for a better understanding of the biodiversity and epidemiology of RV variants and their reservoirs in this region.

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The Study

At autopsy, brain samples were obtained from the patient. Presence of RV antigen on brain tissue was confirmed by the direct fluorescent antibody test (www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm) and direct rapid immunohistochemical test (*3*) (Figure 1). Antigenic typing was performed with a panel of anti-RV nucleoprotein (N) monoclonal antibodies as described (*4*–*6*). Comparison of the human sample with established reaction patterns of RV variants showed that the closest antigenic match was among insectivorous bat patterns, specifically a unique RV variant in Colorado *Myotis* sp. and several *Tadarida brasiliensis* variants (online Appendix Table, available from www.cdc.gov/EID/content/14/12/1906-appT.htm). Patterns obtained from hematophagous bat-, canine-, and terrestrial carnivore–associated RV variants were not consistent with the pattern obtained from the patient.

Total RNA was extracted from infected tissue, and the entire N gene was amplified by reverse transcription–PCR in 2 overlapping amplicons, as described (*7*). Phylogenetic analyses were conducted by comparing full and partial RV N sequences with sequences derived from major extant rabies enzootics in both dogs and vampire bats in Mexico, as well as sequences associated with RVs maintained by other bat species and wild terrestrial carnivores from the United States and the Americas (*8*–*10*) (Figure 2). MEGA and BioEdit software were used to perform the phylogenetic reconstructions and sequence analyses (*11*,*12*).

Rabid dogs and vampire bats are the most common sources of exposure for humans in Mexico (www.salud.gob.mx/unidades/cdi/documentos/rabia.pdf). Residual canine rabies enzootics persist in central and southeastern Mexico, whereas vampire bat rabies is found throughout a wide geographic focus, particularly in the tropical and subtropical areas (*8*,*10*). In addition, at least 20 different lineages of RV that are associated with at least 9 bat species have been described in Mexico (*8*). Nevertheless, phylogenetic analyses of the RV obtained from the brain sample of the patient did not support a close relationship with any of the RV variants previously described. The isolate was found to be most closely related with those from Mexican free-tailed bats (*T. brasiliensis*); overall average identity was 95% but clearly segregated in an independent lineage (Figure 2). Given that the average percentage of genetic divergence among previously sequenced members of the

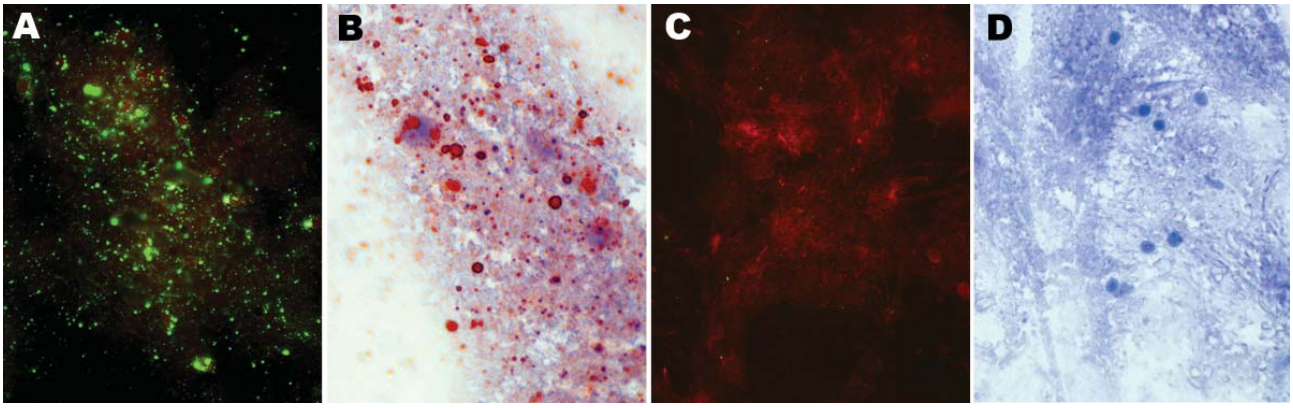

Figure 1. Detection of rabies virus antigen in brain impressions of the patient by direct fluorescent antibody test counterstained with Evans blue, 200× total magnification: A) positive control; B) negative control. Direct rapid immunohistochemistry test counterstained with hematoxylin, 400× total magnification: C) positive control; D) negative control.

T. brasiliensis RV clade ranges between 2.4% for the full N over a 20-year period and 1% for the partial N over a 40-year period, the extent of genetic divergence (5%) between the Oaxaca sample and the *T. brasiliensis* RV clade suggests that this isolate represents a new RV variant. Additionally, although the Oaxaca sample shares a distinctive molecular signature with the *T. brasiliensis* RV clade (i.e., conserved amino acid sequence alanine, aspartic acid, and threonine located at positions 377–379 within the N gene), the histidine at position 321, which is unique and highly conserved in members of the *T. brasiliensis* RV lineage, was changed to glutamine in the Oaxaca patient.

Although this patient's history indicated that he had been bitten near his home (San Vicente Coatlan, district of Ejutla, Oaxaca) by a *costoche* (*2*) (gray fox, *Urocyon cinereoargenteus*), the genetic and phylogenetic analyses did not support a close relationship to any known RV associated with terrestrial carnivores. RVs of major rabies epizootics associated with dogs and other terrestrial carnivores in Mexico and the United States are genetically distinct (average genetic distance 14%–16%) from those in bats throughout North America. Also, although the RV associated with the human case was nested within the monophyletic assemblage of bat RV variants, RV variants phylogenetically closest to this case were still genetically distant. RV variants associated with North American *Tadarida* and vampire bat rabies in Mexico were from 5% to 7% divergent from that of the human case.

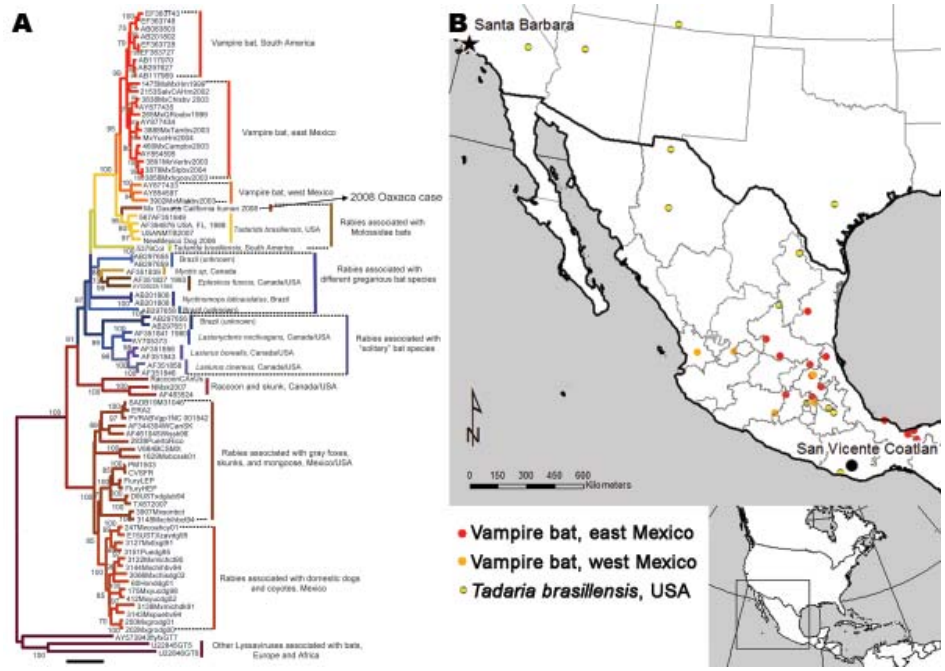

Figure 2. A) Phylogenetic tree of complete lyssavirus nucleoprotein genes, comparing the patient isolate with representative rabies virus variants associated with common New World animal reservoirs. B) Map showing the locations of representative samples associated with rabies transmitted by *Tadarida brasiliensis* and vampire bats used in the analysis.

Results of partial RV N gene sequence analyses indicated that at least 2 other human rabies cases—one in California in 1995 and the other in Nuevo Leon, Mexico, in 1999—were associated with the *T. brasiliensis* variant. These cases segregated within the monophyletic assemblage that includes enzootic rabies in *T. brasiliensis* bats collected over a period of ≈40 years in the United States and Mexico; the Oaxaca human case sample fell outside the *T. brasiliensis* clade, forming an independent lineage that was statistically supported in both the partial and full N phylogenetic reconstructions. These results plus the amino acid change found at position 321 in the RV associated with this case reinforced the concept of a new RV lineage associated with an unknown animal reservoir.

Conclusions

In 2008, a Mexican immigrant with a history of fox bite (*2*) died in California of infection with an RV variant most closely associated with RVs associated with insectivorous bats (*T. brasiliensis*). Both the molecular and phylogenetic characterizations of this RV suggest that this is a new lineage. Although the primary reservoir or most likely origin of this RV was determined to be an insectivorous bat (unknown species), the history of carnivore exposure suggests that a secondary transmitter (vector) could have been involved in the transmission chain, as has been reported in other cases (*13*). One cannot, however, rule out the possibility that the unknown reservoir species of this new RV lineage is, in fact, a different bat species (which could have been involved in the primary transmission after an unnoticed or cryptic exposure) or a terrestrial carnivore (e.g., the biting fox). The establishment of an insectivorous bat-derived RV variant in a terrestrial reservoir (i.e., striped skunk) in northern Arizona has been described (*14*). Enhanced epidemiologic surveillance and intensified research to characterize RV variants and their reservoirs in the region are needed to resolve this intriguing discovery.

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Avian Influenza Outbreaks in Chickens, Bangladesh

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To determine the epidemiology of outbreaks of avian influenza A virus (subtypes H5N1, H9N2) in chickens in Bangladesh, we conducted surveys and examined virus isolates. The outbreak began in backyard chickens. Probable sources of infection included egg trays and vehicles from local live bird markets and larger live bird markets.

The threat that highly pathogenic avian influenza (HPAI) A virus subtype H5N1 poses to poultry and public health has intensified (*1*). As the virus becomes established in poultry in developing countries, the number of human cases increases (*1*,*2*). Countries in the Asian Association for Regional Co-operation are especially vulnerable to virus perpetuation because of insufficient biosecurity, rearing of chickens and ducks together, selling of live birds, and deficient disease surveillance. To prevent human infection with avian influenza (H5N1), knowledge of avian influenza epidemiology is needed. We therefore describe the epidemiology of HPAI outbreaks in chickens in Bangladesh.

The Study

Through July 10, 2007, we investigated 52 outbreaks caused by HPAI virus (H5N1) and 3 outbreaks caused by low-pathogenicity avian influenza (LPAI) virus (H9N2) in chickens in Bangladesh. After a high number of chicken deaths on a farm was reported to an *upazila* (a lower administrative unit of Bangladesh) veterinarian, the sick chickens on the farm were examined. From each of 55 outbreaks, 2 dead chickens were sent to a field disease investigation laboratory or to the Central Disease Investigation Laboratory, where oropharyngeal swabs were tested for avian influenza A virus antigen. From cases with positive results, tracheal samples were referred to the National Reference Laboratory for Avian Influenza (NRL-AI) for viral RNA extraction and purification (*3*), reverse transcription–PCR that used a primer set of hemagglutinin (H) genes (*4*), and end-product visualization. When NRL-AI confirmed H5, the farm was considered HPAI affected and was reported to the Department of Livestock Services. Tracheal samples from chickens involved in 37 outbreaks, including those that were A-antigen positive but H5 negative, were sent to the Veterinary Laboratory Agency in the United Kingdom for confirmation. A farm on which influenza subtype H9N2 was found was considered LPAI affected. All farms affected with HPAI or LPAI virus were called avian influenza–affected farms. A district or *upazila* with at least 1 avian influenza–affected farm was considered an infected district or infected *upazila.*

To collect information about the farms, we used a pretested questionnaire administered by 2 veterinarians. The form had space where veterinarians could add additional comments on the probable virus sources for infections by backward tracing (window ≤21 days of onset of clinical signs) and sources of spread by forward tracing (window between onset of clinical signs and culling), which they obtained by interviewing the affected farmers and allied personnel. Farm geographic coordinates were recorded. Through another questionnaire, we collected data on commercial and backyard farms and outbreaks from the *upazila* livestock offices. All avian influenza data stored at the Department of Livestock Services head office, field disease investigation laboratories, the Central Disease Investigation Laboratory, and NRL-AI were also collected.

Summary statistics were computed and plotted by using Excel (Microsoft, Redmond, WA, USA), Arc View 9.1 (Environmental Systems Research Institute, Redlands, CA, USA), and STATA 7 (Stata Corp., College Station, TX, USA). A 7-day rolling mean of avian influenza–affected farms, according to date of clinical onset of disease, was calculated from January 12, 2007, and plotted as a bar chart on day 4 for each value. Attack rates of farms were calculated separately for *upazilas* of every infected district (Table 1).

We found that the H cleavage site of the selected influenza subtype H5N1 isolates (determined from the Veterinary Laboratory Agency) contained polybasic amino acids, which are characteristic of HPAI A viruses (Table 2). According to categories established by the Food and Agriculture Organization (*5*), 5 breeder and 2 layer farms had production system 2, 28 layer farms and 1 broiler farm had system 3, and 20 backyard farms had system 4. The

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Table 1. Attack rates (infected *upazila*[*s*] for the infected districts) of avian influenza outbreaks in Bangladesh, 17/64 districts, January–July 2007

District	No. farms, infected/total*		Attack rate (95% CI)†	
	Commercial	Backyard	Commercial	Backyard
Dhaka	14/744	0/0	0.019 (0.027–0.009)	
Dinajpur	0/29	1/124,872		0.000008 (0.000018–0.000002)
Gaibandha	1/69	0/52,950	0.014 (0.041–0.009)	NA
Gazipur	3/1,956	0/0	0.001 (0.0032–0.0002)	NA
Jamalpur	5/144	0/58,962	0.034 (0.063–0.005)	NA
Jessore	2/298	3/331,295	0.006 (0.014–0.002)	0.000003 (0.000008–0.000002)
Joypurhat	0/124	1/101,152	NA	0.000009 (0.000027–0.00009)
Lalmonirhat	0/85	2/76,582	NA	0.00002 (0.00005–0.00001)
Magura	0/82	1/83,607	NA	0.00001 (0.00003–0.00001)
Naogaon	0/109	2/80,752	NA	0.00002 (0.00005–0.00001)
Narayangonj	7/925	0/0	0.007 (0.012–0.002)	NA
Nilphamari	0/119	5/277,984	NA	0.00001 (0.00003–0.00001)
Noakhali	1/97	0/0	0.010 (0.029–0.009)	NA
Rajbari	1/395	2/311,279	0.002 (0.006–0.002)	0.00006 (0.000014–0.000002)
Rangpur	0/95	2/143,263	NA	0.00001 (0.00003–0.00001)
Tangail	1/227	0/91,650	0.004 (0.012–0.004)	NA
Thakurgaon	0/1,137	1/73,391	–	0.00001 (0.00003–0.00001)
Total	35/5,635	20/1,807,739	0.006 (0.008–0.004)	0.00001 (0.000014–0.000006)

*Infected, avian influenza–infected farms; total, farms in the infected *upazila*(*s*) of a district.
†CI, confidence interval; NA, not applicable.

7-day rolling means of the numbers of avian influenza–affected farms are shown in Figure 1; the temporal and spatial spreads, in Figure 2. The index farm was recorded on January 15, 2007, at a local live bird market in Sarishabari *upazila* in Jamalpur district. We hypothesized that the infection probably came from chickens in nearby backyard farms because high numbers of deaths in this population went uninvestigated.

The outbreaks peaked on March 26, 2007, when 11 affected farms in 3 districts—Dhaka, Gazipur, and Narayangogj—formed a cluster, indicating a common source. The source may have been larger live bird markets, which probably infected chickens of the 15 districts. The first avian influenza–affected backyard farm was reported on March 22, 2007, the date when avian influenza was confirmed in Bangladesh. Of the 20 backyard farms, 14 were in 7 northern districts.

The overall attack rates for the *upazilas* of the infected districts were 6/1,000 commercial farms and 1/100,000 backyard farms (Table 1). Uninvestigated deaths of backyard chickens could result in underestimation of the attack rate.

Among the 9 probable sources of infection, egg trays and contaminated vehicles from larger live bird markets and local live bird markets accounted for 47% of probable virus sources, eggs for 48%, and apparently healthy chickens for 5%. One avian influenza–affected farm disposed of ≈1,000 dead chickens in an open field before diagnosis was confirmed. For the backyard chickens, sources of spread were selling chickens (5%), giving chickens to relatives or neighbors (15%), moving birds through local poultry vendors, and hiding birds during culling operations (10%).

On the index farm, chickens in 1 shed were infected, but chickens in 2 other sheds <40 yards away remained clinically unaffected through the time of culling (71 days after clinical onset). Although the media reported that a corporate-run poultry farm, Biman Poultry Complex, was the first avian influenza–affected farm in Bangladesh, our investigation found it to be the third. Of the 5 breeder farms, 2 had imported chicks from the United States and 1 from

Table 2. Samples sent to the Veterinary Laboratory Agency, UK, during outbreaks of highly pathogenic avian influenza, Bangladesh, 2007*

Month†	No. samples sent	Influenza virus subtype, no. positive		Hemagglutinin cleavage site motif
		H5N1	H9N2	
Jan	0	NA	NA	NA
Feb	0	NA	NA	NA
Mar	0	NA	NA	NA
Apr	13	12	1	PQGERRRKKRGLF
May	10	9	1	PQGERRRKKRGLF
Jun	8	6	1	PQGERRRKKRGLF
Jul	6	5	NA	NA
Total	37	32	3	NA

*NA, not applicable.
†Month when the first clinical outbreak was reported.

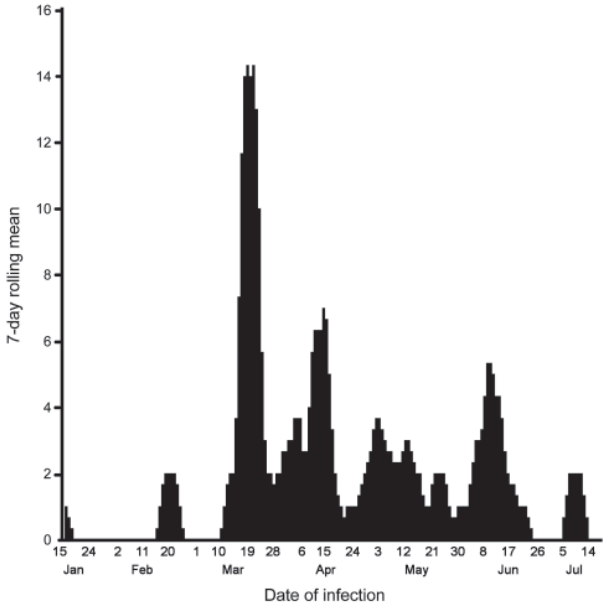

Figure 1. Seven-day rolling mean of occurrence of avian influenza–affected farms in outbreaks of highly pathogenic avian influenza, Bangladesh, January–July, 2007.

the United States and France, but these chicks had arrived >21 days before clinical onset of HPAI.

On May 22, 2008, the Directorate General of Heath Services, Bangladesh, declared that a sample collected from a child in January 2008 was diagnosed by the US Centers for Disease Control and Prevention as positive for influenza virus (H5N1). Before this time, no human infection with influenza virus (H5N1) had been reported in Bangladesh. Lack of human cases may have resulted from early immunologic response (*6*,*7*), genetic variation in receptors (*8–10*), poor surveillance of disease in humans, or using antiviral drugs during culling of birds.

Conclusions

Our investigation showed that the epicenter of the HPAI outbreaks in Bangladesh was the Sarishabari *upazila* of Jamalpur district and that the primary source of infection was backyard chickens. Phylogenetic analysis on 1 influenza virus (H5N1) isolate showed that it belongs to the subclade 2.2 of the Qinghai lineage (*11*), most closely related to viruses isolated from Afghanistan, Mongolia, and Russia (*11*). Therefore, the virus might have entered Bangladesh through migratory birds (*12–14*). The presence of influenza virus subtype H9N2 in chickens on 3 farms, however, raises the question of when this virus was introduced to Bangladesh. An earlier introduction or emergence of LPAI virus (H9N2) in backyard chickens cannot be ruled out because ≈18% of backyard chickens tested during 2000–2003 were seropositive for avian influenza virus (*15*).

This study illustrates the progression of HPAI in Bangladesh. Further study is needed to provided more evidence for the sources we have identified.

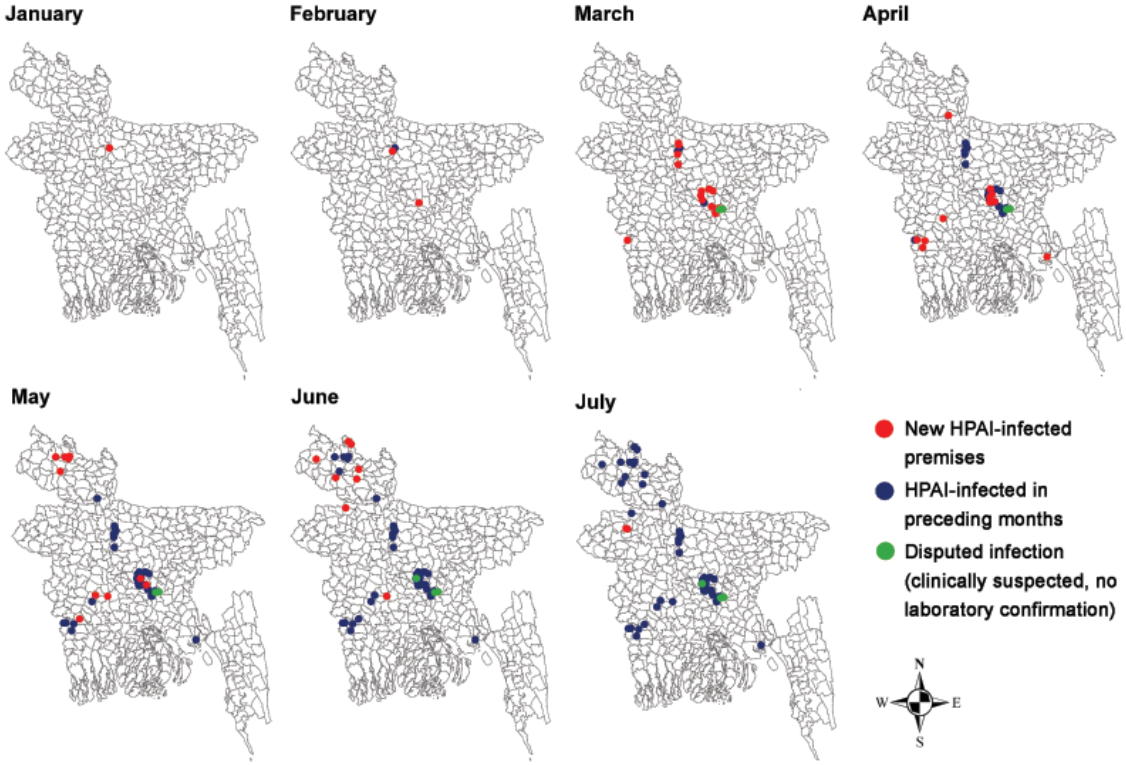

Figure 2. Monthly spread of highly pathogenic avian influenza (HPAI) outbreaks in chickens, Bangladesh, January–July, 2007.

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Outbreak of Trichinellosis Caused by *Trichinella papuae*, Thailand, 2006

Chowalit Khumjui, Pravit Choomkasien, Paron Dekumyoy, Teera Kusolsuk, Wandee Kongkaew, Mutita Chalamaat, and Jeffrey L. Jones

In 2006, the Thailand Ministry of Public Health studied 28 patients from a village in northern Thailand. All had myalgia, edema, fever, and gastrointestinal symptoms; most had eaten wild boar. A muscle biopsy specimen from a patient showed nonencapsulated larvae with a cytochrome oxidase I gene sequence of *Trichinella papuae*.

Trichinellosis is a foodborne parasitic zoonosis distributed worldwide that has not always been recognized for its importance, particularly in resource-poor countries (*1*). In northern Thailand, the main source of infection is domestic pigs kept by villagers (*2*). In addition to raising pigs, villagers hunt wild boar and barking deer to supplement their diet. The causative agent of most trichinellosis outbreaks in Thailand has been *Trichinella spiralis* (*3*). However, in 1994, an outbreak of *T. pseudospiralis* occurred (*4*).

T. papuae was described in 1999 (*5*) and has only been detected in Papua New Guinea (*6*). No human outbreaks of trichinellosis caused by *T. papuae* have been reported. On July 18, 2006, a cluster of 3 patients hospitalized with myalgia, edema, and eosinophilia was reported from Ban-rai district of Uthai Thani Province in Thailand. Local public health teams found an additional 16 villagers with similar symptoms and launched a full investigation on July 19.

The Study

The investigation team reviewed medical records, interviewed hospitalized patients, and performed active case finding in the implicated village by visiting the houses of the known patients and inquiring about symptoms of family members and neighbors. A suspected case-patient was defined as a resident of village A who had myalgia and facial, periorbital, trunk, or limb edema during May–June 2006. A confirmed case-patient was defined as a person who met the criteria of a suspected case-patient and who had eosinophilia and a positive serologic test or muscle biopsy result for *Trichinella* spp. A request for volunteers to serve as controls for the investigation was announced by the village president. A control was defined as a resident of village A who did not meet the case definition and who agreed to give an interview, allowed specimen collection, and had negative serologic results for *Trichinella* spp.

Physicians in the Thai Bureau of Epidemiology's Field Epidemiology Training Program and the Uthai Thani Provincial Health Offices conducted a case–control study to determine the source of infection. A questionnaire was used to collect demographic characteristics, signs and symptoms, date of illness onset, laboratory results, and suspected exposures before onset of illness. An environmental study was conducted to investigate suspected food items, the surrounding area, and wild and domestic animals. Data analysis was performed by using Epi Info 2002 version 2 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

For laboratory investigation, samples were collected from case-patients and controls for complete blood counts and serologic testing. In the hospital, laboratory results, including complete blood counts and creatine phosphokinase (CPK), were reviewed and blood for detection of antibodies to *Trichinella* spp. was collected. A muscle biopsy of 1 case-patient was performed.

Species identification by PCR was performed at the Department of Helminthology, Mahidol University. Briefly, a genomic DNA sample was obtained by using a tissue protocol (QIAamp DNA Mini Kit; QIAGEN, Valencia, CA, USA). The partial cytochrome oxidase subunit I (COI) region was amplified by primers, which were designed from partial COI sequences of mitochondrial DNA from *T. zimbabwensis* and *T. papuae* in GenBank (accession nos. DQ007900 and DQ007899, respectively). Primers for amplification were Tri-COIF (forward: 5′-GTTTATAT(C/T)(C/T)TAGTACTA CC-3′) and Tri-COIR (reverse: 5′-GC(G/A)TTTGATAGTCT(A/G)ACTCC-3′). DNA alignment analyses were conducted by using ClustalW version 1.83 (www.ebi.ac.uk/clustalw), and nucleotide substitutions were identified by using BioEdit version 7.0.1 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA) (*7*).

The investigation team also collected blood from domestic boar for antibodies to *Trichinella* spp. and a sample of fermented barking deer meat for larvae examination. No uncooked wild boar meat was available for laboratory analysis. All larvae and human serum specimens were sent to the Department of Helminthology, Mahidol University,

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Thailand. Immunoblot tests for human trichinellosis were conducted by using the 109-kDa diagnostic band (sensitivity 100%, specificity 100%) (*8*). Boar serum specimens were also tested. Studies were exempted from human subjects review because they were conducted under the authority of the Ministry of Public Health to investigate outbreaks of illness.

The study village is located in a mountainous area and had a population of 273. Of the 82 participants, 28 (3 hospitalized patients and 25 other villagers) had an illness that met the case definition (4 were suspected cases). Of the 28 case-patients (median age 34 years, range 14–55 years), 18 were male. The attack rate in the village was 10.3% (28/273); there were no deaths. The outbreak occurred during May 24–June 26, 2006, and the epidemic curve was characteristic of a common point source (Figure 1). All case-patients had myalgia. Other clinical symptoms included trunk/limb edema (83.3%), weakness (75.0%), periorbital/facial edema (70.8%), fever (37.5%), nausea/vomiting (29.2%), jaw pain (20.8%), abdominal pain (12.5%), and diarrhea (8.3%). Mebendazole (plus prednisolone if the symptoms were severe) (*9*) was prescribed for all symptomatic patients.

The environmental study found that villagers were avid hunters of deer, boar, and other local game; they also raised domesticated pigs (the village had 8 domesticated pig sties, each containing 3–10 pigs). Villagers shared domesticated pig meat from various sties every 2–3 weeks. On May 20, a successful wild boar hunt resulted in distribution of wild boar meat to villagers. On May 21, domesticated pig meat was shared among villagers. A successful hunt for barking deer also obtained meat that was prepared in various styles, including cooked, raw, and fermented, and was distributed to villagers during April and May.

After eating suspected foods on May 20–22, the first case-patient developed symptoms on May 24 (diarrhea and abdominal pain). Eighty-two blood samples were collected during the investigation; 32 (39%) had antibodies to *Trichinella* spp. Among 28 blood samples from case-patients, all had eosinophilia (>10% eosinophils, mean 28.6%, SD 13.5%) and 21 had leukocytosis, (mean 14,500 cells/mm^3, SD 4,230 cells/mm^3). All 3 hospitalized case-patients had elevated CPK levels (median 830 U/L, range 506–1,208 U/L, reference <50 U/L). One domesticated pig

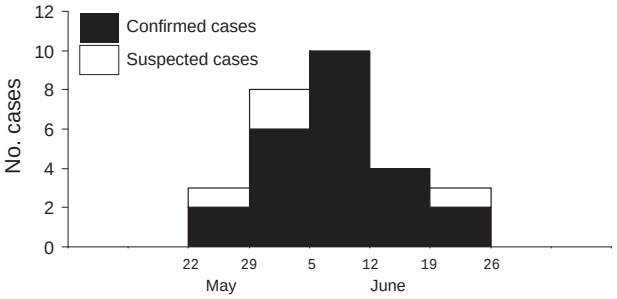

Figure 1. Epidemic curve showing distribution of cases of infection with *Trichinella* spp., by onset date, Village A, Uthai Thani Province, Thailand, May–June 2006.

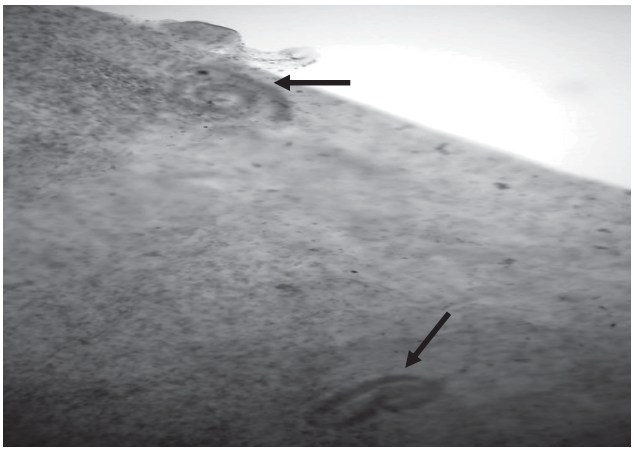

Figure 2. Nonencapsulated *Trichinella* spp. (*Trichinella papuae*) larvae (arrows) in the left gastrocnemius muscle of 1 case-patient, Uthai Thani Province, Thailand, 2006 (magnification ×40).

was randomly selected from each of 8 pigsties and tested by ELISA for antibodies to *Trichinella* spp.; all pig samples were negative. A human gastrocnemius muscle biopsy specimen from a hospitalized case-patient was positive for nonencapsulated *Trichinella* spp. larvae (Figure 2), which provided a definitive diagnosis of trichinellosis. The parasite had a COI partial gene sequence of *T. papuae* (*7*). The case-patient had not traveled outside of Thailand. The fermented barking deer meat was negative for larvae.

Of 28 persons whose illness met the case definition, 24 (3 hospitalized case-patients and 21 villagers) had antibodies to *Trichinella* spp. and were included in the analytic

Table 1. Univariate analysis of 3 suspected food items in an outbreak of trichinellosis, Uthai Thani Province, Thailand, May–June 2006*

	Case-patients (n = 24)		Controls (n = 46)		
Meat ingested	Exposed	Not exposed	Exposed	Not exposed	OR (95% CI)
Wild boar	18	6	11	35	9.6 (3.0–30.1)
Domestic pig	5	19	5	41	1.8 (0.6–5.9)
Barking deer†	19	5	31	15	2.2 (0.6–8.4)

*OR, odds ratio; CI, confidence interval.
†Consumed on May 22, 2006.

Table 2. Association of wild boar meat and risk for trichinellosis, Uthai Thani Province, Thailand, May 24–June 26, 2006*

Wild boar meat	Case-patients (n = 24)	Controls (n = 46)	OR (95% CI)
Uncooked	15	5	17.5 (3.9–86.0)
Cooked	3	6	2.9 (0.4–19.5)
Not eaten	6	35	1

*OR, odds ratio; CI, confidence interval.

study. Of the 54 villagers who did not meet the descriptive case definition, 46 had negative serologic test results and were included as controls. Eating uncooked wild boar from the hunt showed the highest risk for illness (odds ratio [OR] 9.6, 95% confidence interval [CI] 3.0–30.1) (Table 1). Associations were not significant for eating uncooked domesticated pig (OR 1.8, 95% CI 0.6–5.9) or uncooked barking deer (OR 2.2, 95% CI 0.6–8.4). Subgroup analysis showed that persons who ate uncooked wild boar had a high risk for illness (OR 17.5, 95% CI 3.9–86.0) (Table 2). Although the OR for becoming ill after eating cooked wild boar was increased, it was much lower than that for consuming raw wild boar (OR 2.9, 95% CI 0.4–19.5).

Conclusions

Eating undercooked wild boar meat was strongly implicated as the source of this trichinellosis outbreak. The villagers were instructed about the importance of thoroughly cooking meat potentially contaminated with *Trichinella* spp. These findings indicate that the geographic range of *T. papuae* is greater than previously thought.

Acknowledgments

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Multicenter Study of Brucellosis in Egypt

Hassan Samaha, Meshref Al-Rowaily, Ramadan M. Khoudair, and Hossam M. Ashour

Brucellosis causes appreciable economic losses in livestock. Examination of milk and tissues from animals in Egypt for *Brucella* spp. showed increased prevalence rates of serologically reactive animals. All isolates were *B. melitensis* biovar 3. One *Brucella* sp. was isolated from milk of serologically nonreactive buffaloes.

Brucellosis is one of the major zoonotic infections worldwide (*1*). It is caused by gram-negative coccobacilli of the genus *Brucella* and affects cattle, sheep, goats, and other livestock (*2*,*3*). Since the discovery of *Brucella melitensis* by David Bruce in 1887, several species have been identified, such as *B. abortus* (which infects cattle), *B. melitensis* (which infects sheep and goats), *B. suis*, *B. neotomae*, *B, ovis*, and *B. canis* (*2*,*4*). Although brucellosis has been controlled in most industrialized countries, it remains a major problem in the Mediterranean region, western Asia, Africa, and Latin America (*1*). It can cause appreciable economic losses in the livestock industry because of abortions, decreased milk production, sterility, and veterinary care and treatment costs (*2*).

Brucellosis was first reported in Egypt in 1939 (*5*). Control programs for brucellosis in Egypt have used 2 methods: vaccination of all animals and slaughter of infected animals with positive serologic results. The difficulty of accurately detecting all infected animals, especially carriers, is a major limitation of these programs. To enhance efficiency of brucellosis-specific prophylaxis, early detection of brucellosis by highly sensitive and specific methods is needed.

Egypt has mixed populations of sheep, goats, cattle, and buffaloes. The number of buffaloes in Egypt is higher than in any other country in the Near East region (*5*). In addition to high prevalence rates of *B. melitensis* infections in sheep and goats, *B. melitensis* infections of cattle and buffaloes have increased in Egypt (*5*). Our investigation sought to determine the epidemiology of brucellosis in several governorates in Egypt by using different serologic tests, as well as bacteriologic tests, to identify *Brucella* spp. organisms isolated from milk and tissue specimens of sheep, cattle, goats, and buffaloes.

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The Study

We studied 4,482 animals (1,966 cattle, 1,237 buffaloes, 813 sheep, and 366 goats) from production and breeding farms in various governorates in Egypt during 2007; the animals had no history of having been tested for brucellosis. Milk and tissue samples obtained from all animals were examined for *Brucella* spp. We used serologic tests recommended by the National Brucella Committee, which represents the general organization of veterinary services, veterinary laboratories, and universities in Egypt (*5*). The buffered acidified plate antigen (BAPA) test, the Rose Bengal plate test, the standard tube agglutination test, and the Rivanol test were used as described (*6*–*8*).

Direct culture of milk under aseptic conditions was conducted as follows: ≈20 mL of milk was centrifuged at 1,620 × *g* for 10 min, and the sediment cream mixture was placed on *Brucella* spp. agar plates containing an antimicrobial drug supplement. Tissue specimens obtained from internal organs, supramammary lymph nodes, and udders were cultured in the same media and incubated at 37°C in an atmosphere of 10% CO_2. Cultured plates were examined for *Brucella* spp. growth on day 4 and daily for 4 weeks. Suspected colonies were further identified and subcultured on *Brucella* spp. agar slants. We identified *Brucella* spp. isolates according to morphologic characteristics, microscopic appearance, and reactions with positive sera. *Brucella* spp. isolates were typed according to their CO_2 requirement, H_2S production, growth in the presence of dyes, reaction with monospecific sera (immunoglobulin [Ig] A and IgM), and bacteriophage typing (Tiblisi phage; Central Veterinary Laboratory, Wybridge, UK) as described (*7*).

Results obtained for different animal groups are shown in Table 1. Prevalence of brucellosis in cattle was 5.44% by the BAPA test; highest prevalence was in Benisuef (7.77%) and Monofia (7.14%). Prevalence of brucellosis in buffaloes was 4.11% by the BAPA test; highest prevalence was in Benisuef (6.93%) and Qalioubia (5.34%). Prevalence of brucellosis in sheep was 5.41% by the BAPA test; highest prevalence was in Benisuef (6.91%) and Giza (5.81%). Prevalence of brucellosis in goats was 3.55% by the BAPA test; highest prevalence was in Monofia (6.35%) and Benisuef (5.75%).

Prevalence of a serologic reaction was 4.98% for cattle, 3.52% for buffaloes, 4.8% for sheep, and 2.19% for goats by the Rose Bengal plate test. Prevalence of a serologic reaction was 4.73% for cattle, 3.44% for buffaloes, 4.8% for sheep, and 2.19% for goats by the standard tube agglutination test. Prevalence of a serologic reaction was 4.48% for cattle, 3.37% for buffaloes, 4.8% for sheep, and

Table 1. Serodiagnostic test results for brucellosis in animals, Egypt, 2007*

Serum source	Location	No. tested	Serologic test, no. positive (%)			
			BAPA	RBP	SA	Rivanol
Cattle	Alexandria	333	17 (5.11)	15 (4.5)	13 (3.9)	13 (3.9)
	Behera	374	11 (2.94)	11 (2.94)	10 (2.67)	9 (2.41)
	Monofia	280	20 (7.14)	18 (6.43)	17 (6.07)	15 (5.36)
	Qalioubia	221	14 (6.33)	12 (5.43)	12 (5.43)	11 (4.98)
	Giza	346	15 (4.34)	15 (4.34)	14 (4.05)	14 (4.05)
	Benisuef	309	24 (7.77)	22 (7.12)	21 (6.8)	21 (6.8)
	Assiut	103	6 (5.83)	5 (4.85)	6 (5.83)	5 (4.85)
	Total	1,966	107 (5.44)	98 (4.98)	93 (4.73)	88 (4.48)
Buffaloes	Alexandria	137	6 (4.38)	6 (4.38)	6 (4.38)	6 (4.38)
	Behera	397	7 (1.76)	5 (1.26)	5 (1.26)	5 (1.26)
	Monofia	210	10 (4.76)	8 (3.81)	6 (2.86)	7 (3.33)
	Qalioubia	131	7 (5.34)	6 (4.58)	7 (5.34)	6 (4.58)
	Giza	198	8 (4.04)	8 (4.04)	8 (4.04)	7 (3.54)
	Benisuef	231	16 (6.93)	14 (6.06)	14 (6.06)	14 (6.06)
	Assiut	33	1 (3.03)	0	0	0
	Total	1,337	55 (4.11)	47 (3.52)	46 (3.44)	45 (3.37)
Sheep	Behera	210	11 (5.24)	10 (4.76)	10 (4.76)	10 (4.76)
	Monofia	81	2 (2.47)	0	0	0
	Qalioubia	133	6 (4.51)	6 (4.51)	6 (4.51)	6 (4.51)
	Giza	172	10 (5.81)	9 (5.23)	9 (5.23)	9 (5.23)
	Benisuef	217	15 (6.91)	14 (6.45)	14 (6.45)	14 (6.45)
	Total	813	44 (5.41)	39 (4.8)	39 (4.8)	39 (4.8)
Goats	Behera	55	1 (1.82)	0	0	0
	Monofia	63	4 (6.35)	2 (3.17)	2 (3.17)	2 (3.17)
	Qalioubia	103	3 (2.91)	2 (1.94)	2 (1.94)	2 (1.94)
	Giza	58	0	0	0	0
	Benisuef	87	5 (5.75)	4 (4.6)	4 (4.60)	4(4.6)
	Total	366	13 (3.55)	8 (2.19)	8 (2.19)	8(2.19)

*BAPA, buffer acidified plate antigen; RBP, Rose Bengal plate; SA, standard tube agglutination.

2.19% for goats by the Rivanol test. The highest prevalence for cattle, buffaloes, sheep, and goats by any of the 4 tests was in Benisuef, except for the BAPA test in goats, which showed highest prevalence rates in Monofia.

Isolation of the causative agent is still the standard diagnostic method for brucellosis (*9*). Thus, for definitive and confirmative diagnosis of serologically reactive animals, bacteriologic isolation and identification of *Brucella* spp. were performed. Results of bacteriologic isolation from milk and tissues all animals are shown in Table 2. A total of 47 isolates of *Brucella* spp. were identified; all isolates were *B. melitensis* biovar 3. Isolation of *Brucella* spp. confirmed active brucellosis in the animals tested. A *Brucella* spp. was also isolated from milk samples from serologically nonreactive buffaloes in Benisuef.

Conclusions

We observed an increase in animals serologically reactive for *Brucella* spp. in Egypt in 2007 (Table 1). Prevalence rates in cattle, buffaloes, sheep, and goats were generally higher in Benisuef than in other governorates. Variations in infection in different governorates may be attributed to

Table 2. Prevalence of *Brucella* spp. in milk or tissues of animals, Egypt, 2007*

Location	Cattle, no. positive/no. tested				Buffaloes, no. positive/no. tested				Sheep, no. positive/no. tested		Goats, no. positive/no. tested	
	Milk		Tissue		Milk		Tissue		Tissue		Tissue	
	SRA	SNRA	SRA	SNRA	SRA	SNRA	SRA	SNRA	SRA	SNRA	SRA	SNRA
Alexandria	2/10	0/11	1/5	0/5	1/6	0/19	1/5	0/5	0	0	0	0
Behera	2/9	0/9	1/5	0/5	1/5	0/20	1/5	0/5	1/5	0/5	0	0/5
Monofia	4/20	0/12	1/5	0/5	1/7	0/18	2/5	0/5	0	0/5	0/2	0/5
Qalioubia	2/20	0/10	0/5	0/5	2/6	0/3	1/5	0/5	1/5	0/5	1/2	0/5
Giza	4/20	0/10	0/5	0/5	1/7	0/6	1/5	0/5	1/5	0/5	0	0
Benisuef	6/20	0/21	2/5	0/5	1/10	1/15	0/5	0/5	1/5	0/5	1/4	0/5
Assiut	1/5	0/7	1/5	0/5	0	0/5	0	0	0	0	0	0
Total	21/104	0/80	6/35	0/35	7/41	1/86	6/30	0/30	4/20	0/25	2/8	0/20

*SRA, samples from serologically reactive animals; SNRA, samples from serologically nonreactive animals.

environmental factors and stress, which may modulate susceptibility to infection.

Increased prevalence of brucellosis in cattle and buffaloes in Egypt can be attributed to raising sheep and goats with cattle or buffaloes in villages. Most sheep or goat flocks in Egypt are mobile. Movement of infected sheep or goats can contaminate pastures and spread brucellosis to other animals (e.g., cattle or buffaloes) in other herds or areas. This movement is a major risk factor for failure of brucellosis eradication programs. Elimination or control of infection in sheep and goat flocks can reduce spread of the disease in cattle and buffaloes.

All *Brucella* isolates were *B. melitensis* biovar 3. This finding is consistent with reports of *B. melitensis*, particularly biovar 3, being the main cause of brucellosis in animals and humans in many countries (*5*). Isolation and identification of 1 *Brucella* spp. from milk samples of serologically nonreactive buffaloes in Benisuef emphasize the need to routinely check milk samples. Some microorganisms, which can escape identification by not causing appreciable serologic responses, can localize in the udder and be isolated from milk samples.

We recently reported prevalence of human brucellosis in Egypt as high as 8% in high-risk populations (*10*). Our findings emphasize the need for continuous national surveillance programs for control and prevention of brucellosis in Egypt and other affected countries. Measures should be established to control spread of brucellosis, especially in mobile flocks. These measures should include identification of infected animals by periodic examination of flocks or newly purchased animals, application of testing and slaughter policies, adoption of vaccination programs, and strict quarantine measures. Sheep farmers should also be notified about transmission of brucellosis from sheep to cattle and buffaloes. Educational programs about brucellosis are important for livestock owners and consumers.

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Mycobacterium bovis Infection in Holstein Friesian Cattle, Iran

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To identify strains of *Mycobacterium bovis* circulating in Iran, we used region of difference, spoligotypes, and variable number tandem repeats to genotype 132 *M. bovis* isolates from Holstein Friesian cattle. Despite wide geographic origins, the strains were genetically homogeneous. Increased distribution of cattle herds and inadequate control measures may have contributed to strain dispersion.

Estimates suggest that globally >50 million cattle are infected with *Mycobacterium bovis*, causing an annual loss of ≈$3 billion US (*1*). In Asia, 94% of the 460-million cattle herd (33% of the world's cattle) are in areas with either no or only partial tuberculosis (TB) control programs (*2*). In 2006, the prevalence of bovine TB in Iran was 0.12% (Iranian Veterinary Organisation [IVO], unpub. data), yet few studies have been conducted on *M. bovis* in Iran (*3*–*5*). To identify the strains of *M. bovis* in Iran, we used region of difference (RD) typing, spoligotyping, and variable number tandem repeats (VNTR) typing.

The Study

From 1996 through 2003, we collected necropsy specimens from TB-test reactor cattle from abattoirs in 21 of the 28 Iranian provinces where bovine TB has been reported. Specimens were all respiratory and gastrointestinal lymph nodes and any lungs, spleens, or livers that were visibly affected. All specimens were cultured for *M. tuberculosis* complex bacteria and incubated for ≥10 weeks. Of the 470 animals tested, results were positive for 216; however, because of delays in exporting samples to the United Kingdom, only 132 samples contained reculturable isolates with sufficient growth for DNA extraction. Molecular speciation was determined by RD-PCR (RD1, RD5, RD9, RD10, and RD11) (*6*). Spoligotyping was conducted according to the method of Kamerbeek et al. (*7*). VNTR-PCR was conducted according to the 6-locus method of Frothingham and Meeker-O'Connell (exact tandem repeat [ETR]-A through ETR-F loci) (*8*) plus QUB11B and VNTR3232 loci (*9*).

RD-PCR showed that all 132 isolates were *M. bovis*. Spoligotyping identified 8 types (Figure 1). SB0120 was the most common, and 5 others (SB1167–SB1171) were novel patterns and, thus, were specific to Iran. VNTR typing identified 23 profiles (Figure 2).

Conclusions

RD typing of the 132 isolates confirmed that they were all wild type *M. bovis;* none were the *M. bovis* BCG vaccine strain because they carried the RD1 region. This finding is noteworthy because unauthorized vaccination of cattle with BCG has been reported in Iran (IVO, unpub. data). Although previous studies in Iran have reported the isolation of *M. tuberculosis* from tuberculin-positive cattle (*3*), our RD9 and RD10 analyses indicated that no isolates were *M. tuberculosis.* This finding suggests that *M. tuberculosis* is unlikely to be abundant, if even present, on cattle farms of Iran. Similarly, RD5 and RD11 analyses indicated that no isolates were *M. africanum* or *M. microti.*

The spoligotypes were either identical to the BCG-like (SB0120) pattern (41% of isolates) or were simple variants of it by the deletion of 1 or occasionally 2 single or contiguous blocks of spacers (Figure 1). Because spoligotype changes have been attributed solely to the deletion of spacer units, the BCG-like strains here are believed to be ancestral (*11*).

VNTR typing of the 132 *M. bovis* isolates at 8 loci identified 23 different profiles (Figure 2), 4 of which represented 80% of the isolates. The homogeneity of *M. bovis* isolates in Iran was further exemplified by the low diversity seen at ETR-E (2 alleles, 1 allele represented by only 1 isolate) and VNTR3232 (1 allele); these findings contrast with findings of greater heterogeneity, particularly at VNTR3232, reported elsewhere (*12*). Given the large geographic area covered by cattle in the present study, this level of homogeneity was unexpected. This finding is paralleled in the United Kingdom, where ETR-E is virtually monotypic and is believed to indicate a minimal effect of penetrating exotic strains (*11*).

In combination, spoligotyping and VNTR typing stratified the 132 isolates into 26 groups (Figure 2). Most isolates with a particular VNTR profile were found to be a subset of isolates with a specific spoligotype. Thus, VNTR could be used to subtype isolates identified by spoligotyping; presumably because of the more rapid rate of polymorphism changes in VNTR than in spoligotype.

Spoligotyping and VNTR typing showed high similarities for all isolates. Such homogeneity, in combination with the geographic restriction of several of the spoligotypes to Iran (at least in current databases), does not easily support the hypothesis that most strains currently circulat-

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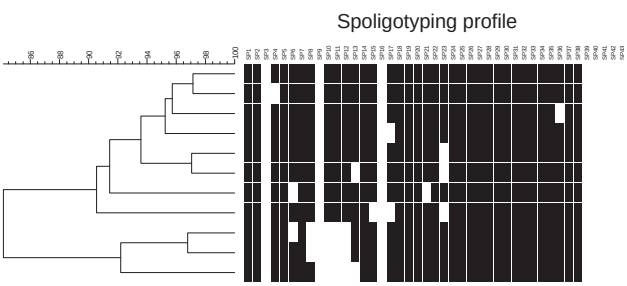

Spoligotype/ no. isolates	Countries reporting
SB0120 (BCG-Lie)/55	France, Belgium, Germany, Netherlands, Italy, Spain, Argentina, Tunisia, Brazil, Iran
SB0822 (Cousins)/1	France, Iran
SB0934/2	France, Iran
SB1169/1	This study
SB1167/49	This study
SB1171/1	This study
SB0265 (Cousins)/4	France, Belgium, Spain, Canada, Iran
SB1168/22	This study
SB0140 (Cousins)/4	UK, Republic of Ireland, France, Australia, Argentina, Venezuela, Mexico, Iran
SB0288/1	France, UK, Australia, Iran
SB1170/1	This study

Figure 1. Spoligoprofiles of Iranian isolates and dendrogram of profile relatedness by the Dice and unweighted pair group method with arithmetic mean and previously published locations of profiles. The three spoligotypes from Iran were previously reported by Cousins et al. (*10*).

ing in Iran have been imported from abroad. Since the introduction of European breeds in the 1930s, Iran's cattle herd has expanded constantly; expansion during the past 4 decades has been ≈1.8% annually (IVO, unpub. data). Given the susceptibility of these European breeds to bovine TB and the initial absence of effective disease control, as the Holstein Friesian herd increased in number, infections with *M. bovis* likely increased in parallel. The homogeneity and localization of the *M. bovis* strains to Iran would be a direct consequence of this dramatic increase in number of bovine TB–susceptible cattle from what has effectively been a genetic bottleneck for *M. bovis*.

The subsequent test-and-slaughter program in Iran may have contributed to the clonality of the *M. bovis* population. This situation would be similar to that in the United Kingdom, where typing of *M. bovis* strains from human patients (presumably infected with *M. bovis* from cattle) suggests that *M. bovis* was more diverse 50 years ago than it is today (*13*,*14*). It is believed that bovine TB control measures throughout the United Kingdom over the past 100 years reduced the *M. bovis* population size and diversity and led to geographic localization of *M. bovis* strains (*9*,*11*). The lower heterogeneity of isolates in Iran perhaps reflects a shorter timescale of events there than in the United Kingdom. Spoligotypes are reported to change over timescales as long as 60 years (*15*); the expansion of the *M. bovis* population in Iran over ≈50 years and the generation of 2–3 sequential spoligotype changes during this time is certainly compatible with these timescales. The absence of geographic regionalization of strains in Iran may also reflect the shorter timescale of events in Iran than in the United Kingdom and insufficient time for significant diversification of new strains. The extensive movement of cattle around Iran would also be expected to reduce regionality of strains.

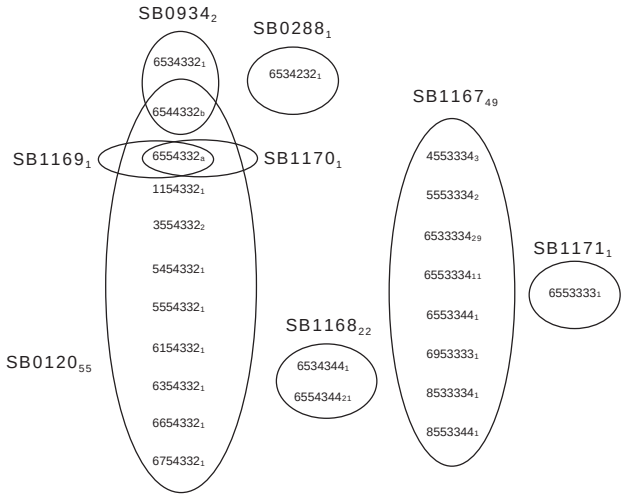

Figure 2. Variable number tandem repeat (VNTR) profiles and their abundance and distribution with spoligotypes. VNTR profiles are listed in the following order: exact tandem repeat (ETR)-A, B, C, D, E, F, QUB11B. The number of isolates of each spoligotype and of each VNTR profile are indicated by the subscript number; "a" indicates 42 SB0120 isolates, 1 SB1169 isolate, and 1 SB1170 isolate; "b" indicates 1 SB0120 isolate and 1 SB0934 isolate.

In a relatively short time, *M. bovis* has emerged as a major cause of cattle illness and economic loss in Iran, notably as a result of the ever-increasing numbers of susceptible hosts. Other causes may be changes in farming practices, such as intensification, and the continued escape of *M. bovis* from the test-and-slaughter scheme, possibly as a result of selection for less easily detectable strains. Without strengthened control measures, *M. bovis* is unlikely to disappear. Indeed, more infective animals in a growing population of susceptible animals increase the risk for other species and for humans.

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Hemoplasma Infection in HIV-positive Patient, Brazil

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Hemotrophic mycoplasmas infect a variety of mammals. Although infection in humans is rarely reported, an association with an immunocompromised state has been suggested. We report a case of a *Mycoplasma haemofelis*–like infection in an HIV-positive patient co-infected with *Bartonella henselae*.

Hemoplasmas are a group of bacteria that infect animals. They are small epicellular parasites that adhere to the host's erythrocytes. Diseases caused by these bacteria range from acute hemolytic anemia to asymptomatic infection. It is generally thought that most *Mycoplasma* spp. are host specific. However, there are occasional reports of infection in an animal species not perceived as primary hosts. These infections may have a pathologic effect, particularly when predisposing conditions, such as immunodeficiency, are present (*1*). We report a case of *Mycoplasma haemofelis*–like infection in an HIV-positive patient with disseminated *Bartonella henselae* infection.

The Study

A 34-year-old HIV-positive man was hospitalized in Brazil in September 2006 with a 30-day history of night sweats; loss of appetite; productive cough; muscle pain; and cervical, axillary, and inguinal lymphadenomegaly. Results of pulmonary, cardiovascular, and abdominal examinations were normal. Abnormal lymph nodes were 2 cm in diameter, firm, and not tender. The patient owned 5 cats and showed signs of multiple cat scratches and bites on his hands and arms. He had received an HIV-positive diagnosis 5 years earlier.

At admission, his CD4 cell count was 286 cells/mm^3 (reference range 500–1,500 cells/mm^3), and viral load was 38,100 copies/mL. Additional laboratory findings included anemia, hematocrit 29% (reference range 38%–50%); leukopenia, 3,300 leukocytes/μL (4,300–10,000 leukocytes/μL); thrombocytopenia, 108,000 platelets/μL (150,000–450,000 platelets/μL); aspartate aminotransferase 66 U/L (15–40 U/L), alanine aminotransferase 79 U/L (10–40 U/L), and lactate dehydrogenase 657 U/L (240–480 U/L). Blood cultures yielded no bacterial growth; sputum cultures were negative for acid-fast bacilli, bacteria, and fungi. Test results were negative for hepatitis B and C, human T lymphotropic virus type 1, syphilis, chlamydia, and cryptococcus infections. Bone marrow and lymph node biopsy and culture results were negative for mycobacterial or fungal infections.

Abdominal computed tomography showed hepatomegaly, splenomegaly, and hypoechoic lesions on the spleen. Transesophageal echocardiography showed no lesions compatible with infective endocarditis. An inguinal node biopsy showed granuloma with necrosis suggesting cat-scratch disease, and no signs of acid-fast bacilli or fungi. Many bacilli suggestive of *Bartonella* spp. were observed by Warthin-Starry staining, and antibodies against *B. henselae* (titer 256) were detected in serum. Treatment with doxycycline was initiated and symptoms subsequently subsided. The patient was discharged and instructed to continue antiretroviral therapy and oral doxycycline.

In June 2007, the patient was hospitalized with fever, malaise, weight loss, and lymphadenomegaly. Echocardiography showed mitral vegetations. Multiple hepatic hypoechoic lesions were found by abdominal computed tomography. Lymph node biopsy specimens showed tiny bacilli by Warthin-Starry staining. The patient had prematurely discontinued antiretroviral and antimicrobial drug treatment, which may have predisposed him to endocarditis and hepatic peliosis. He was treated with doxycycline and gentamicin for *Bartonella* spp. infection. His symptoms disappeared and an echocardiogram 17 days later showed resolution of mitral vegetations. He was discharged and instructed to continue antiretroviral therapy and oral doxycycline for *Bartonella* spp. infection. Ten months after discharge, the patient returned for a follow-up visit while taking recommended therapy. He had no clinical signs and his laboratory findings were improved.

During the patient's first hospitalization in 2006, blood was collected into tubes containing EDTA and 2 aliquots of

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DNA were extracted (DNeasy Blood and Tissue Kit; QIAGEN, Valencia, CA, USA) at the Hospital de Clínicas de Porto Alegre. DNA was tested by PCR for *Bartonella* spp. infection (*2*) and by additional PCR protocols for feline hemoplasmas, including *Mycoplasma haemofelis* (*3*), "Candidatus *M. haemominutum*" (*4*), and "Candidatus *M. turicensis*" (A.P. Santos, unpub. data). These bacteria infect cats and possible infection of this patient was investigated. Positive controls for *M. haemofelis* included DNA extracted from naturally (GenBank accession no. EU930823) and experimentally infected cats (*3*). Three positive controls for *B. henselae* (type 1, type 2, and Houston strain) were used. Negative controls included ultrapure water and DNA extracted from blood of a healthy person and a noninfected cat. All negative controls were negative by PCR.

Amplicons of the expected size were obtained in *Bartonella* spp. and *M. haemofelis* PCRs. The 393-bp PCR product for *M. haemofelis* (Figure) was purified (Zymoclean Gel DNA Recovery Kit; Zymo Research, Orange, CA, USA), cloned (pGEM-T EasyVector; (Promega, Madison, WI, USA), and sequenced (Purdue Genomics Core Facility, West Lafayette, IN, USA). The fragment was 99% homologous with *M. haemofelis* 16S rDNA gene sequences in the GenBank database. To assess the sequence of the 16S rRNA gene, we designed species-specific primers based on the *M. haemofelis* sequence (forward primer 5′-ATG CAA GTC GAA CGG ATC TT-3′; reverse primer 5′-TCC AAT CAG AAT GTT CAC TC-3′). PCR product amplified from the patient's blood was purified and sequenced. A 1,214-bp sequence was submitted to GenBank (accession no. EU888930); it was 99% homologous with the sequence for *M. haemofelis*.

The possibility that the patient's cats might be involved in zoonotic transmission was also investigated. Two weeks after the patient's first blood collection, blood was collected from the 5 cats, and DNA was extracted at the Veterinary Hospital of the Universidade Federal do Rio Grande do Sul, Porto Alegre. Two of the cats were positive by PCR for *M. haemofelis* and all 5 cats were positive for *Bartonella* spp. The cats were not tested for other infectious agents.

PCRs for hemoplasmas were performed in duplicate at 2 laboratories (Universidade de São Paulo, São Paulo, Brazil and Purdue University, West Lafayette, IN, USA) by using split aliquots. PCR results were reproducible. During the patient's second hospitalization, the same PCRs were used and the patient was positive for *Bartonella* spp. but negative for hemoplasmas.

Conclusions

Hemoplasma infections may occur more frequently than is generally recognized, given that these organisms fail to grow in culture and only a few laboratories are equipped to detect and identify hemoplasmas (*1*). Disease associations with latent mycoplasma infections in immunocompromised and nonimmunocompromised patients are now emerging. Increasing numbers of human patients with compromised immune systems living near cats increases the possibility that hemoplasma infections may also emerge in this population.

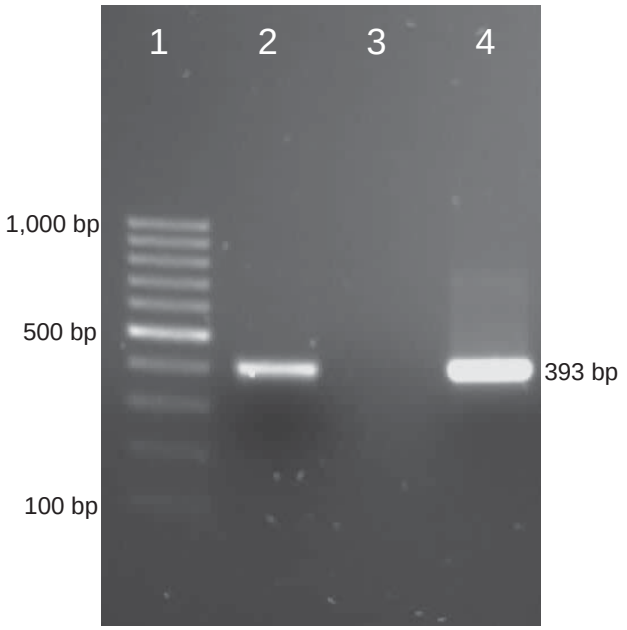

Figure. PCR results for detection of a *Mycoplasma haemofelis*–like organism in an HIV-positive patient. Lane 1, 100-bp marker; lane 2, positive control (DNA from blood of an *M. haemofelis*–positive cat); lane 3, negative control (water); lane 4, DNA from blood of the patient.

There are no molecular studies to date documenting hemoplasma infection in humans. However, it has been suggested that such infections may be seen in immunocompromised patients (*5*). A hemotrophic mycoplasma infection was reported in patients with systemic lupus erythematosus (SLE) (*6*). A 417-bp sequence detected in 1 SLE patient also showed 99% homology with *M. haemofelis* (*7*). Sequence data from another hemotrophic mycoplasma infection in an anemic human patient were reported in GenBank. However, the sequence of 178 bp of the 16S rRNA gene (accession no. EU014880) was more closely related to *M. suis* and *M. wenyonii* (96%–100%) and only 75% homologous to *M. haemofelis* (*8*).

Epidemiologic studies have linked cat bites and scratches and flea-infested cats with transmission of *B. henselae* to humans (*9*). *B. henselae* and *B. quintana* are causative agents of bacillary angiomatosis, bacillary peliosis, and cat-scratch disease in humans. Peliosis hepatis and lymph node angiomatosis, as seen in this patient, have been associated with *B. henselae* infection (*10*). *M. haemofelis* DNA has also been detected in cat fleas (*Ctenocephalides felis*);

C. felis may be involved in transmission of *M. haemofelis* among cats (*11*). Additional studies have documented experimental transmission by administration of infected blood intravenously, intraperitoneally, and orally. Hemoplasma DNA is present in saliva and feces of cats, which suggests that aggressive interactions among cats involving biting may lead to transmission of the organism (*12*). To our knowledge, there is only 1 other report that cats can be co-infected with *M. haemofelis* and *B. henselae* (*13*).

As with other *Mycoplasma* spp., hemoplasmas might act as a cofactor in HIV infection, contributing to acceleration of the course of the disease (*14*). Further studies are needed to establish the role and prevalence of hemoplasma infection in AIDS patients, as well as the zoonotic potential of *M. haemofelis*.

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Occupational Exposure to *Streptococcus suis* among US Swine Workers

Tara C. Smith, Ana W. Capuano, Brenda Boese, Kendall P. Myers, and Gregory C. Gray

Despite numerous cases of human infection with *Streptococcus suis* worldwide, human disease is rarely diagnosed in North America. We studied 73 swine-exposed and 67 non–swine-exposed US adults for antibodies to *S. suis* serotype 2. Serologic data suggest that human infection with *S. suis* occurs more frequently than currently documented.

Streptococcus suis is one of the most important pathogens affecting the swine industry. The gram-positive, encapsulated bacterium causes a wide range of clinical disease syndromes in pigs and other domestic animals. Despite the recognition that *S. suis* infection may result in a life-threatening meningitis or toxic-shock syndrome, little is known about human pathogenesis. A recent outbreak in People's Republic of China caused by a serotype 2 strain resulted in 38 deaths among 215 infected humans, an 18% mortality rate (*1*). The bacterium has caused sporadic human illness in other countries as well, including the United Kingdom (*2*), and has been identified as a leading cause of bacterial meningitis in Hong Kong Special Administrative Region (*3*) and Vietnam (*4*).

Although human infection with *S. suis* has been occasionally documented in North America (*5*), the first human case was not reported in the United States until 2006, when a farmer from New York sought treatment for meningitis. The source was an area farm where the patient had recently purchased piglets (*6*). Several investigators have suggested that the infrequent diagnosis of human *S. suis* infection is due to underdiagnosis or misdiagnosis, rather than a true absence of disease (*5*,*7*,*8*).

The Study

To test the hypothesis that human infections with *S. suis* occur more commonly than currently recognized, we examined archived serum samples from 73 swine-exposed and 67 non–swine-exposed adults living in Iowa (*9*). These persons had all previously completed occupational history questionnaires detailing their pig exposure and use of personal protective gear. Use of materials was approved by the Institutional Review Board at the University of Iowa. Antibodies to serotype 2 *S. suis* were measured by an ELISA that used whole *S. suis* cells (serotype 2, strain 30–336–06) as antigen (*10*).

The ELISA optical density readings for the 73 swine-exposed study participants and 67 non–swine-exposed participants were first compared to the positive-control mean option density read per plate per dilution. Optical densities greater or equal to mean positive-control optical density were considered positive. To be conservative, the titer was defined as the last positive dilution before the first negative. Again, when a conservative approach was used, the lowest titer among duplicates was considered as the final antibody titer. We used the Fisher exact test to test the null hypothesis that the exposed group does not have higher incidence of antibody titer >10 than the non–swine-exposed group does. We tested a similar hypothesis for specific risk groups exposed to swine (such as nursing or finishing swine, use of gloves) compared with groups not exposed to swine. Risk factor analyses were performed with exact logistic regression. Seven (9.6%) of 73 swine-exposed study participants were positive, and 1 (1.5%) of the 67 non–swine-exposed participants was positive.

Study participants who work with both finishing and nursery swine had 8.8× the odds of having a titer ≥10 when compared to nonexposed study participants (exact 95% confidence interval 1.1–406.3). We identified no positive persons in the group that worked solely with nursery swine, a somewhat unexpected finding because most *S. suis* disease occurs in young pigs. However, our study had relatively few persons who worked exclusively with nursery swine (11/73); most participants worked with both nursery and finishing swine. Additionally, no farm-level data on prevalence of *S. suis* where these persons were employed were collected; therefore, whether those persons worked on farms where *S. suis* had been confirmed is not known. Other factors such as age, gender, use of tobacco products, and use of gloves when working with animals were not statistically significant (Table).

Discussion

In this cross-sectional pilot study, we found that more swine-exposed persons had higher titers of antibodies to *S. suis* than did non–swine-exposed persons. These data suggest that human infection with *S. suis* is more common in the United States than currently thought.

Two possible reasons stand out regarding the lack of human *S. suis* disease in the United States. One possibility is underdiagnosis or misdiagnosis, rather than a true ab-

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Table. Characteristics of total swine-exposed study population compared with those who had antibody titers ≥10 against *Streptococcus suis* serotype 2

Variable	Swine exposed	
	Total (n = 73)	Titer ≥10 (n = 7)*
Gender		
Male	56	6
Female	17	1
Nursery/finishing swine		
Nursery	11	0
Finishing	1	0
Nursery and finishing	59	7
No	2	0
Years working with swine		
5–10	1	0
>10	70	7
Years living on a swine farm		
0	2	0
5–10	1	0
>10	66	7

*Final antibody titer reflects the lowest positive antibody titer among duplicates. An antibody titer was considered positive when its optical density was greater or equal to mean positive control optical density.

sence of the disease (*11*). Supporting this hypothesis are reports showing that *S. suis* has been mistaken for enterococci, *Listeria* spp., viridans streptococcus, or *Streptococcus pneumoniae* (*7*,*8*,*11*). Because of this potential misclassification, previous publications have asserted that *S. suis* should be considered in the differential diagnosis of septicemia, especially when complicated by meningitis in adults with a recent history of contact with pigs or unprocessed pork (*12*).

A second possibility is that *S. suis* strains colonizing swine in the United States may be less virulent than Asian strains and therefore unlikely to cause overt human disease even when transferred between species. This possibility is supported by molecular analyses showing that many US strains belong to sequence type (ST) 25, whereas most virulent serotype 2 isolates have been ST1 (*13*). Finally, these hypotheses are not mutually exclusive; both underdiagnosis/misdiagnosis and the circulation of lower-virulence strains may be occurring, resulting in fewer diagnoses of human *S. suis* infections in North America (*11*).

S. suis infection is an important occupational disease in humans in many countries. In research conducted in *S. suis*–endemic countries, the annual incidence of *S. suis* meningitis was ≈3 cases/100,000 swine-exposed people: roughly 1,500× higher than the rate in the nonexposed population (*14*). Because of this risk, it has been recommended that persons in daily contact with pigs or pig meat should use protective gloves to avoid skin trauma and subsequent risk for exposure to the bacterium. Because no human vaccine against *S. suis* exists, suitable preventive measures coupled with education and supervision of those who come in contact with live swine or unprocessed pork are important to decrease the transmission of this organism to humans. However, few studies have been conducted to detect subclinical cases of *S. suis* infection; therefore, the true incidence of infection among the swine-exposed is unknown.

Because our findings only examined 1 serotype of *S. suis*, our results may not accurately reflect antibody prevalence. Because we used a whole-cell ELISA, some antibody reactions may be due to cross-reacting antibodies to other serotypes of *S. suis* or other species of *Streptococcus.* However, using a slightly different method and population, other investigators found a higher seroprevalence, particularly among farmers and meat inspectors (*15*). This difference may stem partly from the fact that we used a conservative criterion for considering a sample positive, which may further underestimate seroprevalence in our group. For example, 4 study participants (3 swine exposed) were classified as having a titer <10 because the first dilution (1:10) was negative in 1 repeat test. However, these participants' serum samples were repeatedly positive in the other 7 dilutions. Acquisition of human positive control serum as a standard to test our assay would enable us to make more definitive comparisons.

Finally, because the samples analyzed for this pilot study were not collected to specifically assess *S. suis* infections, more definitive future prospective studies seem indicated. One limitation of this serologic study is that it does not enable us to distinguish antibodies generated as a result of true infection versus exposure to *S. suis* antigens present in manure or dust in the facility, for example. Additionally, because the questionnaire did not include information on pork consumption or handling of raw pork, those factors could not be examined as potential risks. Future studies might include targeted questionnaires, attempts of bacterial isolation, and serial sera collections to examine serologic evidence of infection.

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Multiple *Francisella tularensis* Subspecies and Clades, Tularemia Outbreak, Utah

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In July 2007, a deer fly–associated outbreak of tularemia occurred in Utah. Human infections were caused by 2 clades (A1 and A2) of *Francisella tularensis* subsp. *tularensis*. Lagomorph carcasses from the area yielded evidence of infection with A1 and A2, as well as *F. tularensis* subsp. *holarctica*. These findings indicate that multiple subspecies and clades can cause disease in a localized outbreak of tularemia.

Tularemia is a zoonotic disease caused by *Francisella tularensis*, a highly infectious, gram-negative coccobacillus found in lagomorphs (rabbits and hares), rodents, and arthropods throughout the Northern Hemisphere. Humans become infected through contact with infected animal tissues, ingestion of contaminated food or water, inhalation of contaminated aerosols, and bites of arthropods, especially ticks and deer flies.

In North America, tularemia is caused by 2 subspecies of *F. tularensis*, subsp. *tularensis* (type A) and subsp. *holarctica* (type B). The distribution of type A and type B strains appears largely overlapping within the United States, with some geographic distinctions (*1*,*2*). Ecologically, the 2 subspecies are thought to be maintained in distinct but incompletely defined cycles, with type A strains frequently associated with lagomorphs and type B strains more commonly associated with rodents and aquatic environments (*3*).

Type A strains can be further divided into 2 major clades by various molecular subtyping techniques (*1*,*2*,*4*–*7*). These clades, designated here as A1 and A2, differ in their overall geographic distribution and clinical features. A1 strains (also known as A.I. and A-east) are usually found east of the Rocky Mountains. A2 strains (also known as A.II. and A-west) are common in the intermountain region of the western United States, are associated with lower mortality rates in humans, and are the only strains currently linked to transmission by deer flies (*Chrysops* spp.) (*2*).

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The Outbreak

In July 2007, an outbreak of ulceroglandular tularemia occurred in Utah among visitors to the southwest shore of Utah Lake; an epidemiologic investigation implicated deer fly bites as the source of infection (*8*). Clinical isolates were obtained July 9–14 from skin lesions of 5 patients. Isolates were identified as *F. tularensis* subsp. *tularensis* (type A) by biochemical analysis (glycerol fermentation). Molecular subtyping of isolates was performed by using *Pme*I pulsed-field gel electrophoresis (PFGE) as previously described (*2*). PFGE gels were normalized by comparison to the *Salmonella enterica* serotype Braenderup (H9812) reference strain by using BioNumerics software (v. 4.0, Applied Maths BVBA, Sint-Martens-Latem, Belgium) (*2*). A dendrogram was constructed by comparison with *Pme*I PFGE patterns for A1 (SCHU S4, MA00–2972) and A2 (ATCC 6223, WY96–3418) control strains (*1*,*2*,*4*–*7*,*9*) (Figure 1). The 5 clinical isolates fell into the 2 major type A clades; 2 isolates were identified as A2 (UT07–4632, UT07–4633), and 3 were identified as A1 (UT07–4262, UT07–4263, UT07–4265) (Figure 1). PFGE patterns for the 3 A1 isolates were indistinguishable from each other, as were patterns for the 2 A2 isolates (Figure 1).

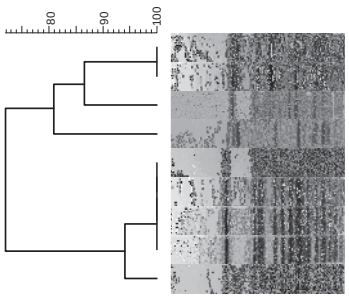

Figure 1. Dendrogram based on *Pme*I pulsed-field gel electrophoresis (PFGE) patterns of *Francisella tularensis* type A isolates. The dendrogram was constructed by using Dice similarity coefficients (1.5% optimization and 1.5% tolerance) and unweighted pair group method with averages. Strains WY96–3418, ATCC 6223, SCHU S4, and MA00–2972 were included as known A1 and A2 controls for creation of the dendrogram. Control strains were previously identified as either A1 or A2 by multiple methods including multilocus variable number tandem repeat analysis, PFGE, housekeeping gene sequence analysis, whole genome sequencing, and Indel analysis (*1*,*2*,*4*–*7*,*9*).

A brief search of the exposure area yielded desiccated carcasses of 10 black-tailed jackrabbits (*Lepus californicus)* and 2 desert cottontail rabbits (*Sylvilagus audubonii*). The carcasses were found within a few hundred meters of each other, in an overall area <0.8 km across. Living deer flies (*Chrysops* spp.) were collected from the same area. DNA was extracted from lagomorph bone marrow and flies by using the QIAamp DNA MiniKit (QIAGEN, Valencia, CA, USA) and tested with real-time PCR *F. tularensis* multitarget type A and type B assays (*10*,*11*). Although all deer fly samples were negative, 11 of 12 lagomorph carcasses tested positive for *F. tularensis* by the multitarget assay (3 of 3 targets positive; crossing threshold (C_t) range 13–38). Among the infected samples, 9 tested positive for type A (C_t range 13–36) and 2 tested positive for type B (C_t range 19–24). The subtyping results were verified by sequencing of the succinate dehydrogenase gene (*sdhA*), which distinguishes type A and type B strains on the basis of a single nucleotide polymorphism (*12*). Type B strains have a G at nt 465 of the *sdhA* gene sequence, whereas type A strains have an A at this position. To further distinguish the type A samples between clade A1 or clade A2, conventional PCRs were used (*13*). Sufficient *F. tularensis* DNA was present to type infections for 5 of the 9 type A–positive lagomorph carcasses; 4 yielded a PCR product consistent with the A1 clade (570 bp), and 1 yielded a product consistent with the A2 clade (396 bp) (Figure 2) (*13*).

Conclusions

Few studies have reported on the diversity of *F. tularensis* subsp. or clades present during outbreaks of tularemia, in part because molecular methods for strain discrimination have only recently been described (*1*,*2*,*4*–*7*). In this discrete deer fly–associated outbreak, we found human infections caused by both A1 and A2 strains, and evidence that A1, A2, and type B strains were circulating among lagomorphs in the exposure area. These findings demonstrate that multiple subspecies and clades can cause disease in a localized outbreak of tularemia and that deer-flies are associated with transmission of A1 strains.

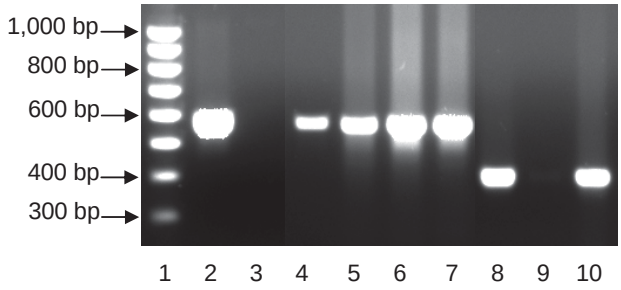

Figure 2. PCR typing of *Francisella tularensis*, clades A1 and A2, in dessicated lagomorph carcasses. Lane 1, 100-bp ladder (Bio-Rad, Hercules, CA, USA); lane 2, A1 positive control (Schu S4); lane 3, A1 negative control (NM99); lane 4, UT07–5156 (A1); lane 5, UT07–5152 (A1); lane 6, UT07–5157 (A1), lane 7, UT07–5159 (A1), lane 8, A2 positive control (NM99); lane 9, A2 negative control (Schu S4); lane 10, UT07–5161 (A2).

Published reports indicate that A1 and A2 strains are generally segregated into areas east and west of the Rocky Mountains, respectively, with some overlap in coastal California (*1*,*2*). In contrast, our results demonstrate that A1 strains are present in areas of the intermountain west and at elevations >1,200 m. This finding is supported by identification of an additional case of human tularemia in Utah caused by an A1 strain in 1998 (Centers for Disease Control and Prevention, unpub. data). On a local level, our results indicate that A1, A2, and type B strains can coexist naturally within the same ecosystem, a paradox when compared with the segregation that appears to exist on a larger scale. Overall, these observations underscore the need for future studies to define the ecologic and evolutionary factors underlying the distributions of *F. tularensis* strains in North America.

Although the role of deer flies as vectors of *F. tularensis* is well established, the dynamics of deer fly–associated outbreaks have not been well researched. Transmission of *F. tularensis* by deer flies is believed to be entirely mechanical, through contamination of the mouthparts. Long-term maintenance of *F. tularensis* has not been shown to occur in deer flies, and it is therefore not surprising that the deer flies we collected 3 weeks after the outbreak tested negative for this organism. Our findings suggest that deer flies nonselectively acquire and transmit whatever strains are circulating in enzootic hosts. We postulate that, in this instance, an abundance of deer flies led to extensive feeding on many hosts, resulting in the simultaneous transmission of multiple strains. High mortality rates among lagomorphs may have forced deer flies to seek alternate hosts, specifically muskrats, which are associated with type B strains and have been linked to outbreaks among trappers at Utah Lake (*3*).

The co-occurrence of multiple subspecies and clades may be unique to arthropod-associated outbreaks of tularemia and not characteristic of outbreaks resulting from other modes of *F. tularensis* transmission, such as contaminated water. Further work is needed to determine whether our findings will apply to other deer fly–associated outbreaks or for outbreaks of tularemia associated with ticks, which are known to maintain as well as transmit *F. tularensis*. Notably, while investigating a tick-borne outbreak of presumed type B infections in South Dakota, Markowitz and colleagues found evidence of both type A and type B strains in *Dermacentor variabilis* ticks collected from dogs (*14*). Outbreaks involving multiple serotypes have been observed with other vector-borne pathogens, including dengue virus (*15*), which suggests that amplification and transmission of multiple strains in a focal area may represent a general feature of some vector-borne diseases.

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Mycobacterium bovis Strains Causing Smear-Positive Human Tuberculosis, Southwest Ireland

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Mycobacterium bovis caused 3% of human tuberculosis cases in southwest Ireland during 1998–2006. Of 11 *M. bovis* strains genotyped, 9 belonged to common animal spoligotypes. Seven strains were from sputum and potential sources of human-centered disease transmission. Ten-locus variable-number tandem repeat typing gave unique strain profiles and would detect disease outbreaks.

Bovine tuberculosis occurs worldwide (*1*–*3*). It is caused by *Mycobacterium bovis*, a cattle-adapted member of the *M. tuberculosis* complex. *M. bovis* has the broadest host range of pathogenic mycobacteria, infecting domestic and wild mammals, and is classified as a Hazard Group 3 infectious agent (*1*). Human infection follows ingestion of unpasteurized milk or inhalation of droplet nuclei (*1*). In many countries, the risk for *M. bovis* infection in humans has been reduced by a test-and-slaughter program in which infected cattle are identified and culled. This program has eradicated *M. bovis* cattle infection from 11 states of the European Union (*3*). However, the Republic of Ireland and its neighbor the United Kingdom have failed to eradicate bovine tuberculosis (*1*,*4*). In the 1980s, 4%–6% of all cases of laboratory-confirmed tuberculosis in southwest Ireland were caused by *M. bovis* (*5*). Our study results suggest that this remains a problem in Ireland.

Molecular typing systems for pathogenic mycobacteria are important for epidemiologic control because they enable case-linking and outbreak tracing (*6*). We report a molecular epidemiology study that used spoligotyping, mycobacterial interspersed repetitive units–variable-number tandem repeat (MIRU-VNTR) typing, and region of difference (RD) typing of *M. bovis* strains isolated from human residents of Ireland.

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The Study

During 1998–2006, the microbiology laboratory at Cork University Hospital obtained *M. tuberculosis* complex isolates from 501 patients (equivalent to 68.5% of notified cases) residing in southwest Ireland (counties Cork and Kerry); 15 were *M. bovis* isolates (3%). Eleven of these *M. bovis* strains were available for testing. Seven additional isolates obtained over this period (from inoculation abscesses) were identified as *M. bovis* BCG and not analyzed. Strains were identified as *M. tuberculosis* complex by using Accuprobe (Gene-Probe, San Diego, CA, USA) and as potential *M. bovis* strains by pyruvate dependence. Definitive identification was performed at the Mycobacterial Reference Unit in London and was based on absence of niacin production and nitrate reductase activity, thiophen-2-carboxylic acid hydrazide negativity, and pyrazinamide resistance (*1*). DNA extraction was performed as described (*2*). DNA controls were *M. tuberculosis* H37Rv and *M. bovis* AF2122/97. A 6-locus panel VNTR (exact tandem repeat [ETR]-A to ETR-F) (*7*) was used initially, then a 10-locus VNTR panel was used (Table 1) (*8*). Spoligotyping was as described by Kamerbeek (*9*). RD analysis was conducted by the method of Brosch et al. (*10*) for RD1, RD4, RD9, RD10, RD12, RDpan, RD17, N-RD17, and N-RD25. This study was reviewed and approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Ref ECM 3).

Spoligotype signatures were recorded as binary numbers (1 and 0 denoting presence and absence of oligonucleotide spacer, respectively). Signatures were matched against the *M. bovis* spoligotype database (www.mbovis.org) and the global spoligotype database, SpolDB4. Unmatched spoligotypes were sent to the *M. bovis* spoligotype database curator for an authoritative name assignment (SB number). The discriminatory power of VNTR and spoligotyping was calculated by using the Hunter Gaston Discriminatory Index. Two or more spoligotype or VNTR patterns with 100% identity were considered a cluster.

Eight spoligotypes (Table 2) were identified among 11 isolates. Two clusters were identified: 3 strains of SB0140 (ST683) and 2 strains corresponding to SB0139 (ST680) (Table 2). SB0140 (also known as spoligotype A1 or ST1), is the most common spoligotype in animals in Ireland and the United Kingdom (*1*,*11*) (Table 2). SB0139 was previously detected as an isolate from a cow in Northern Ireland in 2000 (SpolDB4; R. Skuce, pers. comm.). Other spoligo-

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Table 1. Heterogeneity of each MIRU-VNTR locus of *Mycobacterium bovis* isolates, southwest Ireland, 1998–2006*

VNTR locus	Isolate	No. tandem repeat copies												HGDI	Allelic variants
		0	1	2	3	4	5	6	7	8	9	10	11		
1895	QUB1895	0	0	1	2	8	0	0	0	0	0	0	0	0.47	3
2163a	QUB11a	0	0	0	2	0	0	0	0	0	0	4	5	0.69	3
2163b	QUB11b	0	1	0	2	8	0	0	0	0	0	0	0	0.47	3
2165	ETR-A	0	0	0	0	1	2	0	8	0	0	0	0	0.47	3
2461	ETR-B	0	0	0	1	1	8	0	1	0	0	0	0	0.49	4
2687	MIRU24	0	6	5	0	0	0	0	0	0	0	0	0	0.55	2
2996	MIRU26	0	0	0	1	1	7	2	0	0	0	0	0	0.60	4
3232	QUB3232	1	0	0	0	0	0	0	0	7	1	1	1	0.62	5
3336	QUB3336	0	0	1	6	3	0	0	1	0	0	0	0	0.67	4
4052	QUB26	0	0	2	1	8	0	0	0	0	0	0	0	0.47	3

*MIRU, mycobacterial interspersed repetitive units; VNTR, variable-number tandem repeats; HGDI, Hunter Gaston Discriminatory Index; QUB, Queen's University Belfast; ETR, exact tandem repeat.

types we identified that had been previously reported in isolates from animals in Ireland were SB0130 (ST691), also described as D1 or ST7; SB0142 (ST679), also described as A2 or ST2*;* and SB1047 (not found in SpolDB4). Three strains were not found in either of the databases. These strains were assigned spoligotypes SB1185, SB1186, and SB1187 through the *M. bovis* spoligotype database. SB1186 and SB1187 differed from the ubiquitous SB140 by absence of spacer 35 and spacers 35 and 37, respectively. Previously reported SB0139, SB0142, and SB1047 differed from SB0140 by absence of spacers 33, 33–34, and 35–36, respectively. SB1185 differed from the established animal strain SB0130 by the absence of spacers 20–21.

Six-locus VNTR (ETR-A–ETR-F) showed little variation. The predominant profile (5 strains) was 7-5-5-4*-3-3.1. All strains except CUH-HB005 had ETR A–F profiles, matching spoligotype SB0140 strains isolated from cattle in England and Wales (*12*). A 10-locus expanded panel derived from loci recently described for Irish zoonotic *M. bovis* strains (*8*) improved strain discrimination by producing

Table 2. Molecular characteristics of strains and clinical and demographic parameters of 11 patients from whom *Mycobacterium bovis* was isolated, southwest Ireland, 1998–2006*

Study no.	Spoligotype	SpolDB4†	Mbovis.org‡	Other name§	VNTR pattern¶	Patient data		
						Sex/ age, y	Sample site	Sputum smear
HB002	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	ST683	SB0140	A1	4-11-4-7-5-1-5-8-3-2	F/81	Sputum	+
HB008	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	ST683	SB0140	A1	3-10-4-5-5-1-5-8-3-4	F/86	Sputum	–
HB010	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	ST683	SB0140	A1	4-3-4-7-5-1-5-8-3-4	F/33	Sputum	+
HB011	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■□■■■■■□□□□□	ST680	SB0139	NA	4-3-4-7-3-1-5-8-3-4	F/29	Sputum	+
HB012	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■□■■■■■□□□□□	ST680	SB0139	NA	4-10-4-7-4-1-5-8-3-4	M/62	Sputum	+
HB006	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■□■■■□□□□□	ND	SB1186	NA	4-11-4-5-5-2-5-9-4-4	M/60	Urine	NA
HB007	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■■□□■■□□□□□	ND	SB1047	NA	4-10-3-7-7-2-4-x-4-2	F/74	Neck abscess	NA
HB003	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■□□■■■■□□□□□	ST679	SB0142	D1	3-11-4-7-5-1-6-8-3-4	M/37	Spinal disc aspirate	NA
HB009	■■□■■□■□□□□□■■■□■■■■■■■■■■■■■■■□■□■□□□□□	ND	SB1187	NA	4-11-4-7-5-2-5-10-4-4	F/80	Sputum	+
HB001	■■□■■■■■□■□■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□□	ST691	SB130	A2	2-11-3-7-5-2-3-8-2-3	M/16	Sputum	+
HB005	■■□■■■■■□■□■■■■□■■■□□■■■■■■■■■■■■■■■□□□□□	ND	SB1185	NA	4-10-1-4-5-2-6-11-7-4	M/68	Testis biopsy sample	NA

*VNTR, variable number tandem repeats; +, smear positive by auramine and Ziehl-Neelsen stains; –, smear negative by auramine and Ziehl-Neelsen stains; NA, not applicable; ND, not described; x, not determined.
†Spoligotype designation as described in the SpolDB4 database (www.pasteur-guadeloupe.fr/tb).
‡Spoligotype assignment as described in the Mbovis.org database (www.mbovis.org).
§Spoligotype assignment (*11*).
¶VNTR profile based on a 10-loci scheme for *M. bovis* strains from Ireland in this order: QUB1895, QUB11a, QUB11b, ETR-A, ETR-B, MIRU 24, MIRU 26, QUB 3232, QUB 3336, and QUB26 (*8*).

different profiles for every isolate (Table 1). Hence, VNTR typing was able to split the clustered spoligotypes into individual profiles. Analysis of regions of difference confirmed that none of the strains were derived from *M. bovis* BCG.

Conclusions

A recent outbreak report describing sputum-positive *M. bovis* disease transmitted by person-to-person contact (*6*) underlines the need for precise genetic markers of *M. bovis* to aid epidemiologic traceback. We studied strains of *M. bovis* isolated from humans in the Republic of Ireland, and we have defined an optimal set of markers using a combination of spoligotyping and VNTR. We detected a group of isolates (Table 2) of spoligotype SB0140, which is predominant in animal strains of *M. bovis* reported from the Republic of Ireland (51.8% of isolates) (*11*) and the United Kingdom (*1*). It forms the single largest group of *M. bovis* strains isolated from humans in the United Kingdom (30%) (*1*). This group was not reported in a recent series of *M. bovis* isolates from humans in Italy (*13*) or France (*14*). Indeed, none of the spoligotypes in our survey and these reports overlap. Predominant strains by spoligotype in animals and those infecting humans in the same country are known to be linked (*1*,*13*,*14*). We found 3 novel spoligotypes similar to SB0140 in a small group of patients, showing that a wider variety of strains infect humans than animals, as described in similar studies (*1*,*2*,*13*).

In our study, 81% of patients infected were >30 years of age (Table 2), comparable with findings of a previous survey of the southwest Ireland population (*5*). Primary infection of this group is likely to have been several decades before diagnosis, and our isolates probably represent reactivation of disease acquired earlier in life, effectively a record of past prevalence in animals. Spoligotype SB0140 strains were isolated from 2 patients who were >80 years of age, showing that the current predominance of SB0140 in animals (*11*) is therefore of long duration in Ireland, potentially going back 8 decades.

Molecular typing by insertion sequence (IS) *6110*–based restriction fragment length polymorphism is inadequate for *M. bovis* because of low copy numbers of IS*6110*. A combination of MIRU-VNTR and spoligotyping gives better discrimination (*15*). Traditional 6-locus VNTR (ETR-A–ETR-F) has been described for typing of *M. tuberculosis* complex strains (*7*) including *M. bovis* (*2*), but an expanded panel with an additional 10 loci applied to our strains greatly improved discrimination and enabled individual identification of each isolate.

Seven of our 11 isolates were from sputum, and 6 were detected on direct smear with potential for transmission. A VNTR-typing scheme based on the loci established on *M. bovis* isolates from animals (*8*) would detect *M. bovis* clusters derived from foodborne outbreaks or horizontal transmission of disease between humans in Ireland. Our study provides ways to markedly improve the ability to identify and contact-trace future clusters of *M. bovis* infection in humans.

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Emergence of *Francisella novicida* Bacteremia, Thailand

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We report isolation of *Francisella novicida*–causing bacteremia in a woman from Thailand who was receiving chemotherapy for ovarian cancer. The organism was isolated from blood cultures and identified by 16S rDNA and PPIase gene analyses. Diagnosis and treatment were delayed due to unawareness of the disease in this region.

Francisella novicida, a rare human pathogen, has recently been considered to be a subspecies of *F. tularensis* on the basis of DNA similarity (*1*,*2*). The reservoir and transmission route of *F. novicida* were not clearly defined. Since the first isolation of *F. novicida*, to our knowledge, only 5 patients with suspected infection have been reported (*3*–*5*). *F. novicida*, however, has neither been isolated nor associated with human disease in Thailand. We report a case of *F. novicida* infection in a Thai patient who was undergoing chemotherapy.

The Study

In October 2007, a 37-year-old woman from Thailand sought treatment at Siriraj Hospital (a 2,400-bed university hospital in Bangkok, Thailand) with a history of fever for 1 week. She was a hairdresser residing in a suburban area of Prachuap Khiri Khan, a southern province of Thailand. She denied history of blood transfusion, animal contact, and travel abroad. She had not been aware of being bitten by insects recently. There was no incidence of unusual animal death in the area in which she resided. Five months before seeking treatment, she received a diagnosis of advanced stage clear cell adenocarcinoma of the ovary with metastasis to peritoneum, spleen, uterus, and multiple abdominal lymph nodes. Chemotherapy was planned. Initial laboratory screening showed increased liver enzyme levels and abnormal hepatitis markers confirming chronic active hepatitis B virus infection. Chemotherapy was delayed while she was treated with lamivudine. A follow-up visit in early September showed that her liver function biochemistry results had returned to within normal limits. Chemotherapy with carboplastin and paclitaxel was then initiated.

At the time of admission, 25 days after the start of chemotherapy, the patient had fever (39°C), blood pressure 90/60 mm Hg, and pulse rate 75 beats/min. She also had an episode of gastrointestinal hemorrhage with melena. It was believed that fever and gastrointestinal bleeding were complications from chemotherapy; thus, microbiologic investigation was not promptly initiated. Abnormal laboratory findings included anemia (hemoglobin 80 g/L) and leukocytosis with marked neutrophilia (Figure). Urine and stool cultures showed insignificant growth.

Two samples of blood cultures from peripheral lines were obtained using BacT/Alert FA bottles (bioMérieux, Durham, NC, USA) on day 10 of hospital admission and incubated in the continuous monitoring BacT/Alert 3D system (bioMérieux). Both blood culture bottles grew small pleomorphic gram-negative coccobacillus after incubation for 2 days. Samples from positive bottles were subcultured onto 5% (vol/vol) sheep blood agar, MacConkey agar, and chocolate agar. A slow-growing bacterium was recovered on both blood agar and chocolate agar after 2-day incubation at 35°C with 5% CO_2. The organism was negative for catalase and oxidase. Bacterial identification was delayed because the organism was unidentifiable based on the conventional biochemical keys and the Vitek 2 system (bioMérieux, Marcy L'Etoile, France). Using the API 20NE (bioMérieux), the organism was identified as *Mannheimia haemolytica*/*Pasteurella trehalosi* with good confidence level (94.5% probability).

The identification of this isolate was further determined by the 16S rDNA gene analysis that used screening primers SQE1 and SQE3 (*6*) and the 1,445-bp gene sequencing procedure as described elsewhere (*7*). The DNA sequence was analyzed by using the standard nucleotide-nucleotide BLAST algorithm (www.ncbi.nlm.nih.gov). The 16S rDNA sequence of this isolate, called strain RB401, was most closely related (99.9% homology) to the *novicida*-like

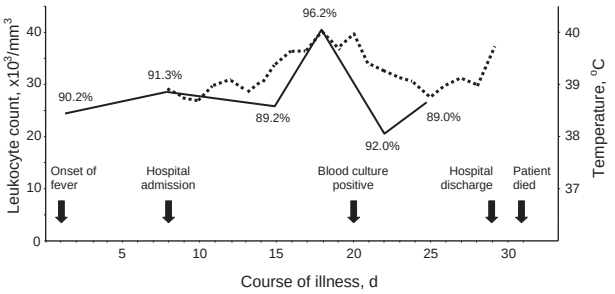

Figure. Course of illness in a Thai patient with *Francisella novicida* bacteremia. Solid line, leukocyte count with percentage of neutrophils; dashed line, temperature.

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subspecies of *F. novicida* strain 3523 (GenBank accession no. AY243028) and was deposited to the GenBank database under the accession no. EU365864.

Identification of strain RB401 was further confirmed by analyzing DNA sequences of TUL4 and PPIase genes as previously described for strain 3523 (*5*). The fragment containing TUL4 amplicon (363 nt) from strain RB401 shared the highest similarity (98%) to the matching region from strain 3523. Comparing the 267-nt coding region sequence of the 3′ end of PPIase gene nt 733259–733525 to the genome sequence of *F. novicida* strain U112 (GenBank accession no. CP000439) showed the highest similarity to all subspecies of *F. tularensis,* e.g., *F. tularensis* subsp. *tularensis* strain WY96-3418 (GenBank accession no. CP000608), and to *F. novicida* strain U112. The PPIase gene sequence of strain 3523 was not available for the alignment. However, the above findings also supported that our isolate was *F. novicida*. The sequences of TUL4 and PPIase genes from strain RB401 were deposited to GenBank under accession nos. EU786119 and EU786120, respectively.

Antimicrobial drug susceptibility testing was performed by disk diffusion method and interpreted based on the CLSI criteria for *Acinetobacter* spp. (*8*). Results showed that strain RB401 was susceptible to piperacillin/tazobactam, third-generation cephalosporins, cefepime, imipenem, meropenem, aminoglycosides, fluoroquinolones, and tetracycline, but resistant to co-trimoxazole. Due to the delay in organism identification and lack of awareness of its significance, antimicrobial treatment (piperacillin/tazobactam 4.5 g intravenously every 8 h) was not initiated until day 19. However, on day 21, the patient requested to be referred to her hometown hospital and died 2 days after referral. The final microbiology report was released 2 days after her death. The serologic test for antibodies against *Francisella* spp. was not available. All laboratory technicians who processed culture and identified the bacterium received doxycycline (100 mg twice a day for 14 days) for prophylaxis, and none reported fever or abnormal symptoms.

Conclusions

F. novicida, often referred to as *F. tularensis* subsp. *novicida*, is rarely attributed to human infection and is not readily recognized in most clinical laboratories. Because of the unreliable results of phenotypic identification methods, unfamiliarity of the bacterium and close genetic relatedness among *Francisella* spp., the organism can be misidentified, thus leading to inappropriate management. A clinical diagnosis of *Francisella* infection is highly nonspecific, and it seems that the underlying disease with immunosuppression was an important factor to contracting the disease in the described case and previous case reports (*3*–*5*). Human *F. novicida* infection has not previously been described from Asia.

There is no clear explanation regarding route of acquisition and the pathogenic role of this organism. Additionally, there is no evidence for human-to-human transmission. In our case, clinicians and microbiologists did not suspect *F. novicida* infection. Given that our patient had severe complicated underlying diseases while the potentially low-virulence organism was recovered, it was thus difficult to claim that the patient's death was solely due to *F. novicida* infection. Notably, the 16S rDNA, TUL4, and PPIase sequences of our isolate are most closely related to the strain earlier reported from Australia (*5*). These areas are not known to be endemic for *F. novicida,* and therefore, these strains represent the emergence of *F. novicida* in the Asia-Pacific region. The earlier case-patient was successfully treated with flucloxacillin and doxycycline, followed by dicloxacillin and doxycycline (*5*).

F. novicida is not considered to have a fastidious growth requirement. The standard protocol of 5-day incubation for automated blood culture (*9*) is supposedly sufficient for detection of *F. novicida* bacteremia. Identification of *F. novicida*, however, is often difficult because the bacterium can be easily misidentified as a non-*Francisella* species or as a highly pathogenic *F. tularensis*. It was also indicated that this organism could not be detected by a direct fluorescent antibody test used for the identification of *F. tularensis* types A and B (*5*). As a preliminary result, our 16S rDNA sequence was closely related to subspecies of *F. tularensis*, and thus doxycycline prophylaxis for the organism-exposed laboratory personnel could not be avoided. There is high risk for laboratory-acquired tularemia when handling *F. tularensis* cultures (*10*).

In retrospect, prophylactic treatment may have been unnecessary because our isolate proved to be *F. novicida,* which is not known to cause laboratory-acquired infections. Rapid methods for *Francisella* species identification are needed for better consideration of antimicrobial prophylaxis. Most *F. tularensis* strains were susceptible to aminoglycosides, quinolones, and tetracyclines (*11*). Our isolate was similarly susceptible to these agents, which suggests that they may be considered as therapeutic options for *Francisella* spp. Initial treatment with piperacillin/tazobactam was based on the preliminary report of gram-negative bacilli, but later antimicrobial susceptibility testing showed this isolate to be susceptible to this agent. However, previous reports (*11*–*13*) suggested that piperacillin/tazobactam and other β-lactams are generally considered ineffective in vivo against *Francisella* spp. and likely result in therapeutic failure. Given that the organism is believed to be of low virulence, particularly that it does not pose a substantial risk for immunocompetent persons, its role as a human pathogen remains controversial. Further studies to gain better understanding of pathogenic mechanism and transmission route of *F. novicida*

are necessary to provide appropriate guidelines for the treatment and prophylaxis.

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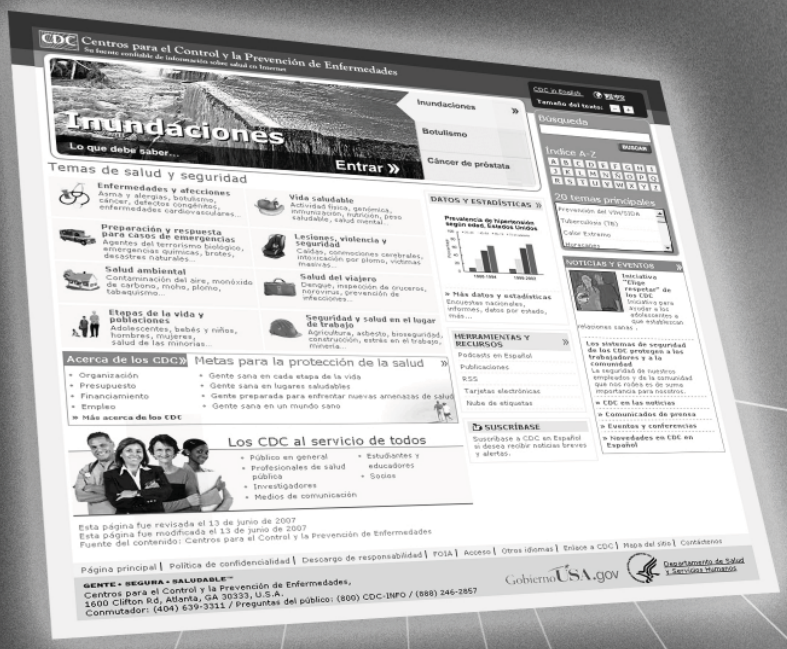

Índice A–Z

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R	S	T	U	V	W	X	Y	Z

Temas de salud y seguridad

Enfermedades y afecciones
Asma y alergias, botulismo cáncer, defectos congénitos, enfermedades cardiovasculares...

Preparación y respuesta para casos de emergencias
Agentes del terrorismo biológico, emergencias químicas, brotes, desastres naturales...

Salud ambiental
Contaminación del aire, monóxido de carbone, moho, plomo, tabaquismo...

Etapas de la vida y poblaciones
Adolescentes, bebés y niños, hombres, muheres, salud de las minorías...

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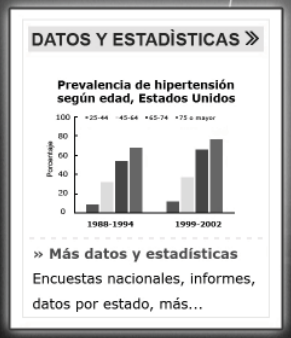

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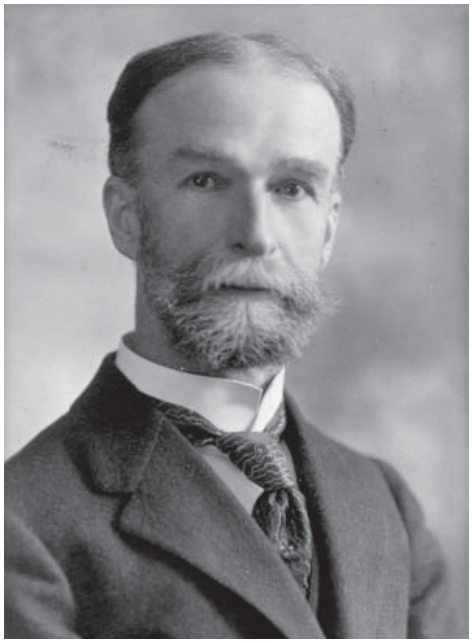


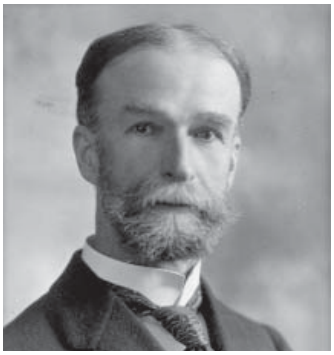
Who is this man and what did he accomplish?

The theme of this month's issue of EID is zoonotic diseases. Consistent with this theme, here is a photograph of a physician who greatly advanced medicine and public health through his investigations of diseases of animals. He and his colleagues were the first to show that insects can transmit disease.

Who is he?

A) Sir David Bruce
B) Robert Koch
C) Sir William Osler
D) Sir Ronald Ross
E) Theobald Smith

Decide first. Then turn the page.



Theobald Smith

Myron Schultz

This is a photograph of Theobald Smith (1859–1934). Smith was a pioneer epidemiologist, bacteriologist, and pathologist who made many contributions to medical science that were of far-reaching importance. He is best known for his work on Texas cattle fever, in which he and his colleagues discovered the protozoan agent and its means of transmission by ticks. This was the first time that an arthropod had been definitively linked with the transmission of an infectious disease.

Theobald Smith was born in 1859. He was the son of a German immigrant, who kept a small tailoring shop in Albany, New York. At age 18, Smith earned a tuition-free scholarship to Cornell University. He graduated from Cornell in 1881 with a Bachelor of Philosophy degree, and he received his MD at Albany Medical College in 1883. Realizing that his 2 years of study had not prepared him for the practice of medicine, Smith returned to Cornell for graduate study. Smith's mentor at Cornell, Professor Simon Gage, helped him secure his first job at the newly formed Bureau of Animal Industry (BAI) in Washington, DC. Smith also established a department of bacteriology at Columbian University (now George Washington University), where he taught from 1886 to 1895. This was the first department of bacteriology at a medical school in the United States.

When he went to Washington, Smith knew very little bacteriology. He had not been able go to Europe to study with men like Pasteur, Koch, or Virchow. Because he could read and speak German almost as well as English and he could read French easily, he was able to study the papers of these masters and teach himself. Within a year of his arrival in Washington, Smith introduced Koch's methods. At this time, he also began his life-long work on tuberculosis. At a later time, he successfully challenged Koch's concept that human and bovine tuberculosis were caused by the same organism.

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Smith's work at BAI was extremely productive. BAI was created within the Department of Agriculture in 1884, when efforts by the states to stem the rising tide of animal diseases proved inadequate. The major problems were hog cholera, bovine pleuropneumonia, Texas cattle fever, turkey blackhead, and bovine tuberculosis. During his first 2 years at BAI, Smith discovered a new species of bacteria (*Salmonella enterica*, formerly called S*almonella choleraesuis*), which he thought was the cause of hog cholera. It was later shown that hog cholera was in fact a viral infection and Smith's bacillus was a constant but secondary invader. Although this genus of bacteria was discovered by Smith, Daniel E. Salmon, Smith's chief, claimed credit for the discovery, and the genus *Salmonella* is named after him. In 1886, Smith, collaborating with Salmon, presented the first proof that killed bacteria could be used to induce active immunity in experimental animals. This established the basis for the later development of protective immunization for human bacterial enteric diseases such as typhoid and cholera. Smith was the first person to use the fermentation tube to study bacterial physiology and classification, especially focusing on the details for differentiating aerobes, facultative anaerobes, or anaerobes and on characterizing fresh isolates thought to belong to these groups.

A few years after beginning his work at BAI, Smith turned his attention to Texas cattle fever, a devastating disease that destroyed 90% of herds in some affected areas. It occurred in northern cattle that came in contact with cattle from Texas during cattle drives to stockyards in Kansas, Missouri, Iowa, and Illinois. It was a problem of great economic and political importance. Cattle ranchers had long held a vague but persistent impression that ticks were in some manner the cause of the disease. Smith had the good sense to listen to the cattle ranchers and formulate a hypothesis based on these impressions that he tested with searching experiments to subject it to scientific scrutiny. Some confusion exists about the part that Smith played in the Texas cattle fever discovery. Smith is widely cited as the sole person who discovered that ticks were the vectors

of Texas cattle fever, when in fact, it was a collaborative effort of Smith with his colleagues, Fred L. Kilbourne and Cooper Curtice, both veterinarians. Smith never claimed this work as solely his own, even though popular accounts entirely credited him.

In 1889, Smith described little bodies in the erythrocytes of infected cattle; he later recognized (1891) them as protozoa, which he eventually named *Piroplasma bigeminum* (now called *Babesia bigemina*). Following this discovery, Smith and Kilbourne conducted experiments in which they placed southern cattle in pens with northern cattle. In some instances, ticks were left on the infected animals; in other enclosures, the ticks were removed. The researchers also kept native cattle in fields in which infected ticks had been left on the ground. These transmission experiments established beyond question the role of ticks (*Boophilus* spp.) as the carrier of this disease. Smith's 301-page monograph about the laboratory and field experiments, BAI Bulletin No. One (1893), is regarded as one of the classics of medical literature. In these experiments, it was also demonstrated that the infection could pass in ticks from adults to nymphs, a new and extraordinary phenomenon of parasitism. This research was conducted by Curtice. Delineation of the tick's life cycle soon paved the way for control of the disease by dipping cattle to kill the ticks.

The discovery by Smith et al. that insects can transmit disease represents one of the fundamental steps forward that altered the entire course of medical science and public health. It presaged the discovery in the next few years of the insect transmission of trypanosomiasis of cattle (nagana) in 1895 by David Bruce, malaria in 1897 by Ronald Ross, yellow fever in 1900 by Walter Reed and his colleagues, and typhus in 1909 by Charles Nicolle.

In 1895, Smith reported that blackhead, an economically devastating enterohepatitis of turkeys, was caused by a protozoan called *Amoeba meleagridis* (now *Histomonas meleagridis*). Later, while at the Rockefeller Institute for Medical Research, Smith resolved the puzzle of transmission by discovering that embryonated eggs of the intestinal roundworm *Heterakis papillosa* (now *Heterakis gallinae*) could transmit the amoebas. This mechanism of transmitting a protozoan remains unique in the annals of parasitology.

In 1895, Smith moved to Cambridge, Massachusetts, to accept a dual appointment, serving as professor of comparative pathology at Harvard University and director of the Massachusetts State Antitoxin and Vaccine Laboratory. As director of this laboratory, Smith undertook many practical and theoretical studies on the production of tetanus and diphtheria antitoxins. He was one of the first to demonstrate the production of immunity by killed cultures of disease organisms and to show that a mixture of diphtheria toxin and antitoxin confers immunity. Under Smith's administration, diphtheria antitoxin production increased from 1,700 to 33,000 doses within 4 years. Human deaths were reduced from 25% to 11% during the first year. Smith concluded that during the first 7 years of production of the antitoxin in the state laboratory, 10,000 lives had been saved by its use. Among Smith's many fundamental contributions to immunology, the most important was demonstrating that animals develop hypersensitivity to bacteria upon repeated injections. What is now called anaphylaxis was long known as the "Theobald Smith phenomenon."

In 1915, Smith joined the Rockefeller Institute for Medical Research as director of the Department of Animal Pathology, in Princeton, New Jersey. He remained there until his retirement in 1929. One of Smith's accomplishments during this period was to clearly establish the criteria to distinguish between types of tubercle bacilli that affect humans and bovines. While Robert Koch asserted that bovine bacilli could not invade the human body, Smith took the position (which Koch later accepted) that the bovine organism could infect humans but was not the usual source of human infection. The bovine and human tubercle bacilli differ in many ways in their pathogenicity for animals and humans.

Regarding Smith's personality, he was known as a patient, tenacious researcher whose experiments were meticulously planned. Hans Zinsser describes him as "a simple person who had the qualities of unpretentious probity and an instinctive integrity." Smith authored 305 scientific publications, addresses, and government reports, usually as sole author. The last of Smith's publications was his scientific credo, a treatise on parasitism and disease in which he put into context the great mass of his individual findings. When he was 74, a dinner was given in his honor in Philadelphia. He made many sage remarks about the nature of research. Among them he said, "In general, a fact is worth more than theories in the long run. The theory stimulates, but the fact remains and becomes fertile. The fertility of a discovery is perhaps the surest measure of its survival value."

Among those who knew him, Smith was considered to be one of the most notable figures in American medicine of his period. Smith received 12 honorary degrees from leading universities and 11 medals. Among them was the Copley Gold Medal of the Royal Society, regarded as one of the highest scientific awards in the world at that time.

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Suggested Reading

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Bartonella henselae Antibodies after Cat Bite[1]

To the Editor: *Bartonella henselae* is the causative agent of cat-scratch disease, which is the most common form of human bartonellosis (*1*). In immunocompromised patients, e.g., HIV-infected patients, *B. henselae* can give rise to longstanding fever, bacillary angiomatosis, and peliosis hepatitis (*2*). Domestic cats are the reservoir for *B. henselae*, and cat fleas transmit the organism between cats (*3*). The seroprevalence and culture findings of *Bartonella* spp. in cats have been shown to be low in Sweden (*4*,*5*) compared with warmer areas (*6*). Cat-scratch disease is most often spread from cats to humans by scratches, but other forms of transmission, including cat bites, have been suggested (*7*).

To determine seroprevalence of antibodies against *B. henselae* in Sweden, we used data from a recently published prospective study of patients with infected cat bites (*8*). In addition to the information about bites, information about cat scratches was collected by retrospective review of the patients' medical records. Serum samples were taken during the patient's first visit to a hospital and at a follow-up visit about 2 weeks later. The study was approved by the local ethics committee.

Immunoglobulin G against specific *Bartonella* spp. was detected by the immunofluorescence antibody test (*1*). Cell-cultivated antigens were prepared from the following strains: *B. henselae* Houston-1 (ATCC 49882), *B. henselae* Marseille (CIP 104756), *B. henselae* Berlin1 (M. Arvand), *B. henselae* K68 (E. Olsson-Engvall), *B. elizabethae (*ATCC 49927*)*, and *B. grahamii* (ATCC 700132). The cutoff values for a positive immunofluorescence antibody test result were chosen as ≥128 for *B. henselae* and *B. grahamii* and ≥256 for *B. elizabethae*. The titers are expressed as the reciprocal of the end-point dilution. For controls, we also analyzed serum from 117 blood donors with these antigens. The χ^2 test was used for statistical analysis.

We analyzed antibodies to *Bartonella* spp. in serum from 71 patients (51 women and 20 men), median age 47 years (range 15–85 years). Only 11 patients had fever. Cat scratches were reported for 17 patients. A single serum sample was obtained from 37 of the 71 patients, and an additional convalescent-phase sample was obtained from 34 patients after a median of 16 days (range 6–54 days).

Antibodies against any *B. henselae* strain were found for 24/71 (34%) patients, against *B. elizabethae* for 9/71 (13%), and against *B. grahamii* for 12/71 (17%). A total of 13/71 (18%) patients showed reactivity to *B. henselae* only. Antibodies to any *Bartonella* spp. were found for 28/71 (39%) of the patients. As many as 13/24 (54%) serum samples with antibodies against *B. henselae* reacted to antigens of only that species. More patients (19/71; 27%) reacted to the antigen from the cat in Sweden, K68, than to other strains. The least common reactivity found in this study was against the *B. henselae* Marseille strain.

Of the 117 controls, 1 (0.8%) had antibodies against *B. henselae* K68 antigens, 3 (2.6%) against Berlin1, 2 (1.7%) against Marseille, 1 (0.8%) against Houston-1, and 4 (3.4%) against any *Bartonella* spp. The difference between patients and controls was significant ($p<0.001$).

Seroconversion was reported for 6 of the 34 patients (18%) from which 2 serum samples were analyzed (Table). Among those who seroconverted for *B. henselae,* 1 had fever and only 2 reported having been scratched. Two of the patients who seroconverted were treated with doxycycline, and 1 was treated with ciprofloxacin. In addition, 1 patient with Sjogren disease was initially treated with penicillin, and later a hemangioma-like exanthema developed. Because of severe acne, the patient was treated with doxycycline for 6 months. The other patients who seroconverted were treated with penicillin or amoxicillin.

Seroconversion for *B. henselae* occurred in 4 patients, of which only 2 had reported a scratch. Three of these patients reacted to *B. henselae* Berlin1, and 1 reacted to the Houston-1 strain. Because symptoms did not differ between the patients who did seroconvert and those who did not, these findings could indicate subclinical infection.

The prevalence of immunoglobulin G against *B. henselae* in particular was shown to be much higher than that previously reported in Sweden (*9*). Earlier studies used only 2

[1]Presented in part at the 4th International Conference on Bartonella as Emerging Pathogens, 2004 Aug 26–28, Uppsala, Sweden.

Table. Titers against *Bartonella* spp. antigens in 6 patients who seroconverted

Patient sex/ age, y	Reciprocal titer		Interval, d	Antigen	High titer to other antigen
	Acute-phase serum	Convalescent-phase serum			
F/77	32	128	6	*B. henselae* Berlin1	*B. henselae* K68
F/50	32	256	17	*B. henselae* Berlin1	0
M/74	128	512	19	*B. grahamii*	*B. henselae* K68
F/62	32	128	44	*B. elizabethae*	*B. henselae* K68
M/56	32	128	15	*B. henselae* Houston-1	*B. grahamii*
F/23	32	128	10	*B. henselae* Berlin1	*B. grahamii*

B. henselae antigens (Houston-1 and Marseille) compared with the 4 different *B. henselae* antigens used in the present study. Most reactivity to *B. henselae* in the present study was directed against the Swedish isolate K68 (27%); only 0.8% of controls had antibodies against that antigen.

An increased prevalence of antibodies against *B. henselae* after exposure to cats has been reported from Spain (*10*). Because seroconversion against *B. henselae* occurred in 2 patients who had not been scratched, cat bites may contribute to transmission of *B. henselae*.

Acknowledgments

We thank Mardjan Arvand and Eva Olsson-Engvall for providing antigens of *B. henselae* Berlin1 and *B. henselae* K68, respectively.

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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.

Fatal Case of Israeli Spotted Fever after Mediterranean Cruise

To the Editor: Israeli spotted fever (ISF) is caused by *Rickettsia conorii* subsp. *israelensis*. This recently described subspecies is genetically close to *R. conorii* subsp. *conorii*, the agent of Mediterranean spotted fever (MSF) (*1*,*2*). ISF is likely transmitted by the dog tick *Rhipicephalus sanguineus* (*3*). This tick, which is also the vector of *R. conorii* subsp. *conorii*, has low affinity for hosts other than dogs. Therefore, like MSF, cases of ISF will likely be sporadic (*4*,*5*). ISF was first reported in Israel (*1*) and was also recently described in Portugal and Italy (*6*–*8*). The clinical manifestations of ISF are similar to those of other spotted fever group infections, but an inoculation eschar is rarely observed and a history of tick exposure is not always present (*4*–*6*,*9*). The incubation period is ≈7–8 days after the tick bite (*4*).

We describe a 63-year-old man who had fatal ISF despite adequate therapy. The patient, who lived in Switzerland, took a cruise on the Mediterranean Sea, sailing for a week along the coasts of Crete, Libya, and Malta (Figure). With his wife, he visited several archeological sites in Libya (Cyrene, Apollonia, Ptolemais, Leptis Magna, Sabratha). Three days after returning to Switzerland, the patient reported loss of appetite, epigastric pain, and loose stools. Four days later, a fever (40°C) and generalized rash developed. The patient was hospitalized 6 days after symptom onset. At that time, he had fever (38.3°C), hypotension (85/55 mm Hg), tachycardia (100/min); a maculopapular rash involving the trunk, limbs, palms, and soles; and petechial lesions on the right arm. The patient was confused and exhibited bilateral dysdiadochokinesis. Laboratory investigations yielded the following

results: C-reactive protein level 183 mg/L; leukocyte count 4.9 $\times 10^9$/L; platelet count 23 $\times 10^9$/L; creatinine 741 μmol/L; sodium 127 mmol/L; aspartate aminotransferase 299 U/L; alanine aminotransferase 156 U/L; γ-glutamyl transpeptidase 160 U/L; pH 7.45; and lactate 4.5 mmol/L. A rapid blood test for malaria (OptiMAL-IT, DiaMed, Cressier, Switzerland) had negative results, and peripheral blood smears did not show any *Plasmodium* spp. During his trip, the patient had not had contact with animals and had no history of tick bite. His wife was asymptomatic.

The initial differential diagnosis mainly included typhoid fever, leptospirosis, malaria, HIV seroconversion, and MSF. Treatment with intravenous doxycycline, 100 mg twice a day, and ceftriaxone, 2 g every 24 h, was immediately started. The patient was admitted to the intensive care unit because of hypotension and received vasopressors. Twenty-four hours later, renal function deteriorated, and the patient was transferred to our university hospital in Lausanne, Switzerland, for hemodialysis. Antimicrobial drug treatment was switched to intravenous imipenem, 500 mg 4 times a day, and clarithromycin, 500 mg twice a day. On day 8 after symptom onset, he had pulseless electrical heart activity probably caused by metabolic acidosis. He died on day 11 after symptom onset.

Blood was sterile but was not cultured for rickettsiae, and stool culture showed no pathogens. Using serum obtained on day 6, we conducted a *Rickettsia* spp. microimmunofluorescence test; results were negative. However, results of a skin biopsy, conducted after 2 days of antibiotherapy, were positive by 2 PCRs targeting the *omp*A and *glt*A genes (*10*). Sequencing allowed postmortem identification of the etiologic agent as *R. conorii* subsp. *israelensis.* The sequences of *omp*A exhibited 99.8% (532/533 bp) similarity with *R. conorii* subsp. *israelensis* strain ISTT-CDC1^T (GenBank accession no. U43797), 98.3% (524/533 bp) with *R. conorii* subsp. *caspia* strain A-167^T (U43791) and 96.8% (540/558 bp) with *R. conorii* subsp. *conorii* strain Malish^T (AE008674). The sequences of *glt*A exhibited 100% (177/177 bp) similarity with *R. conorii* subsp. *israelensis* (U59727), 99.4% (176/177 bp) with *R. conorii* subsp. *caspia* (U59728), and 98.3% (174/177 bp) with *R. conorii* subsp. *conorii* (AE008677).

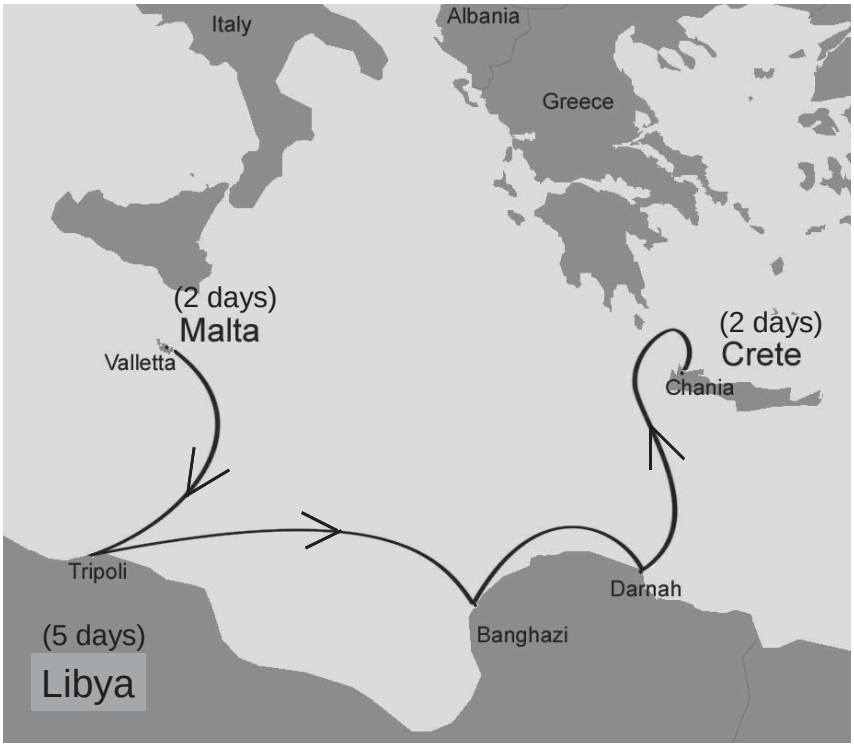

Figure. Cruise path on the Mediterranean Sea along the coasts of Crete, Libya, and Malta.

Autopsy showed severe pulmonary edema and liver ischemia. The brain had petechial hemorrhages in the left cerebellum and right frontal lobe, as well as a small recent ischemic infarction in the right caudate nucleus.

In this case of ISF, delayed medical consultation and thus late initiation of antimicrobial drug therapy (6 days after symptoms onset), the patient's age, and his chronic alcohol abuse probably contributed to the fatal course. The virulence of *R. conorii* subsp. *israelensis* might also be higher than that of *R. conorii* subsp. *conorii* (*6*,*7*).

ISF has not been described in any of the countries visited by the patient. Given the incubation time of spotted fever (7–8 days), he was probably infected in Lybia, where he spent days 6–10 before symptom onset. Geographic distribution of ISF can thus be extended to North Africa. Since ISF and MSF share the same vector (*Rh. sanguineus)*, disease-endemic areas probably overlap.

This report also points out the importance of early empirical treatment. Rickettsiosis should be suspected in febrile travelers, especially when they have a rash, even in the absence of history of tick exposure and inoculation eschar. Counseling before travel to areas endemic for spotted fever rickettsioses should include preventive measures for tick bites and recommendations to immediately seek medical advice in case of fever.

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Streptococcus suis Meningitis without History of Animal Contact, Italy

To the Editor: *Streptococcus suis*, a major swine pathogen worldwide, is emerging as a zoonotic agent capable of causing a variety of serious infections in swine as well as in persons exposed to pigs or to pork products. These infections include meningitis, septicemia, pneumonia, endocarditis, arthritis, and septic shock (*1*,*2*). Despite recent outbreaks among persons in China, *S. suis* disease in humans is a rare, probably underdiagnosed infection that usually occurs as sporadic cases (*1*,*2*). Persons in close occupational or accidental contact with pigs or pork products and those who eat uncooked or undercooked pork may be at higher risk than others. However, most infected persons are likely healthy carriers, and *S. suis* is believed to induce overt disease (especially meningitis) in only some circumstances (*2*). We describe a case of *S. suis* meningitis in a 68-year-old man from Sardinia, Italy, who had no reported contact with swine, other animals, or any animal products; the patient also had cancer, which was discovered incidentally during the workup.

In November 2007, the patient was hospitalized with a 48-hour history of fever, headache, nausea, and general malaise. Physical examination showed impaired consciousness, nuchal rigidity, and a temperature of 39.5°C. Laboratory findings were 20,700 leukocytes/mm^3 with 92% neutrophils, glucose 95 mg/dL, and C-reactive protein 375 mg/L. Cerebrospinal fluid (CSF) analysis demonstrated 240 leukocytes/μL with 80% polymorphonuclear cells, glucose 24 mg/dL, and protein 277 mg/dL. A computed tomography scan of the head showed no abnormal findings. Gram stain of CSF showed gram-positive cocci, mostly in pairs (Figure).

Empirical therapy consisted of intravenous ceftriaxone (2 g twice a day) and oral chloramphenicol (2 g once a day). On day 5, α-hemolytic streptococci were isolated from CSF on sheep blood agar and identified as *S. suis* by using APIStrep (bioMérieux, Marcy l'Etoile, France). Serotyping, performed by slide agglutination with specific antiserum (Statens Serum Institute, Copenhagen, Denmark), identified the isolate as serotype 2.

Antimicrobial drug–susceptibility testing, performed according to guidelines of the Clinical and Laboratory Standards Institute (www.clsi.org), indicated susceptibility to penicillin, ceftriaxone, chloramphenicol, levofloxacin, and vancomycin and resistance to erythromycin (MIC >128 mg/L) and tetracycline (MIC 16 mg/L). Erythromycin resistance was constitutive and was mediated by the *erm*(B) determinant; tetracycline resistance was mediated by *tet*(W). Multilocus sequence typing (http://ssuis.mlst.net) assigned the *S. suis* isolate to sequence type (ST) 1.

The patient, a retired welder, denied any recent occupational or even occasional contact with swine or other animals and had no history of eating raw or undercooked pork. The patient's condition improved; chloramphenicol was discontinued on day 10, but the 14-day course of ceftriaxone was completed. On day 6, the patient

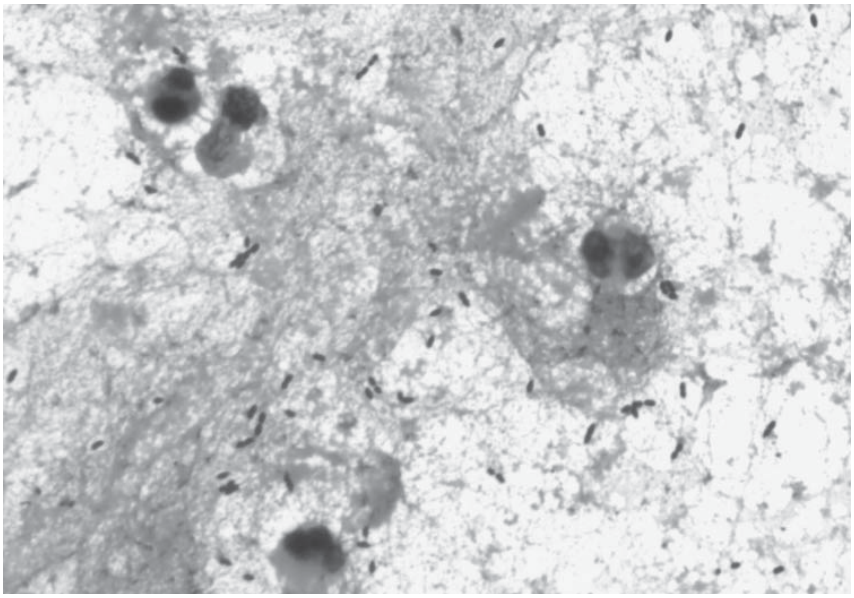

Figure. Gram-positive cocci, mostly in pairs, in cerebrospinal fluid from a 68-year-old man with *Streptococcus suis* meningitis. Magnification ×1,000.

became afebrile but had dizziness and deafness; a formal audiology evaluation on day 9 showed severe bilateral sensorineural high-frequency hearing loss (–80 dB) that improved after a short course of dexamethasone. However, the patient was not discharged because of the lung mass found on initial chest radiograph. Computed tomography scan, bronchoscopy, and histopathologic findings led to diagnosis of the mass as an advanced-stage squamous cell carcinoma.

The meningitis had common and uncommon features. The common features were hearing loss, a typical outcome of *S. suis* meningitis independent of early antimicrobial drug administration (*1*,*2*); serotype 2, the most frequent and virulent serotype in swine and in humans (*1*,*2*); ST1, belonging to the ST1 complex, strongly associated with *S. suis* meningitis isolates (*2*,*3*); and *erm*(B)-mediated erythromycin resistance, widespread in this species (*4*). The uncommon features were tetracycline resistance mediated by *tet*(W), increasingly detected in gram-positive and in gram-negative bacteria (*5*) but never previously reported in *S. suis* or in other major streptococcal pathogens, where common determinants are *tet*(M) and *tet*(O); and lack of evidence for recent contact with swine, other animals, or swine (pork) products.

Two previous cases of human *S. suis* meningitis in Italy (*6*,*7*) and other recent cases from Europe (*8*,*9*) were related to occupational exposure. However, the patient reported here also had cancer, and malignancy has been indicated as a predisposing factor for the development of severe *S. suis* disease in humans (*2*). These findings appear to be consistent with the recent suggestion of new epidemiologic patterns of infection caused by this organism (*2*). *S. suis* may become an opportunistic pathogen in persons who are under stress or who have immunodeficiency, and it has been increasingly isolated from mammalian species other than pigs, from birds, and from the environment. As also discussed in a recent survey (*10*), the possibility cannot be excluded that a patient with *S. suis* infection may be unaware or have no memory of previous exposure to animals. Alternatively, because asymptomatic carriage of *S. suis* has been documented in humans (*2*) and is believed to contribute to its transmission (*10*), the possibility should also be considered that the infection may be a reactivation, possibly favored by malignancy, of latently colonizing *S. suis*.

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Equine Herpesvirus Type 9 in Giraffe with Encephalitis

To the Editor: Herpesviruses have been isolated from many mammals. Herpesvirus infection in natural hosts is often mild and is usually followed by a latent infection; however, cross-species herpesvirus infections cause severe and fatal diseases. Equine herpesvirus (EHV)–1 causes abortion, respiratory disease, and, occasionally, neurologic disorders in horses. EHV-1 infection is usually limited to equine species, although it has also been found in other species (*1*), in which it causes fatal encephalitis. Recent sequence analyses suggested that the equine herpesviruses isolated in the United States from onagers (*Equus hemionus*), Grevy's zebras (*E. grevyi*), and Thomson's gazelles (*Gazella thomsoni*) are a subtype or variant of EHV-1 (*2*). With respect to epizootiology, the nonequine animals affected by EHV-1 or EHV-1–related virus were kept in enclosures adjacent to those of zebra species (Grevy's or Burchell's).

Another EHV-related virus was isolated from 2 Thomson's gazelles that had encephalitis and were kept with zebras (*3*). The virus was later found to be a new type of EHV, EHV-9, although it was serologically cross-reactive with EHV-1 (*3*). Recently, neutralizing antibodies against EHV-9 were found among Burchell's zebras in the Serengeti ecosystem (*4*).

A herpesvirus was recently isolated from a reticulated giraffe (*Giraffa camelopardalis reticulate*) with neurologic symptoms; the giraffe was from a zoo in the United States (*5*). Nonsuppurative encephalitis was found by histopathologic examination of the giraffe brain. Several Burchell's zebras that were apparently healthy and later determined to be seropositive for EHV-1 were housed in the same pen as the giraffe. The isolated virus was identified by PCR and a monoclonal antibody assay as EHV-1 (*5*). In the present study, we analyzed 4 gene sequences of the giraffe herpesvirus to show its relatedness to EHV-1 and EHV-9.

We amplified portions of 4 genes from giraffe herpesvirus DNA by PCR. The DNA polymerase catalytic subunit (open reading frame [ORF] 30) gene was amplified by using herpesvirus universal primers (*6*). The genes for glycoprotein B (gB) (ORF33), glycoprotein 2 (gp2) (ORF71), and glycoprotein D (gD) (ORF72) were amplified by using primers specific for EHV-9. The ORF33 primers were gB-F (5′-GGCACAATAGTCCTAGCATG TCTGTTGCTG-3′) and gB-R (5′-AAATATCCTCAGGGCCGGAAC TGGAAAGTG-3′). The ORF71 primers were gp2-F (5′-CCCCGTTGATG AGTTTTGCGTAGAGGTCTA-3′) and gp2-R (5′-GCCACCACTGGTTG TAAAGGCCAAGAGATA-3′). The ORF72 primers were gD-F (5′-TTTACAACCACTGGTGGCGT GTGTGCAGAA-3′) and gD-R (5′-TATCTCCAAACCGCGAAGCTT-TAAGGCCGT-3′). The amplified products were used as templates for direct sequencing (Dragon Genomics, Mie, Japan). The sequences were edited with Phred, Phrap, and Consed (www.phrap.org/phredphrapconsed.html), and the phylogenic trees were constructed with PHYLIP (*2*,*7*). Accession numbers of the sequences (submitted to the DNA Data Bank of Japan) are given in the Figure.

We used PCR to amplify a part of the gB gene of the giraffe herpesvirus, and we used EHV-1 specific primers for sequencing. However, we could not obtain amplicons (data not shown). Therefore, the more conserved gene, ORF30, was sequenced. The sequence of the 1,066-bp segment of the giraffe herpesvirus ORF30 gene was 99.5% identical to EHV-9 and 94.6% identical to EHV-1, which indicates that the giraffe herpesvirus was most closely related to EHV-9. Therefore, EHV-9 ORF33–specific primers were used to amplify the corresponding region of the giraffe herpesvirus. The sequence of the giraffe herpesvirus ORF33 was 98.8% identical to EHV-9 and 95.9% identical to EHV-1. Also, the sequence of the other envelope glycoproteins (ORF71 and ORF72) of the giraffe herpesvirus were 99.8% and 99.6% identical to EHV-9 and 91.6% and 96.3% identical to EHV-1. A phylogenic tree of maximum likelihood showed that EHV-9 and the giraffe virus formed a genetic group that was apparently distinguished from other genetic groups of EHV (Figure).

Herpesviruses have caused clinical disease in zoo animals, including a case of EHV-9 infection in Thomson's gazelles (*3*) and a recently described endotheliotropic betaherpesvirus in-

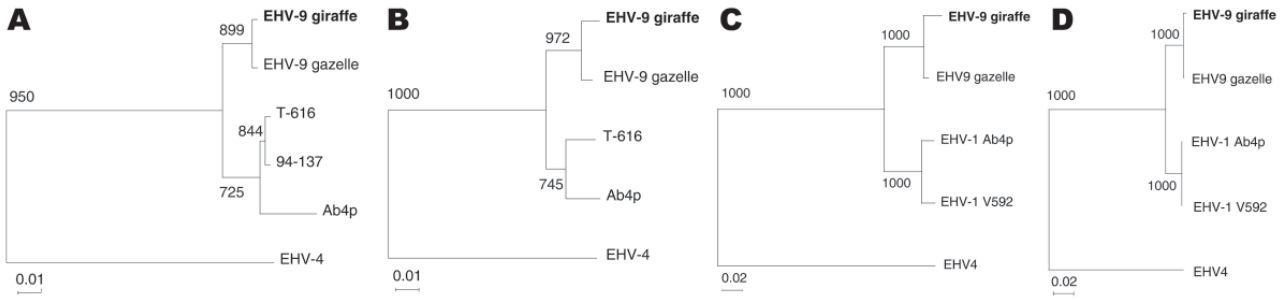

Figure. Phylogenic trees of giraffe herpesvirus and other related viruses. A) Open reading frame (ORF) 30, B) ORF33, C) ORF71, and D) ORF72. EHV-9 giraffe, equine herpesvirus (EHV) type 9 isolated from reticulated giraffe (*5*) (AB453826); EHV-9 gazelle, EHV-9 isolated from a Thomson's gazelle in Japan (*3*) (AP010838); T-616, EHV-1 isolated from a zebra fetus in the United States (EU087295); 94-137, EHV-1 isolated from a Thomson's gazelle in the United States (EU087297); Ab4p, EHV-1 isolated from horses (AY665713); EHV-4, EHV-4 isolated from horses (AF030027). Accession numbers of the sequences are AB439722 for ORF30, AB439723 for ORF33, AB453825 for ORF71, AB453826 for ORF72 of giraffe herpesvirus (DNA Data Bank of Japan, National Institute of Genetics, Japan), and AP010838 for EHV-9 genome sequence (H. Fukushi, unpub.data). Boldface indicates the sequence of EHV-9 derived from the giraffe. Scale bars indicate number of nucleotide substitutions per site.

fection in Asian and African elephants (*8*). The distribution and severity of herpesvirus encephalitis often differ between natural and accidental hosts in terms of enhanced neurovirulence. For example, herpes simplex virus causes a severe and fulminating encephalitis in rabbits but only herpetic stomatitis in humans; herpesvirus B infection is fatal to humans but not to other primates (*9*). These findings may explain why the giraffe had lesions while the zebras in the same enclosure did not.

Alphaherpesviruses can evade the immune system and become latent within lymphoid tissues, peripheral leukocytes, and trigeminal ganglia; they have the potential for reactivation and shedding after immune suppression or stress (*10*). Thus, the fact that the zebras were apparently healthy and seropositive for EHV-1 raises the possibility that the virus was reactivated and shed by one of the zebras, resulting in systemic infection and disease in the giraffe (*5*). This cross-species transmission of equine herpesviruses raises the possibility of latent infection and transmission of the disease from zebras to other animal species kept in zoos; the results could be devastating. Zebras might be EHV-9 carriers in zoos. Cross-species transmission must be considered in terms of screening susceptible animals for subclinical infection in terms of husbandry and housing issues for irreplaceable species.

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Reexamination of Human Rabies Case with Long Incubation, Australia

To the Editor: Long incubation periods are an occasional feature of infection with rabies virus and should be considered in human cases of acute encephalitis in rabies-free countries where there has been a history of travel to rabies-endemic areas (*1*). Until 1987 Australia had recorded only 1 case of travel-acquired rabies. However, in 1990 an extreme case of long-incubation rabies was diagnosed. The patient was a 10-year-old girl of Vietnamese origin in whom rabies developed after she had lived continuously in Australia for almost 5 years (*2*). A thorough investigation of the case history by public health officers concluded that the likely source of infection was Vietnam, which the girl left in July 1984, suggesting an incubation period >6.5 years. Preliminary sequence analysis of a fragment of the rabies virus genome extracted from postmortem samples taken from the patient confirmed that the likely origin was Southeast Asia (*3*).

Since that time, 2 major developments occurred that suggested that the case should be reexamined. The first was the discovery of a bat lyssavirus in Australia (*4*). In addition to being closely related to rabies virus, Australian bat lyssavirus (ABLV) had been isolated from 2 patients with fatal cases in Australia, one of whom was deduced to have had a potential incubation period of 27 months (*5*). The second development was the increase in phylogenetic investigations of rabies viruses in many countries across Asia including Sri Lanka, the Philippines, India, People's Republic of China (*6*), Indonesia, and, importantly, Vietnam (*7*).

After the patient's death, a decomposed sample of the patient's brain was sent to the Australian Animal Health Laboratory at Geelong. Although live virus was not isolated from this sample, several short sequences of the viral genome were amplified by PCR. From one of these, a 200-bp sequence of the nucleoprotein gene was derived that confirmed the clinical diagnosis of rabies. This sequence was compared to a small panel of virus sequences known at the time. Although limited, with no representative sequences from Vietnam, this early comparison indicated that the virus was of Asian origin (*3*).

To reinvestigate this case, we reassessed one of the other virus sequences originally generated from this case with a panel of rabies virus sequences from Southeast Asia, including viruses from Vietnam, China, and examples of ABLV. The original DNA fragment that yielded this sequence was also amplified from postmortem brain samples and the 306-bp sequence (GenBank accession no. EU854576) corresponded to positions 71–376 of the Pasteur virus genome (NC_001542) encoding the first 102 aa of the viral nucleoprotein. Further sequences were obtained from published studies submitted to GenBank. Sequences were aligned and edited with DNAstar Lasergene version 7 suite of programs (DNAstar Inc, Madison, WI, USA). Neighbor-joining and maximum likelihood analysis were undertaken by using PHYLIP version 3.5 as previously described (*8*). A sequence derived from a human case of European bat lyssavirus type 2 (EF157977) was used as the outgroup. One thousand replicates were used to assess bootstrap support for the phylogenic analysis.

The phylogenetic tree produced by this investigation indicates that the 1990 sequence falls within the rabies virus clade and is clearly distinct from the 4ABLV sequences included (Figure). These findings exclude the possibility that the young Vietnamese girl was infected with ABLV, now known to be endemic within Australian bats, after she arrived in Australia. The virus from the patient belonged within the rabies virus clade, forming a separate lineage most closely related to a subgroup of viruses found in China

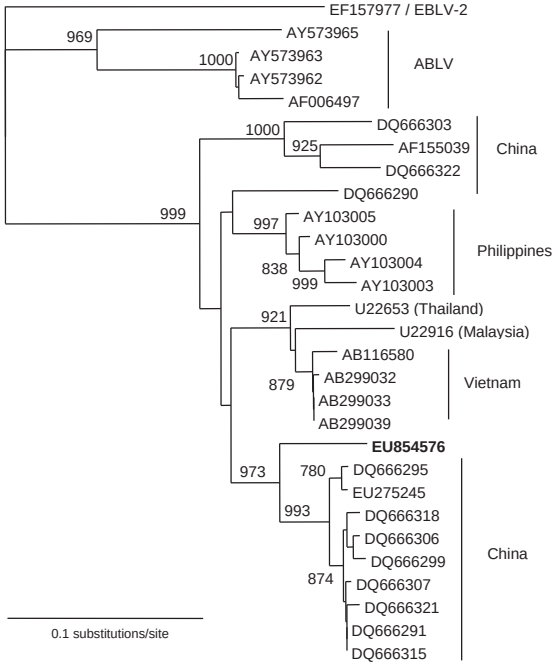

Figure. Phylogenetic analysis of lyssavirus nucleoprotein sequences (306 bp) derived from viruses of Asian origin. European bat lyssavirus type 2 has been used as the outgroup. The human sequence is shown in **boldface**.

(*6*). The sequence is distinct from a small number of sequences derived from rabies viruses in Vietnam, which suggests that China is a stronger candidate for the source of the virus than her native country.

Although the case history could not provide evidence for interaction with a dog while her family was in Hong Kong Special Administrative Region, rabies was endemic within the colony at the time that the patient's family was resident. From 1980 through 1984, 5 human cases were recorded (*9*). Only 2 case-patients had clear evidence of a dog bite; histories for the remaining 3 cases provided no evidence for an animal bite. From 1956 to 1979, Hong Kong had been free of rabies, but the disease had reentered the colony shortly after its incidence had increased in the neighboring Chinese province of Guangdong. If Hong Kong was where the young girl was infected, it would indicate an incubation period of 4.5–6 years.

Such long incubation periods are rare for rabies virus infections. An earlier epidemiologic study of 177 cases in Amritsar, India, demonstrated that rabies developed within 6 months of exposure in 90% of human cases (*10*). However, the thorough documentation of a small number of cases (*1*,*2*) suggests that clinicians need to be aware of the importance of including travel history over several years in cases of unexplained encephalitis.

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Human Case of *Bartonella alsatica* Lymphadenitis

To the Editor: Lymph node enlargement is a common medical problem that is usually caused by bacterial, viral, fungal, or protozoal agents (*1*). Malignancies or lymphoproliferative diseases are often found, especially in elderly patients (*1*). *Bartonella henselae*, the main causative agent of cat-scratch disease (CSD), appears to be the most common organism responsible for lymphadenopathy in adults and children (*1*). CSD has also been rarely associated with *B. quintana* (*2*). Recently, the epidemiology of *B. quintana* as an emerging source of human infection has changed because it has been isolated from the dental pulp of a domestic cat (*3*). Feral cats have also been found to be infected by *B. quintana* (*4*). We report a human case of *B. alsatica* lymphadenopathy.

A 79-year-old woman came to a hospital in Agen, France, in February 2008 with a large painless axillary mass that she had noticed 10 days earlier. She reported that ≈1 month earlier she was scratched on her finger while butchering a wild rabbit. On examination, she did not have any other specific findings. Blood cell counts and levels of liver enzymes were normal. A large necrotic lymph node was surgically removed the next day. Her condition was treated with doxycycline (200 mg) for 3 weeks.

Our laboratory received a fragment of the lymph node of the patient and a portion of the rabbit that had been cooked, boiled as a terrine, and stored in a freezer at –20°C in the home of the patient. DNA was extracted from these specimens by using a QIAamp Tissue Kit (QIAGEN, Hilden, Germany). The DNA was used as a template in 3 described PCRs specific for a portion of the *B. alsatica* 16S–23S intergenic spacer (ITS) region, *ftsZ* gene, and 16S rDNA (*5*). All results

for the lymph node were positive for *B. alsatica*, and amplification products of the expected size were obtained from this extract. Sequences obtained shared 100% similarity with the corresponding 16S rDNA, ITS region, and *ftsZ* gene fragment of *B. alsatica*. However, the terrine specimen was negative for 16S rDNA, the ITS region, and the *ftsZ* gene. All negative controls showed typical results. *B. alsatica* have not been tested or found in our laboratory for several years.

B. quintana subsp. Oklahoma, *B. henselae* subsp. Houston (ATCC 49882), *B. vinsonii* subsp. *berkhoffi* (URBVAIE25), *B. vinsonii* subsp. *arupensis* (ATCC 700727), and *B. alsatica* (CIP 105477 T) strains were used for immunofluorescence and Western blotting assays (*5*). A serum sample taken at admission was negative for *B. alsatica* by immunofluorescence assay. This result was accepted because serologic results may be negative during the onset of the disease (*6*). Western blotting with *Bartonella* spp. antigens (*5*) was positive for *B. alsatica* and after adsorption, only *B. alsatica* antigens retained all antibodies (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/14/12/1951-appF.htm).

Formalin-fixed, paraffin-embedded tissue specimens (3-μm thick) were stained with hematoxylin and eosin. Microscopic examination showed that the normal architecture of the lymph node was destroyed. Histologic changes were dominated by large irregular stellate or round granulomas with central neutrophil-rich necrosis (online Appendix Figure, panel B). Granulomas were composed mainly of macrophages, whereas neutrophils in the necrotic areas were fragmented. These granulomas with abscess formation were similar to those described in CSD. Warthin-Starry staining showed bacteria in the necrotic center of the granulomas (online Appendix Figure, panel C).

Immunohistologic staining was used to demonstrate *B. alsatica* in the lymph node. Immunohistochemical analysis was performed by using a monoclonal antibody against *B. alsatica* with an immunoperoxidase kit previously described (*7*). Briefly, after deparaffinization, the tissue section was incubated with polyclonal-specific antibody to *B. alsatica* (*8*) diluted 1:1,000 in phosphate-buffered saline. Immunodetection was performed with biotinylated immunoglobulins, peroxidase-labeled streptavidin (HistoStain Plus Kit; Zymed, Montrouge, France), and amino-ethyl-carbazole as substrate. Slides were counterstained with Mayer hematoxylin for 10 min. Location of bacteria was superimposable on that in the Warthin-Starry–stained specimens, and clusters of microorganisms were seen in the inflammatory areas (online Appendix Figure, panel D).

We report lymphadenitis caused by *B. alsatica*. Our finding was confirmed by molecular, serologic, and staining methods. *Bartonella* spp. are zoonotic agents that infect erythrocytes of mammals in which they cause chronic bacteremia (*9*). *B. alsatica* was first identified in 1999 in Alsace, France, as an agent of bacteremia in healthy wild rabbits (*10*). However, in 2006, interest in *B. alsatica* increased when it was considered to be a human pathogen because it caused blood-culture–negative endocarditis in a patient who had contacts with rabbits (*5*). The present case confirms that *B. alsatica* could be a human pathogen, especially in persons who live in contact with rabbits and should be considered a cause of lymphadenopathy.

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Molecular Detection of *Ehrlichia chaffeensis* in *Amblyomma parvum* Ticks, Argentina

To the Editor: *Ehrlichia chaffeensis* is an obligate intracellular bacterium in the family *Anaplasmataceae*. It is considered an emerging pathogen in the United States because it is the causative agent of human monocytotropic ehrlichiosis (*1*), a flu-like illness that can progress to severe multisystem disease and has a 2.7% case-fatality rate (*2*).

In Central and South America, human cases of ehrlichiosis with compatible serologic evidence have been reported in Venezuela, Brazil, Mexico, and Chile, although the bacterium has not been isolated (*3*). Recently, molecular evidence of *E. chaffeensis* infection was reported for a symptomatic 9-year-old child in Venezuela (*4*). In Argentina, antibodies reactive to *E. chaffeensis*, or an antigenically related *Ehrlichia* species, were detected in human serum samples during a serologic survey in Jujuy Province, where fatal cases of febrile illness were reported (*5*).

During November–December 2006, we collected ticks by dragging the vegetation and by examining mammal hosts, including humans, in semiarid southern Chaco, Argentina, Moreno Department, Province of Santiago del Estero. Ticks, kept in 70% alcohol, were identified as *Amblyomma parvum* (n = 200), *A. tigrinum* (n = 26), and *A. pseudoconcolor* (n = 13). A sample of 70 *A. parvum* and 1 *A. tigrinum* ticks collected on domestic ruminants and canids were subjected to PCR and reverse line blot hybridization by using the TBD-RLB membrane (Isogen Life Science, Maarssen, the Netherlands) (*6*) to look for *Anaplasma* and *Ehrlichia* spp. DNA was extracted from individual ticks by using the DNeasy Blood and Tissue kit (QIAGEN Valencia, CA, USA); several negative controls (distilled water) for both DNA extraction and PCRs were run alongside the samples in random order throughout the experiments. Primers Ehr-R (5′-CGGGATCCCCA GTTTGCCGGGACTTYTTCt-3′) (*6*) and Ehr-Fint (5′-GGCTCA GAACGAACGCTG-3′; Inst. Biotecnologia, Instituto Nacional de Tecnología Agropecuaria, unpub. data) were used to amplify a 500-bp fragment of the 16S gene of *Anaplasma/Ehrlichia* spp. PCR products were analyzed by reverse line blot hybridization , and 11.3% (95% confidence interval [CI] = 4.9–21.0) showed a positive signal to the specific *E. chaffeensis* probe: 8 *A. parvum* ticks collected from a dog (n = 1), a fox (*Lycalopex gymnocercus*, n = 1), goats (n = 2), and cattle (n = 4). No signals to other probes present in the membrane were recorded (*A. phagocytophylum*, *A. marginale*, *A. centrale*, *A. ovis*, *E. ruminatium*, *E.* sp. *Omatjenne*, *E. canis*). Further sequence analysis of 16S fragments confirmed the result, with our sequences showing 99.6% identity with the corresponding fragment of the *E. chaffeensis* strain Arkansas 16S gene (GenBank accession no. EU826516). To better characterize the positives samples, we then amplified variable-length PCR target (VLPT) of *E. chaffeensis* (*7*). PCR products of variable length were detected by conventional gel electrophoresis analysis (Figure). Distilled water and *R. conorii* DNA were used as negative controls, and *E. chaffeensis* DNA as the positive control. The finding was confirmed by sequence analysis (GenBank accession nos. EU826517 and EU826518)

In view of these positive results, another set of 108 specimens was tested by *E. chaffeensis* VLPT PCR: all the ticks collected on humans (80 *A. parvum*, 1 *A. pseudoconcolor,* and 4 *A. tigrinum*), 18 host-seeking *A. parvum* ticks, and 5 *A. parvum* ticks collected on armadillos of the genera *Tolypeutes* and *Chaetophractus*. *E. chaffeensis* was detected in *A. parvum* ticks only: 5 from humans (6.2%; 95% CI 2.1–14.0; Figure, panel A) and 3 from host-seeking ticks (16.7%; 95% CI 3.6–41.4). In total, *E. chaffeensis* was detected in 9.2% (95% CI 5.4–14.6) of tested *A. parvum* ticks in the study area. Of the 16 positive *A. parvum*, 5 were infesting humans.

Little is known about *E. chaffeensis* epidemiology in South America. In Brazil, wild marsh deer (*Blastocerus dichotomus*) are suspected to be its natural reservoir, but the tick involved in the transmission cycle is not known (*8*). In North America, *E. chaffeensis* sp. is maintained principally by the lone-star tick, *A. americanum,* and the white-tailed deer (*Odocoileus virginianus*) (*2*). However, the possibility of transmission by different ticks and infection among other hosts has been reported; specific antibodies to *E. chaffeensis* were detected in domestic and wild canids and goats (*2*), and recently experimental infection was demonstrated in cattle (*9*). We did find *E. chaffeensis* organisms in ticks collected on both wild and domestic animals, but the possible role of different mammals as reservoir hosts deserves further investigation. Moreover, the finding of polymorphic VLPT gene fragments in our sample indicates the circulation of *E. chaffeensis* genetic variants in the study area. VLPT repetitive sequences vary among isolates

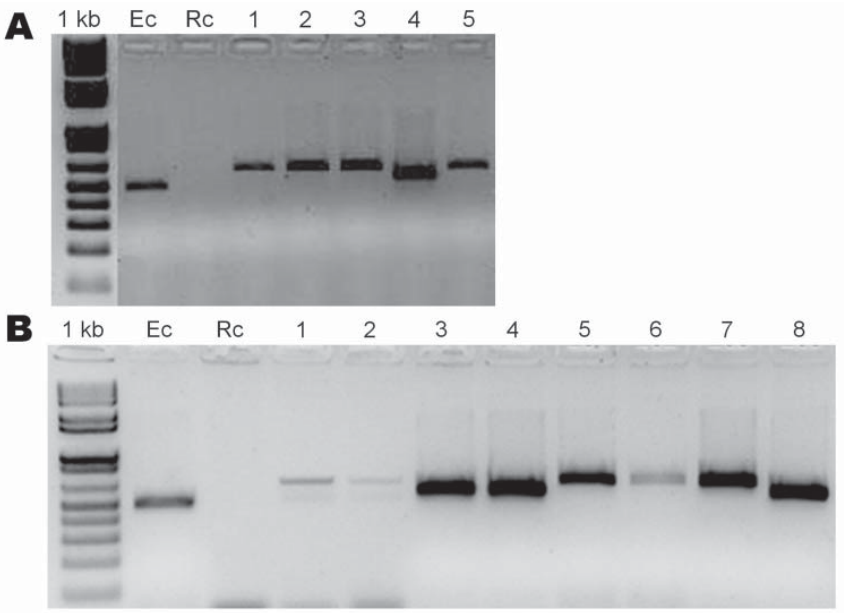

Figure. Agarose gel electrophoresis of PCR products amplified with *Ehrlichia chaffeensis* (Ec) variable-length PCR target primers. Rc, *Rickettsia conorii* (negative control). The sources of DNA templates used for amplification are *Amblyomma parvum* ticks collected from different hosts: A) 1–5 humans; B) 1 dog, 2 foxes, 3–6 cattle, 7–8 goats. Variable amplicon size represents different genotypes that result from differences in the number of tandem repeats in the 5′ end of the variable-length PCR target; PCR products' sizes range from 500 bp to 600 bp.

(*7*); however, it is not known whether genetic variants differ in pathogenicity or are correlated with geographic distribution or host range.

All positive ticks were *A. parvum*, a common tick of domestic animals that frequently feeds on humans in Argentina and Brazil and is considered a potential vector of zoonoses (*10*). In our study area, this tick species was by far the most abundant on humans (93.2%), and our results suggest its potential role as a vector of *E. chaffeensis*.

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Enzootic Angiostrongyliasis in Shenzhen, China

To the Editor: *Angiostrongylus cantonensis* is a zoonotic parasite that causes eosinophilic meningitis in humans after they ingest infective larvae in freshwater and terrestrial snails and slugs, paratenic hosts (such as freshwater fish, shrimps, frogs, and crabs), or contaminated vegetables. With the increase of income and living standards, and the pursuit of exotic and delicate foods, populations around the world have seen angiostrongyliasis become an important foodborne parasitic zoonosis (*1*–*9*).

Shenzhen municipality is situated in the most southern part of mainland People's Republic of China between the northern latitudes of 22°27′ to 22°52′ and eastern longitudes of 113°46′ to 114°37′; it shares a border with the Hong Kong Special Administrative Region, China, in the south. The climate is subtropical, with an average annual temperature of 23.7 °C. The city is 1,952.84 km^2 and has a population of 10 million.

Since 2006, thirty-two sporadic cases of human eosinophilic meningitis caused by consumption of undercooked aquacultured snails have been documented in Shenzhen (Shenzhen Center for Disease Control and Prevention, unpub. data). To identify the source of these infections and assess the risk for an outbreak of eosinophilic meningitis, we conducted a survey to investigate whether *A. cantonensis* occurs in wild rats and snails in Shenzhen.

To examine *A. cantonensis* infection in intermediate host snails, 302 terrestrial snails (*Achatina fulica*) were collected from 10 investigation sites across Shenzhen, and 314 freshwater snails (*Pomacea canaliculata*) were sampled from 6 investigation sites. We examined the snails for *A. cantonensis* larvae by using pepsin digestion standardized procedures (*3*). To survey the prevalence of adult *A. cantonensis* in definitive host rats, we collected 187 *Rattus norvegicus* rats and 121 *R. flavipectus* rats collected from 4 sites where positive snails positive for *A. cantonensis* were found. These rats were examined for the presence of adult *A. cantonensis* in their cardiopulmonary systems.

A. cantonensis larvae were found in 96 (15.6%) of 616 examined snails. Of these, *P. canaliculata* had an average infection rate of 20.7% (65/314), significantly higher ($p<0.01$) than that of *A. fulica* (10.3%, 31/302), an indication that *P. canaliculata* may be the principal intermediate host for *A. cantonensis* in Shenzhen. *A. cantonensis* adults were recovered from the cardiopulmonary systems of 37 (12%) of 308 examined rats. Infection rate for *R. norvegicus* rats was 16.6% (31/187), significantly higher ($p<0.01$) than that for *R. flavipectus* (4.9%, 6/121), an indication that *R. norvegicus* may be the principal definitive host for *A. cantonensis* in Shenzhen, possibly due to the rat's preference for eating snails. Infection rates were higher for female rats (25.6% for *R. norvegicus* and 7.8% for *R. flavipectus*) than for male rats (8.9% for *R. norvegicus*, 2.9% for *R. flavipectus*), possibly because female rats eat more snails to supply proteins for reproduction. This report of enzootic *A. cantonensis* infection in wild rats and snails in Shenzhen demonstrates the existence of natural origins of infection with *A. cantonensis* for humans in this city.

Persons in Shenzhen eat raw or undercooked freshwater and terrestrial snails and slugs. This practice provides opportunities for infection with *A. cantonensis,* particularly given that *P. canaliculata* has been aquacultured intensively for human consumption. The prevalence of *A. cantonensis* in wild rats and snails in Shenzhen poses substantial risk for future outbreaks of human eosinophilic meningitis. Moreover, public health officials, epidemiologists, researchers, clinical technicians, medical practitioners, parasitologists, and veterinarians, as well as the general public, should be aware of such risks, and integrated strategies should be taken to reduce or eliminate such risks.

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Knowledge about Avian Influenza, European Region

To the Editor: Since the first identifications of avian influenza (H5N1) in Europe in late 2005 and early 2006, Eurobarometer survey data obtained during April–May 2006 have provided a unique opportunity to examine the knowledge of respondents across the European Union, Croatia, and Turkey about the risks and transmission of avian influenza. The H5N1 strain of avian influenza virus has caused >240 human deaths in central and Southeast Asia, the Middle East, and Africa (*1*). Four of these deaths occurred in Turkey in 2006. Understanding gaps in the public's knowledge about avian influenza risks and transmission provides guidance on which issues future public health information campaigns may wish to focus. From a public health perspective, a more informed general public will be less likely to unnecessarily alter their travel and food consumption behavior and more likely to take appropriate preventive actions.

A 2006 Eurobarometer survey asked 29,170 residents of the 27 countries in the European Union, Croatia, and Turkey about their knowledge of avian influenza risks (*2*). Eurobarometer surveys are undertaken by the European Commission to monitor the EU public's social and political opinions. The survey was conducted on a multistage random sampling basis. Therefore, the sample is representative of the whole territory surveyed. Each country's population was randomly sampled according to rural, metropolitan, and urban population densities. A cluster of addresses was selected from each primary sampling unit by using country-dependent resources such as electoral registers. Addresses were chosen systematically by using standard random route procedures, beginning with a randomly selected initial address. The survey was conducted by face-to-face interviews in respondents' homes.

Data were collected from March 27 through May 1, 2006. This period is especially interesting when looking at Europeans' knowledge about avian influenza risk because the first European cases of avian influenza (H5N1) were found in October 2005 in Turkey; additional cases were found later that month in Romania, Croatia, and the United Kingdom. Therefore, the period would have included media coverage about avian influenza as well as any targeted public health efforts to inform residents about avian influenza risks. By the end of this survey's fieldwork period, 17 of the 29 countries surveyed had reported influenza virus (H5N1) in birds, 3 in mammals, and 1 in humans (*3*).

Respondents were asked 7 questions about their knowledge of the risks humans face regarding avian influenza (Table). When we looked at these results with the aim of setting future public health information campaign objectives, we considered incorrect or "don't know" responses to indicate public health information campaign failures. Uncertainty regarding avian influenza risks appeared to involve consumption of eggs and vaccinated, cooked poultry and whether the virus can be transmitted between humans. However, for all questions asked, more than half of the respondents answered correctly except when asked about eating poultry that had been vaccinated against avian influenza. This question also had the highest number of "don't know" responses. Respondents are most knowledgeable about the preventive measure of culling chickens, perhaps because of the media attention these events attract. The large percentage of correct answers for some questions points to successes of previous information campaigns and media coverage, but the 40% of respondents answering incorrectly or "don't know" to questions about poultry and egg consumption

Table. Knowledge of human risks associated with avian influenza, European region, 2006*

For each of the following statements tell me whether, in your opinion, it is true or false:			
	Response, no. (%)		
Transmission risks	True	False	Don't know
The avian influenza virus can be transmitted between humans	9,864 (33.8)	**16,574 (56.8)**	2,732 (9.4)
Humans can catch avian influenza by touching contaminated birds	**22,722 (77.9)**	4,473 (15.3)	1,975 (6.8)
Food-related risks			
Even when it is contaminated, poultry is not a health risk if it is cooked	**17,906 (61.4)**	8,536 (29.3)	2,728 (9.4)
The avian influenza virus contained in an egg or present on its shell can be eliminated by prolonged cooking	**17,369 (59.5)**	6,593 (22.6)	5,208 (17.9)
It is not dangerous to eat the meat of a chicken vaccinated against avian influenza	**12,833 (44.0)**	9,272 (31.8)	7,065 (24.2)
Other			
The vaccination against seasonal influenza is also effective against avian influenza	4,265 (14.6)	**20,847 (71.5)**	4,058 (13.9)
If a chicken is contaminated by avian influenza on a farm, all the poultry on that farm must be destroyed immediately	**24,492 (84.0)**	2,725 (9.3)	1,953 (6.7)

*Source: Eurobarometer 65.2 (http://ec.europa.eu/public_opinion/archives/eb/eb65/eb65_ee_exec.pdf). Information in **boldface** refers to the correct answer.

and human-to-human virus transmission leaves areas for further work.

These results support previous findings that knowledge about avian influenza, especially about prevention and human-to-human transmission, has scope for improvement (*4*,*5*). Persons in Europe reported that they have little ability to prevent themselves from getting avian influenza (*6*). Previous research in the Lao People's Democratic Republic examined how consumers' knowledge of avian influenza risk reduced the likelihood that consumers will substitute poultry for other foods during an avian influenza crisis. This research indicates the importance of informing persons about consumption and transmission-related risks to reduce the likelihood of unnecessary behavioral changes that can cause larger macrolevel market effects (*7*).

The state of knowledge about avian influenza in Europe during the outbreak in the spring of 2006 leaves room for further public health information campaign efforts, especially those that increase consumers' understanding of consumption-related avian influenza risks. Persons in Europe appear to be aware of culling procedures and the risks of touching infected birds but have a more limited understanding of how avian influenza in their region should influence their consumption patterns.

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Human *Salmonella* Infection Yielding CTX-M β-Lactamase, United States

To the Editor: In the United States most third-generation cephalosporin resistance among salmonellae is due to AmpC plasmid–mediated β-lactamases. Extended-spectrum β-lactamases (ESBLs) have rarely been reported (*1*). The CTX-M β-lactamases constitute a group of ESBL enzymes that are increasing in prevalence worldwide. Currently, the CTX-M enzymes are classified into 5 different subgroups on the basis of DNA sequence similarities (*2*). We report on a domestically acquired CTX-M–producing *Salmonella* isolate in the United States.

In 2003, public health laboratories in all US state health departments submitted every 20th non-Typhi *Salmonella* (NTS) isolate from humans

to the Centers for Disease Control and Prevention (CDC) for susceptibility testing by the National Antimicrobial Resistance Monitoring System (NARMS). MICs were determined by broth microdilution and interpreted according to Clinical and Laboratory Standards Institute standards (www.clsi.org), when available. Resistance to cefquinome was defined as ≥32 mg/L.

Among the 1,864 human NTS isolates submitted to NARMS in 2003, 105 (5.6%) displayed elevated MICs (≥2 mg/L) to ceftriaxone or ceftiofur, third-generation cephalosporins used in human and veterinary medicine, respectively. Genomic DNA was prepared from the 105 isolates, and a PCR with degenerate primers capable of detecting all CTX-M enzymes identified a single positive *S. enterica* ser. Typhimurium (*3*). The isolate came from a stool sample collected in September 2003 from a white, non-Hispanic, US-born, 3-month-old boy who lived in the state of Georgia. The patient had diarrhea and fever for ≈1 week. Because neither the patient nor his family had traveled internationally in the 3 months before specimen collection, the infection appears to have been domestically acquired. The patient did not receive any antimicrobial agents before illness but was treated for 14 days with cefpodoxime. The infant recovered from the illness without complications.

The isolate displayed resistance to β-lactams, aminoglycosides, phenicols, tetracyclines, and folate pathway inhibitors (Table). Two β-lactamases (isoelectric pH [pI] 7.5 and 8.8) were resolved by isoelectric focusing.

Group-specific PCR primers were used to characterize the presumed $bla_{\text{CTX-M}}$ gene (*4*). Primers TOHO1–2F and TOHO1–1R yielded a 351-bp product, confirming a group II $bla_{\text{CTX-M}}$ gene. To perform sequencing of the entire gene, a ClustalW alignment with representatives from group II was performed to identify primers (DNASTAR, Madison, WI, USA). The sequence of the gene was identical to the sequence of the $bla_{\text{CTX-M-5}}$ gene detected in other isolates of *S. enterica* ser. Typhimurium (GenBank accession nos. U95364 and AF286192) as well as to the *kluA-2* gene of *Kluyvera ascorbata* (GenBank accession no. AJ251722).

Table. MIC values of antimicrobial drugs for the *Salmonella* ser. Typhimurium isolate and its *Escherichia coli* DH10B transformant

Antimicrobial agent	MIC, mg/L		
	S. ser. Typhimurium	*E. coli* DH10B transformant	*E. coli* DH10B
Amikacin	1	1	1
Amoxicillin–clavulanic acid	32	16	4
Ampicillin	>32	>32	4
Aztreonam	32	32	0.12
Cefepime	32	32	≤0.06
Cefotaxime	>64	>64	≤0.06
Cefotaxime–clavulanic acid	0.25	0.12	≤0.06
Cefoxitin	2	8	8
Cefquinome	>32	>32	≤0.06
Ceftazidime	8	8	0.5
Ceftazidime–clavulanic acid	0.5	0.25	0.12
Ceftiofur	>8	>8	0.5
Ceftriaxone	>64	>64	≤0.25
Chloramphenicol	>32	≤2	≤2
Ciprofloxacin	≤0.016	≤0.016	≤0.016
Gentamicin	4	≤0.25	≤0.25
Imipenem	0.5	0.25	0.25
Kanamycin	>64	≤8	≤8
Nalidixic acid	4	1	1
Piperacillin-tazobactam	>64	2	2
Streptomycin	≤32	>64	>64
Sulfisoxazole	>256	≤16	≤16
Tetracycline	>32	≤4	≤4
Trimethoprim-sulfamethoxazole	>4	≤0.12	≤0.12

Presence of other β-lactamase–encoding genes (bla_{TEM}, bla_{SHV}, and bla_{OXA}) was investigated by PCR (*6*–*8*). Amplification with primers OXA-1F and OXA-1R yielded a 595-bp product with a sequence consistent with that of $bla_{\text{OXA-1}}$ (*8*).

To determine whether the CTX-M enzyme was plasmid-borne, plasmids were extracted and transformed into electrocompetent *Escherichia coli* DH10B. The transformant exhibited resistance to cefotaxime but not to ceftazidime (Table). In addition, the transformant exhibited resistance to cefquinome and cefepime. The presence of a $bla_{\text{CTX-M}}$ gene was confirmed by PCR (*3*,*4*). The bla_{OXA} gene could not be amplified from the *E. coli* transformant (*8*).

A CTX-M–producing *Salmonella* isolate has been reported only once previously in the United States (*9*). This was in 1994, when an isolate of *Salmonella* ser. Typhimurium var. Copenhagen with a CTX-M-5 was recovered from a 4-month-old girl adopted from Russia; that infection was not domestically acquired (*9*). We compared the 1994 isolate and the isolate in this study by pulsed-field gel electrophoresis; the isolates showed distinct patterns.

The IS*Ecp1* insertion sequence has been described as a flanking region of several $bla_{CTX\text{-}M}$ genes and has been implicated in the expression and mobilization of the genes (*5*). A recent study by Lartigue et al. showed that a CTX-M-2 progenitor in *K. ascorbata* could be mobilized and transferred to a conjugative *E. coli* plasmid by the IS*Ecp1B* element; enhanced mobilization was observed in the presence of ceftazidime, cefotaxime, and piperacillin (*10*).

This *Salmonella* isolate's resistance to cefepime and cefquinome, fourth-generation cephalosporins, is troubling. Cefquinome is not approved for use in the United States but has been used in Europe for treating food animals since 1994. ESBLs, including CTX-M enzymes, are more common in Europe than in the United States (*1*). Further studies are warranted to clarify the extent to which the use of cefquinome has contributed to high CTX-M prevalence in Europe.

In conclusion, we report a domestically acquired CTX-M–producing *Salmonella* isolate in the United States. Because third-generation cephalosporins are important for treating invasive *Salmonella* infections, continued monitoring of ESBL-producing bacteria is important.

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Yersinia pseudotuberculosis O:1 Traced to Raw Carrots, Finland

To the Editor: Illness caused by *Yersinia pseudotuberculosis* is mainly characterized by fever and acute abdominal pain due to mesenteric lymphadenitis that mimics appendicitis. Secondary manifestations include erythema nodosum and reactive arthritis (*1*). Outbreaks have been reported in the Northern Hemisphere, including Canada (*2*,*3*), Japan (*4*), and Russia (*5*). Several community outbreaks have also been reported in Finland since 1982 (*1*,*6*–*9*). Only in a few of the outbreaks has the vector or source of the infection been identified. Recently, fresh produce, such as iceberg lettuce (*7*) and carrots (*9*), has been implicated by epidemiologic investigations as a source of infection, but mechanisms of contamination of fresh produce have remained unknown.

On April 8, 2004, the National Public Health Institute of Finland was informed of several cases of gastroen-

teritis in schoolchildren in 1 municipality in northern Finland. On April 13, 2004, stool samples from symptomatic schoolchildren confirmed *Y. pseudotuberculosis* infections. At the same time, an increase occurred in *Y. pseudotuberculosis* cases reported to the National Infectious Disease Register (NIDR) from other parts of the country. We conducted epidemiologic, microbiologic, trace-back, and environmental investigations to determine the source of the outbreak and the origin of contamination.

In the school outbreak, a survey concerning symptoms of gastrointestinal illness was conducted among all schoolchildren (7–18 years of age) and personnel (N = 900) of the 7 schools in the municipality. A case was defined as a laboratory-confirmed *Y. pseudotuberculosis* infection in a child or staff member who attended a school that received lunches from the school central kitchen, or as abdominal pain and fever, or erythema nodosum with illness onset from March 8 through March 28, 2004. Of respondents to the survey, 53 met the case definition. Among these respondents, *Y. pseudotuberculosis* was isolated from stool samples of 5 persons.

A case–control study was conducted to identify the source of infection; self-administered questionnaires asked about consumption of items on school menus from March 8 through March 26, 2004. For each of the 53 case-patients identified in the survey, 3 controls were selected from the same class; 39 cases and 107 controls were included the analysis. Univariate analysis showed that a vegetable mixture of carrots and white cabbage served on March 8 and a mixture of cucumber and white cabbage served on March 25 were associated with illness. Multivariate analysis showed that only the carrot–white cabbage mixture was associated with illness.

We also conducted a case–control study by mailing questionnaires to 37 persons with microbiologically confirmed *Y. pseudotuberculosis* infections reported to the NIDR from March 15 through May 7, 2004. These cases were from other parts of the country and were not associated with the school outbreak. For each case, 4 controls matched by age, sex, and municipality were randomly selected from the national population registry. Risk of illness increased with increased frequency of eating fresh carrots.

Carrots served in the school were traced back to the farm level. Samples checked included grated carrots, which were available from the school kitchen. The kitchen had received all vegetables from 1 fresh-food processing plant. Samples were taken from the carrot-peeling line, carrot-peeling leftovers, grated carrots, and other vegetable-processing lines at the plant. Carrots originated from only 2 farms, which were inspected, and samples were obtained for bacteriologic examination. Small mammals at the farms were caught in carrot fields and investigated microbiologically to identify the reservoir of *Y. pseudotuberculosis*. This bacterium was isolated from 1 environmental sample from the carrot-peeling line in the fresh-food processing plant, from spoiled carrots, from fluid draining from spoiled carrots, and from a pooled sample of common shrew (*Sorex araneus*) intestines from 1 farm.

Human and environmental isolates obtained were serotype O:1, subtype O:1b. Pulsed-field gel electrophoresis (PFGE) profiles of isolates from schoolchildren, fluid of spoiled carrots at the infected farm, and shrew intestines were indistinguishable. All 22 isolates from NIDR cases belonged to 2 PFGE genotypes. One genotype had a PFGE profile that was indistinguishable from the profile of the school outbreak isolates and the other genotype differed from these isolates by only 1 fragment.

Our study provides microbiologic and epidemiologic evidence that the school outbreak was caused by carrots contaminated at the production farm. We isolated a *Y. pseudotuberculosis* subtype from human patients that was indistinguishable from isolates from the implicated source and a potential animal reservoir. Although the association between shrews and carrots is uncertain, shrews may have been picked up with carrots by harvesting machinery and ended up dead in wooden storage frames with the carrots. If carrots become contaminated, long storage at cold temperatures favors growth of *Y. pseudotuberculosis* and may result in human infections. Further studies are needed to determine the mechanism of contamination and other natural reservoirs. After the outbreak, the Finnish Food Safety Authority recommended controlling contamination at the farm level by removing spoiled carrots and paying attention to any subsequent spoilage during handling procedures.

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Antibodies against *Rickettsia* spp. in Hunters, Germany

To the Editor: A number of emerging *Rickettsia* species have been recently described (*1*). One of these, *R. helvetica*, was first isolated in Switzerland in 1979 and was implicated in perimyocarditis and nonspecific febrile disease in humans (*2*–*5*). PCR showed its prevalence in 1,187 *Ixodes ricinus* ticks in southern Germany to be 8.9% (*6*). This finding raises the question whether autochthonous transmission of rickettsiae to humans may occur in Germany. To help answer this question, we conducted a cross-sectional study of the presence of antibodies against *Rickettsia* spp. in a population in Germany presumably exposed to ticks.

On February 4–5, 2006, we used convenience sampling to enroll 286 hunters at a national hunting fair in Dortmund, Germany. All study participants gave written, informed consent. The Ethics Committee of the Charité approved the study.

Every participant completed a standardized questionnaire. Serum samples were collected from all hunters and analyzed by immunofluorescence assay for 9 *Rickettsia* species (*R. conorii, R. slovaca, R. helvetica*, *R. massiliae, R. mongolitimonae, R. israelensis, R. aeschlimannii, R. felis,* and *R. typhi*) as described previously (*7*). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using SPSS software version 14 (SPSS, Inc., Chicago, IL, USA). We considered $p<0.05$ to be significant.

Of the 286 hunters, 252 (88.1%) were male; median age was 46 years (range 17–79 years). Positive antibody titers (immunoglobulin [Ig] G, IgM, or both) against any *Rickettsia* spp. were found for 26 (9.1%) hunters (95% CI 6.2–13.0). Antibodies against different *Rickettsia* spp. were found for 18 hunters; species-specific antibodies against *R. helvetica* were found for 2 hunters and against *R. aeschlimannii* for 6 (Table). Seropositive and seronegative hunters did not differ significantly with respect to sex, age, and total years of hunting. Neither hunting nor traveling in a foreign country within the past 5 years was significantly associated with seropositivity. Neither of the 2 hunters with *R. helvetica*–specific antibody titers had traveled outside Germany in the 5 years before the study, but 3 of the 6 hunters with specific titers against *R. aeschlimannii* had traveled and hunted in countries with unknown endemicity for *R. aeschlimannii* (Russia, Romania, Namibia). A total of 212 (74.1%) hunters had received at least 1 tick bite in the year before the study; median was 4 tick bites/year. Living in the southern parts of Germany (below 50°N) was significantly related to seropositivity (OR 4.1, 95% CI 1.3–12.3, $p = 0.02$). Although the 26 persons with positive serologic results for *Rickettsia* spp. reported arthralgia with higher frequency than did seronegative persons (50% vs. 37%, respectively), their reports of arthralgia and of other clinical signs did not differ significantly: temperature >38.5°C (8% vs. 2%), enlarged lymph nodes (12% vs. 9%). No seropositive hunter reported having had an eschar.

This study provides data for Germany on the seroprevalence of *Rickettsia* spp. in persons highly exposed to ticks. Our results suggest that *Rickettsia* spp. are endemic to southern Germany and may cause autochthonous infections. Although most seropositive hunters exhibited reactivity to

Table. Positive immunofluorescence assay results for antigens to 9 *Rickettsia* spp. in 26 hunters, Germany, 2006*

Hunter no.	IgG/IgM								
	R. conorii	*R. slovaca*	*R. helvetica*	*R. massiliae*	*R. mongolitimonae*	*R. israelensis*	*R. aeschlimannii*	*R. felis*	*R. typhi*
106			0/64						
109	**64/128**	**64/128**	**64/128**	**64/128**	**64/128**		**32/32**	**64/0**	**0/128**
111		**0/32**	**0/64**	**0/32**					
113			**0/128**						**0/64**
115		**0/32**	**0/128**	**0/32**	**0/32**	**0/32**	**0/64**	**0/32**	**0/64**
117			**0/32**	**0/32**	**0/32**				**0/32**
124		**0/32**	**0/64**	**0/32**	**0/32**	**0/32**	**0/32**		**0/64**
127			**0/128**						**0/64**
130	**0/64**	**0/64**	**0/128**	**0/32**		**0/32**	**0/64**		**0/64**
131			256/0					**0/64**	
142	**0/64**	**0/64**	**0/64**	**0/64**	**0/64**				
148		**0/64**	**0/64**	**0/64**	**0/32**				
151	**0/64**	**0/64**	**0/64**	**0/64**	**0/64**				
160		**0/32**	**0/32**	**0/32**	**0/32**				
161		**0/64**	**0/64**	**0/32**	**0/32**				
182							128/0		
185							64/64		
224	**64/64**	**64/64**	**64/64**	**64/64**	**64/64**	**64/64**	**64/64**	**64/64**	**64/64**
230		**0/32**		**0/32**	**0/32**				
233		**0/32**	**0/32**	**0/32**	**0/32**	**0/32**	64/16		
236							64/16		
237							64/32		
277			**64/32**				**64/32**		
281							64/32		
285		**0/32**	**0/32**				**0/32**	**0/32**	**0/32**
297		**0/32**	**0/32**	**0/32**	**0/32**	**0/32**	**0/32**	**0/32**	**0/32**

*All specimens were tested for all antigens. **Boldface** indicates nonspecific titers. Cutoff titers for seropositivity (immunoglobulin [Ig] G or IgM) were 128/64 for *R. conorii* and 64/32 for other antigens (*8*). A rickettsial antigen was considered to represent the agent of infection when cross-reactions were absent or when titers of IgG or IgM antibody against this antigen were ≥2 serial dilutions higher than titers of IgG or IgM antibody against other rickettsial antigens.

several rickettsial antigens, some had species-specific titers for *R. helvetica*. Six hunters exhibited specific reactivity to *R. aeschlimannii*. Serologic cross-reactions are frequently noted among spotted fever group rickettsiae, and 1 of the best indicators of species identity remains the geographic origin of the infection (*7*). Until now, *R. aeschlimannii* had not been detected in Germany or neighboring countries. We therefore suggest that the specific titers against *R. aeschlimannii* in our study population may be partly related to traveling or hunting abroad and that the observed seroprevalence for other rickettsial species is most likely caused by *R. helvetica*, or, alternatively, by *R. monacensis*, which was recently isolated from a tick in the English Garden in Munich (*9*). Cutoff titers for IgM and IgG were chosen to achieve a specificity >98%; sensitivity varied between different rickettsial antigens. However, if we assume a sensitivity of only 50% (with a prevalence of 9.1%), the positive predictive value of our test would still be 74%. In addition, a test with high specificity and low sensitivity underestimates the true seroprevalence; the proportion of seropositive hunters in our study group is likely higher.

Although hunters with positive immunofluorescence assay results reported having had symptoms compatible with rickettsioses more frequently than did seronegative hunters, these differences were not significant. A similar situation has been noted for persons who were tested for antibodies against *Borrelia burgdorferi* and human granulocytic anaplasmosis; the findings may reflect the mild and poorly defined clinical picture that is typical for each of these diseases (*10*).

To conclude, we report the presence of *Rickettsia* spp. antibodies in a high-risk group from Germany. Final proof that human rickettsiosis occurs in Germany, however, will require the isolation of the agent from patients.

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Rickettsia sp. in *Ixodes granulatus* Ticks, Japan

To the Editor: The genus *Rickettsia* consists of obligate intracellular bacteria that cause spotted fever and typhus fever; these bacteria are usually transmitted by an arthropod vector. We report isolation of a *Rickettsia honei*–like organism from the *Ixodes granulatus* tick; this organism may be a causative agent of rickettsiosis in Japan. Serotyping and DNA-sequencing analysis distinguished this *I. granulatus* isolate from previously reported *Rickettsia* spp.

During 2004–2005, an investigation of rickettsiosis was conducted in Okinawa Prefecture in the southernmost part of Japan, an area known to be inhabited by *I. granulatus*, a parasitic tick commonly found on small mammals. A total of 43 *I. granulatus* ticks (3 larvae, 27 nymphs, 8 adult females, and 5 adult males) were collected from small mammals (*Rattus rattus*, *R. norvegicus*, *Suncus murinus*, *Mus calori*, and *Crocidura watasei*) for the present study. For the isolation of *Rickettsia* spp., the cell line L929 was used as previously described (*1*). A total of 13 isolates, designated as strains GRA-1 to GRA-13, were obtained from 11 ticks (1 fed larva, 5 fed nymph, 1 fed adult female, 1 fed adult male, 1 unfed nymph molted from engorged larva, 2 unfed adult females molted from engorged nymphs) and from 1 pool of eggs and 1 larva derived from the engorged female tick.

Serotyping was performed by using a microimmunoperoxidase approach according to the method described by Philip et al. (*2*); we used anti-*Rickettsia* mouse serum and several spotted fever group *Rickettsia* antigens: 2 of the present isolates (GRA-1 and GRA-2) and 6 known members of the Asian *Rickettsia* spp. (*R. honei*, *R. japonica*, *R. asiatica*, *R. tamurae*, *R. sibirica*, and *R. conorii*). Differences among antigen reaction titers were calculated, and the results are given as the specificity difference (SPD) value. The SPD value between the present isolates and *R. honei* was 0 or 1, whereas the SPD values were ≥3 for the other spotted fever group *Rickettsia* spp. (Table). According to the criteria for serotyping (*2*), we assumed the isolates to be of the same serotype when the SPD value was ≤2. In addition to serotyping, a sequencing analysis was performed to genetically characterize the isolates. The archive of DNA sequences has been mostly established for the outer membrane protein A gene (*ompA*), citrate synthesis gene (*gltA*), and 17-kDa antigen gene. Thus, we determined these DNA sequences in the isolates and compared the results with those of representative *Rickettsia* spp. The *ompA* sequencing analysis showed a DNA sequence of 491 bp in the 6 isolates from *I. granulatus* (GenBank accession nos. AB444090–AB44095), which yielded the following similarity values: *R. slovaca* (98.0%), *R. honei* and Thai tick typhus *Rickettsia* (97.8%), and *R. honei* subsp. *marmionii* (97.6%). Sequencing of the 1,250-bp fragment of *gltA* of the strain GRA-1 (accession no. AB444098) showed >99% DNA similarity with that of *R. sibirica* (99.3%), *R. slovaca* (99.2%), *R. conorii* (99.2%), *R. honei* (99.1%), and certain types of *Rickettsia* spp. Moreover,17-kDa antigen gene sequencing analysis of a 392-bp fragment of the strain GRA-1 (accession no. AB444097) showed the highest levels of sequencing similar-

Table. Serotype results for *Rickettsia* sp. strains GRA-1 and GRA-2 from *Ixodes granulatus* tick, Okinawa Prefecture, Japan

	Results*							
Mouse antiserum to	GRA-1	GRA-2	TT-118	Aoki	IO-1	AT-1	246	Moroccan
Strains from this study								
Rickettsia sp., GRA-1	**320**†	**320** (0)‡	160 (1)	80 (3)	40 (6)	40 (6)	80 (4)	80 (3)
Rickettsia sp., GRA-2	**320** (0)	**320**	**320** (0)	40 (4)	20 (7)	40 (7)	40 (5)	40 (5)
Reference strains								
R. honei, TT-118	**5,120** (1)	**5,120** (0)	**5,120**	80 (7)	320 (7)	80 (9)	160 (7)	80 (7)
R. japonica, Aoki	2,560 (3)	2,560 (4)	1,280 (7)	**5,120**	320 (9)	320 (10)	320 (9)	32 0 (8)
R. asiatica, IO-1	640 (6)	640 (7)	80 (7)	160 (9)	**5,120**	80 (12)	80 (11)	160 (11)
R. tamurae, AT-1	640 (6)	320 (7)	80 (9)	80 (10)	80 (12)	**5,120**	160 (11)	40 (13)
R. sibirica, 246	1,280 (4)	1,280 (5)	320 (7)	160 (9)	160 (11)	80 (11)	**5,120**	320 (7)
R. conorii, Moroccan	640 (3)	640 (5)	80 (7)	80 (8)	20 (11)	20 (13)	160 (7)	**1,280**

*Highest serum dilutions against each *Rickettsia* antigen (specificity difference between each pair of strains), determined by microimmunoperoxidase method. **Boldface** indicates equivocal titer to homologous antigen.
†Highest serum dilution showing a positive reaction with antigen.
‡Specificity difference between each pair of strains.

ity value with *R. honei* and Thai tick typhus *Rickettsia* (99.5%) compared with those of the sequences of other deposited *Rickettsia* spp. Comprehensive analyses led us to presume that the isolate GRA-1 from *I. granulatus* was a genetic variant of *R. honei*, although further studies are necessary to better define the taxonomic position of our isolates.

The vector for *R. honei* was presumed to be ixodid ticks: *I. granulatus* in Thailand; *Amblyomma cajennense* in Texas, USA; and *Aponomma hydrosauri* in Australia (*3*–*5*). Lane et al. reported that a *Rickettsia* organism from a *Haemaphysalis* tick was closely related to *R. honei* in Australia (*6*). In the present study, we observed that the *Rickettsia* organism was maintained in the tick after molting. Moreover, *Rickettsia* organisms were also isolated from egg and unfed larva. These preliminary findings may suggest that *I. granulatus* is a possible vector for the *R. honei*–like bacterium in Japan.

Recently, a *Rickettsia* sp. was found in *I. granulatus* ticks; its proposed designation was unclassified *Rickettsia* IG-1, according to DNA sequencing from specimens obtained in Taiwan (*7*). With respect to the DNA sequences of *gltA* and *ompA*, our isolates from *I. granulatus* were identical to the *Rickettsia* IG-1.

R. honei, a member of the spotted fever group *Rickettsia*, has been reported as the etiologic agent of Flinders Island spotted fever in Australia (*8*) and also of Thai tick typhus (*3*). *R. honei* is a public health threat for rickettsiosis in these countries. Although the human health implications of the *Rickettsia* sp. found in this study are not yet known, knowledge from this study will be useful in epidemiologic investigation for rickettsiosis in Japan.

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Sin Nombre Virus Infection in Deer Mice, Channel Islands, California

To the Editor: Sin Nombre virus (SNV) is a highly virulent strain of hantavirus associated with rodent hosts in North America (*1*,*2*). Documenting the prevalence of SNV in wild rodent populations is an important component of determining risk for exposure and ultimately providing sound recommendations for epidemiologic management (*3*). Prevalence of SNV is highly variable. Deer mice (*Peromyscus maniculatus*) that inhabit the Channel Islands off the California coast often have rates of SNV that greatly exceed values on the mainland (*2*). Even though these islands have high rates of SNV prevalence and are recreational areas for humans, no surveys of the Channel Islands have been performed to document the dynamics of prevalence since 1994–1996 (*2*,*4*). We visited 4 of the Channel Islands in 2007 to document rates of SNV prevalence in *P. maniculatus*.

From May 3–15, 2007, we visited 4 of the Channel Islands off the California coast: East Anacapa Island, Santa Barbara Island, San Miguel Island, and Santa Rosa Island. On each island, mice were captured by using Sherman live traps from habitats characterized by giant coreopsis (*Coreopsis gigantea*), a shrub native to California, to provide a standardized habitat for comparisons across islands. The number of sampling areas depended largely upon the distribution of *C. gigantea* habitat and logistical considerations during each island visit (Table). Upon capture of the mice, blood samples were taken from the submandibular vein by using Medi-Point animal lancets (Medi-Point International, Inc., Mineola, NY, USA) and stored in sterile micropipette tubes. Samples were stored on ice until shipment to the California Department of Health Services' Viral and Rickettsial Disease Laboratory for processing. *P. maniculatus* serum samples were examined for immunoglobulin (Ig) G antibodies to the SNV nucleocapsid protein by ELISA with Centers for Disease Control and Prevention reagents (*5*).

Detailed information regarding SNV prevalence, sampling location, and sampling effort is presented in the Table. We compare our 2007 data with data collected in 1994 by Jay et al. (*2*) because 1994 was the only other year when all 4 islands used in our study were sampled. Graham and Chomel (*4*) also collected data from San Miguel Island and Santa Rosa Island in 1995 and 1996 (the use of the average prevalence from 1995 and 1996 for these 2 islands does not change any of our results).

There was no significant difference in prevalence of SNV antibodies between our 2007 results and the prevalence found by Jay et al. (*2*) in 1993–1994 (paired *t* test $t = 0.13$, 3; df = 3; $p = 0.91$). Overall, 36 male and 42 female mice were captured; the sex of captured animals was independent of SNV infection (9 males and 6 females positive for SNV; test of independence $\chi^2 = 0.28$, 1 df, $p = 0.59$). We captured only 2 subadult mice on islands where we also detected antibodies to SNV; 1 mouse tested positive, the other tested negative. Although our sample sizes precluded detecting very low rates of SNV infection with confidence on Santa Barbara and East Anacapa Islands, the consistency of our results with those of Jay et al. (*2*) suggests that our sampling was sufficient for comparative purposes.

Several studies now indicate the importance of long-term surveillance of SNV prevalence in wild rodent populations for understanding the factors that may contribute to outbreaks of human disease, e.g. (*6*). These studies often document the generally positive, though often temporally delayed, relationship between population density of *P. maniculatus* and seroprevalence for SNV (*7*). Our results suggest a high degree of temporal stability in prevalence of antibodies to SNV in *P. maniculatus* on the Channel Islands, despite considerable variation in host population density between earlier studies and ours (*4*,*8*). Although we cannot know the prevalence of SNV among *P. maniculatus* on the Channel Islands in periods between the studies by Jay et al. (*2*), Graham and Chomel (*4*), and our own, SNV prevalence on these islands is quite similar to levels previously recorded both for islands with relatively low prevalence

Table. Sin Nombre virus in *Peromyscus maniculatus* mice on 4 Channel Islands, California, May 3–15, 2007*

Island*	No. trap nights	Prevalence, %	
		2007	1994
East Anacapa†	180	0	0
San Miguel‡	104	26.3	17.9
Santa Barbara§	216	0	0
Santa Rosa¶	216	47.6	58

*The number of captured mice that were sampled for Sin Nombre virus (SNV) was 23 on East Anacapa, 19 on San Miguel Island, 15 on Santa Barbara Island, and 21 on Santa Rosa Island. The 1994 data in the table are from a study by Jay et al. (*2*) and are included for comparison puposes.
†East Anacapa: 34º00'56"N/119º21'49"W.
‡San Miguel: 34º02'18"N/120º20'54"W.
§Santa Barbara: 33º28'30"N/119º02'12"W.
¶Santa Rosa: 34º00'03"N/120º03'30"W.

(i.e., East Anacapa and Santa Barbara Islands) or high prevalence (i.e., San Miguel and Santa Rosa Islands).

Future studies comparing long-term dynamics on islands and related mainlands are needed to examine the possibility that insular systems provide unique opportunities to understand the factors affecting pathogen dynamics and human risk. Given the substantial variation in mouse population density among different habitats within these islands and variation in prevalence among trapping areas in our study (Table) and others (*4*), we also recommend that future studies focus on the diverse array of habitats where *P. maniculatus* is found on the islands to more completely characterize within-island risk.

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Parachlamydia acanthamoebae Infection and Abortion in Small Ruminants

To the Editor: Abortion in ruminants is of worldwide economic importance. Moreover, several abortigenic agents have a zoonotic potential, i.e., *Brucella abortus, Coxiella burnetii,* and *Chlamydophila abortus. C. abortus*, which causes ovine enzootic abortion, may also infect pregnant women who have had contact with *C. abortus*–infected sheep and goats, and such infection can lead to miscarriage (*1*).

Parachlamydia acanthamoebae (*2*) is a *Chlamydia*-related organism considered as an emerging agent of pneumonia in humans. Recently, we reported its role in the setting of bovine abortion (*3*). Here, we investigated the prevalence of *C. abortus* and *P. acanthamoebae* infections in abortions in small ruminants.

Formalin-fixed placenta, fetal lung and liver, or both, were available from abortion products from 144 goats and 86 sheep (n = 211). These specimens had previously been investigated for several abortigenic agents (*4*). Placentas and fetal organs were analyzed by histopathologic examination and by specific real-time PCR and immunohistochemical protocols that detect members of the *Chlamydiaceae* family and *P. acanthamoebae*.

DNA from paraffin blocks was extracted as described (*5*) by using the DNeasy Tissue kit (QIAGEN, Hilden, Germany). The real-time PCR for *Chlamydiaceae* was conducted on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) by using a modified version of Everett's PCR (*6*). Primers Ch23S-F (5′-CTGAAACCAG TAGCTTATAAGCGGT-3′), Ch23S-R (5′-ACCTCGCCGTTTAACTTA ACTCC-3′), and probe Ch23S-p (5′-FAM-CTCATCATGCAAAAGGCA CGCCG-TAMRA-3′) were used to

amplify and detect a 111-bp product specific for members of the family *Chlamydiaceae*. Chlamydial species identification of real-time PCR positive cases was performed with the ArrayTube Microarray (Clondiag, Jena, Germany) as described (*7*).

The *Parachlamydia*-specific real-time PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems), as reported (*8*). This PCR is genus-specific, as demonstrated by the absence of PCR positivity with DNA extracted from other *Parachlamydiaceae* (*Protochlamydia* spp./*Neochlamydia hartmannellae).* To confirm positive results, another specific PCR, which targeted the *tlc* gene, was performed (*9*).

Paraffin sections from specimens positive in real-time PCR were further examined by immunohistochemical tests. A *Chlamydiaceae*-specific mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (Progen, Heidelberg, Germany) and a specific mouse polyclonal antibody against *Parachlamydia* spp. was used as described (*3,5,10*). These antibodies were applied at dilutions of 1:200 and 1:1,000, respectively. Detection was performed with a detection kit (ChemMate; Dako, Glostrup, Denmark). Antigen retrieval was performed by enzyme digestion for 10 minutes (Pronase; Dako) for the *Chlamydiaceae* antibody and repeated microwave treatment in citrate buffer (ChemMate; Dako) for the *Parachlamydia* antibody, respectively. Double immunohistochemical labeling was performed on the sheep abortion specimen identified as simultaneously infected with *Chlamydiaceae* and *Parachlamydia* spp. Immunohistochemical analysis for both pathogens was performed subsequently by using diaminobenzidine as substrate for the *Chlamydiaceae* antibody (brown labeling) and by using 3-amino-9-ethylcarbazole as substrate for the *Parachlamydia* antibody (red labeling). Specificity of PCR and immunohistochemical tests for *Chlamydiaceae* and *Parachlamydia* spp., respectively, was assessed by using negative control placentas taken from 2 healthy ruminants (both specimens were negative in all tests).

Results of real-time PCR showed that 55 (26.1%) of 211 specimens were positive for *Chlamydiaceae*. All 55 cases could be identified as *C. abortus* by ArrayTube Microarray (Clondiag). Of these, 42 (76.4%) could be confirmed by immunohistochemical analysis with the anti-*Chlamydiaceae* antibody.

Of the 211 specimens, only 2 (0.9%) were positive for *Parachlamydia* spp. by real-time PCR, and both cases could be confirmed by immunohistochemical testing with the parachlamydial antibody. These 2 specimens were negative for other common abortigenic agents such as *Toxoplasma gondii, C. burnetii,* and border disease virus (data not shown). One case was recorded among the 144 goat samples investigated. This placenta displayed necrotizing placentitis and was positive for *Parachlamydia* spp. by 16S rRNA-specific real-time PCR (cycle threshold [Ct] 40.5) and immunohistochemical testing, but negative for *Chlamydiaceae*. Results of this PCR was confirmed by another PCR, targeting the *tlc* gene (Ct 36.7),

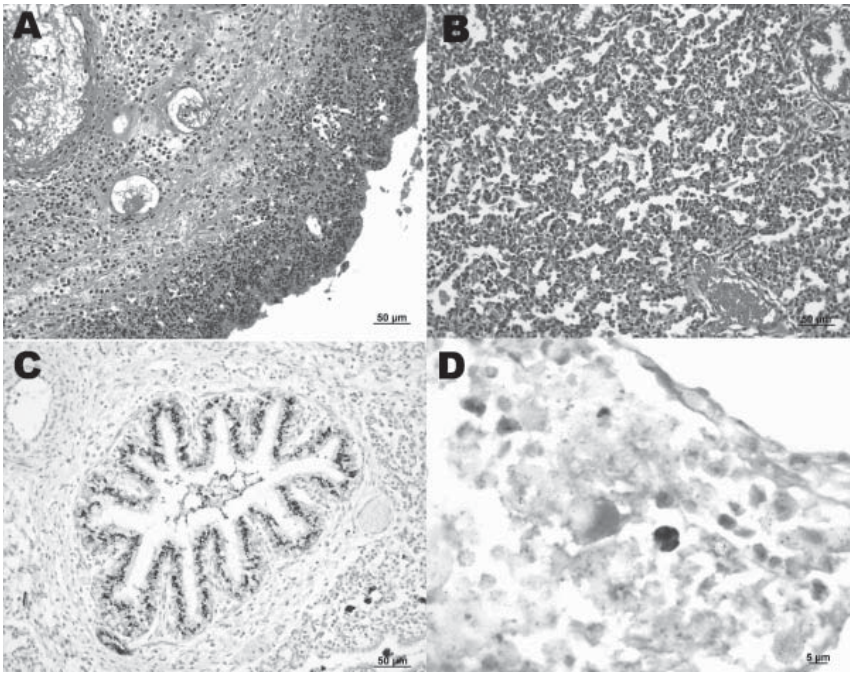

Figure. A) Sheep placenta positive by real-time PCR and immunohistochemistry for *Parachlamydia* spp. and *Chlamydiaceae. Chlamydophila abortus* was identified by ArrayTube Microarray. Necrotizing placentitis and vasculitis are shown (hematoxylin and eosin stain; magnification ×200). B) Fetal lung of the sheep abortion specimen positive by real-time PCR and immunohistochemical tests for *Parachlamydia* spp. and *Chlamydiaceae*; interstitial pneumonia is shown (hematoxylin and eosin stain; magnification ×200). C) Fetal lung that was positive by real-time PCR and immunohistochemical testing for *Parachlamydia* spp. Positive granular material can be seen within the lung tissue. Antigen detection (immunohistochemistry) was carried out with a polyclonal antibody directed against *Parachlamydia* spp. 3-amino-9-ethylcarbazole/peroxidase method (hematoxylin counterstain; magnification ×200). D) Double immunohistochemical labeling of the sheep placenta that was positive by real-time PCR and immunohistochemical tests for *Chlamydiaceae* and *Parachlamydia* spp. The simultaneous presence of *Chlamydiaceae* and *Parachlamydia* spp. granular reaction is shown within necrotic trophoblastic epithelium and neutrophilic exudate (diaminobenzidine/AEC/peroxidase method, hematoxylin counterstain; magnification ×1,000). A color version of this figure is available online (www.cdc.gov/EID/content/14/12/1966-F.htm).

which excluded false-positive results because of amplicon contamination.

The second case was identified among the 86 sheep investigated. Placenta and fetal lung and liver exhibited necrotizing placentitis and vasculitis (Figure, panel A), interstitial pneumonia (Figure, panel B), and mixed cellular periportal hepatitis. Fetal liver was negative by parachlamydial 16S rRNA real-time PCR and immunohistochemical analysis, but the fetal lung was positive by parachlamydial 16S rRNA real-time PCR (Ct 40.7) and immunohistochemical tests (Figure, panel C), but negative with the *tlc* PCR. Fetal lung and liver were positive by real-time PCR for *Chlamydiaceae* (mean Ct for both organs 36.7), but negative by immunohistochemical tests. The placenta was positive for *Chlamydiaceae* by immunohistochemical tests and real-time PCR (mean Ct 23.3), and *C. abortus* was identified by ArrayTube Microarray. Brown (*Chlamydiaceae*) and red (*Parachlamydia* spp.) granular reaction was demonstrated within the necrotic lesions of the placenta by double immunohistochemical labeling (Figure, panel D).

We report *Parachlamydia* infection in small ruminant abortion. *C. abortus* and *Parachlamydia* spp. were simultaneously present in an aborted sheep placenta. *Parachlamydia* spp. could be further detected in the lung of the aborted sheep fetus by real-time PCR and immunohistochemistry. *Parachlamydia* was also detected in a goat placenta. Thus, *Parachlamydia* spp. should be considered as a new abortigenic agent in sheep and goats. Persons in contact with small ruminants should be informed about the zoonotic potential of these abortigenic agents.

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Candidate New Species of *Kobuvirus* in Porcine Hosts

To the Editor: Picornaviruses (family *Picornaviridae*) are small, nonenveloped viruses with single-stranded, positive-sense genomic RNA, currently divided into 8 genera: *Enterovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Teschovirus*, and *Kobuvirus* (*1*). To date, the genus *Kobuvirus* consists of 2 species, *Aichi virus* and *Bovine kobuvirus*, each possessing 1 serotype. Aichi virus (strain A846/88) was first isolated from a stool sample obtained from a person with acute gastroenteritis in 1991 (*2*). Bovine kobuvirus (strain U-1) was detected in bovine

sera and in feces samples from clinically healthy cattle in 2003 (*3*). Human and bovine kobuviruses were first isolated in Japan. Recently, kobuviruses have also been detected in humans in other countries in Asia (*4*), Europe (*5*,*6*), and South America (*5*) and in calves with diarrhea in Thailand (*7*). The Aichi virus and bovine kobuvirus genomes are approximately 8.2–8.3 kb, respectively, and both have a typical picornavirus genome organization, including the L protein following structural (VP0, VP3, and VP1) and nonstructural (2A–2C and 3A–3D) regions (*1*,*3*). Genetic identity between Aichi and U-1 viruses ranges from 47.7% (3′ untranslated region) through 70.8% (3D region) (*3*). In this study, we report a new candidate species of kobuvirus. Porcine kobuvirus was serendipitously detected in fecal samples from domestic pigs in Hungary.

Fecal samples were collected in February 2007 from 15 healthy piglets (*Sus scrofa domestica*) <10 days of age from a farm in Ebes located in eastern Hungary. The aim of the study was to detect porcine calicivirus (norovirus and sapovirus) in domestic pigs by using reverse transcription–PCR (RT-PCR), using the generic primer pairs p289/p290 designed for the calicivirus RNA-dependent RNA polymerase gene (319 nt for norovirus or 331 nt for sapovirus) (*8*). RNA isolation and RT-PCR were performed as described previously (*9*). PCR products were sequenced directly in both directions with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK) by using the PCR primers and run on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Stafford, TX, USA). Phylogenetic analysis was conducted by using MEGA software version 4.0 (*10*). Complete nucleotide sequence of porcine kobuvirus (strain *Kobuvirus*/swine/S-1-HUN/2007/Hungary) was submitted to GenBank under accession no. EU787450.

Two (13.3%) of 15 samples were positive for porcine sapoviruses; however, a consequent nonspecific, ≈1,100-nt, strong, and single PCR product was found in all samples by agarose gel electrophoresis (data not shown). The nucleotide sequence of the 1,065-nt nonspecific PCR product was determined. Genetic and amino acid similarity was found to be bovine (U-1) and human Aichi kobuvirus 3C (87 nt) and 3D (978 nt) regions in GenBank database by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide and amino acid identity of the 3C–3D regions were 73%–79% and 69%–70% to U-1 strain and Aichi virus, respectively. The phylogenetic tree confirmed that S-1-HUN belonged to kobuviruses and formed a distinct lineage (Figure). Cleavage sites for 3C and 3D was Q/S. Highly conserved amino acid motif KDELR in 3D (RNA-dependent RNA polymerase) region and high rate of cytidine (29%) and uracil (26%) nucleoside composition were seen in the 3C and 3D parts of the genome of strain S-1-HUN; both are suspected to be a typical skew of kobuviruses (*3*).

Most picornavirus genera consist of ≥2 species (*1*). Our study reports detection of kobuvirus in domestic pigs. Serendipitously, the generic calicivirus primers p289 and p290, designed for a calicivirus RNA-dependent RNA polymerase region, amplified a kobuvirus 3C/3D region when specimens were tested for porcine caliciviruses by RT-PCR. Comparison of the primers p289 and p290 and the S-1-HUN sequence showed that there are 12- and 11-bp homologous regions between the kobuvirus and the 3′ end of the primer sequences, respectively. Reverse primer p289 designed for calicivirus (norovirus and sapovirus) conserved amino acid 3D motif YGDD, which is also present in kobuviruses.

All apparently healthy animals <10 days of age carried the kobuvirus, which was excreted in the feces. These results indicate a general circulation and endemic infection of kobuvirus on the tested farm. In addition, because of its analogy to other picornaviruses and because bovine kobuvirus was first detected in culture medium that originated from

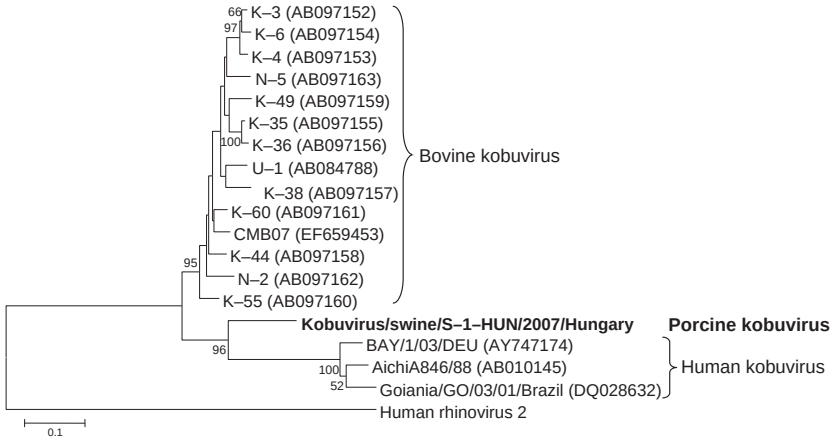

Figure. Phylogenetic tree of porcine kobuvirus (Kobuvirus/swine/S-1-HUN/2007/Hungary, GenBank accession no. EU787450), based upon the 1,065-nt fragment of the kobuvirus 3C/3D regions. The phylogenetic tree was constructed by using the neighbor-joining clustering method; distance was calculated by using the maximum composite likelihood correction for evolutionary rate with help of the MEGA version 4.0 software (*10*). Bootstrap values (based on 1,000 replicates) for each node if >50% are given. Reference strains were obtained from GenBank. The human rhinovirus 2 strain (X02316) was included in the tree as an outgroup. Scale bar indicates nucleotide substitutions per site.

cattle sera (*1*,*3*), we cannot exclude the possibility that the S-1-HUN–like kobuvirus can cause viremia (and generalized infection) in swine. S-1-HUN–like virus may typically cause asymptomatic infections in swine. However, epidemiologic and molecular studies are required regarding the importance of this virus as a causative agent of some diseases of domestic pigs and related animals. Sequence analysis of the complete nucleotide and amino acid sequences of coding (L, P1, P2, and P3: 7,467 nt) and noncoding regions and the genetic organization strain indicate that S-1-HUN is a typical kobuvirus. Phylogenetic analysis shows that S-1-HUN strain is genetically included in the genus *Kobuvirus* but is distinct from Aichi and bovine kobuviruses. Porcine kobuvirus strain S-1-HUN is a candidate for a new, third species of the genus *Kobuvirus*.

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Human Case of *Rickettsia felis* Infection, Taiwan

To the Editor: *Rickettsia felis*, the etiologic agent of flea-borne spotted fever, is carried by fleas worldwide (*1*). In the past decade, several human cases of *R. felis* infection have been reported (*1*–*3*). Clinical symptoms and biological data for *R. felis* infections are similar to those for murine typhus and other rickettsial diseases, which makes clinical diagnosis difficult (*2*). Patients with *R. felis* infections may have common clinical manifestations, such as fever, headache, myalgia, macular rash, and elevated levels of liver enzymes (*4*,*5*).

Reportable rickettsioses in Taiwan include scrub typhus, epidemic typhus, and murine typhus. Although there are no known human cases of infections caused by spotted fever group (SFG) rickettsiae in Taiwan, novel strains of SFG rickettsiae have been isolated as recently described (*6*,*7*). In addition, evidence for *R. felis* infections in cat and cat flea populations has been identified by using immunofluorescence assay (IFA), PCR, and organism isolation (K.-H.Tsai et al., unpub. data). We report an indigenous human case of *R. felis* infection in Taiwan.

In January 2005, a 27-year-old woman living in Fongshan City, Kaohsiung County, in southern Taiwan was admitted to Kaohsiung Medical University Hospital with a 4-day history of intermittent fever (37.8°C–38.0°C), chills, headache, and fatigue. Associated symptoms were frequent micturition and a burning sensation upon voiding. The patient was admitted with a possible urinary tract infection; urinalysis showed pyuria (leukocyte count 25–50/high-power field), compatible with the clinical diagnosis. During the 6-day hospital stay, the patient received daily intravenous first-generation cephalosporin (cefazolin); gentamicin was given only on the

first 3 days. She was discharged with a prescription for oral antimicrobial drugs (cephradine 500 mg every 6 h) to be taken for 7 days. Micturition-associated symptoms subsided after treatment.

The patient also had headaches and glove-and-stocking numbness in both hands because of fever, but denied any associated rash and arthralgia. Although the patient did not recall any arthropod bites, she had noticed some stray dogs and cats nearby and rodents in the neighborhood surrounding her house. Because of acute polyneuropathy-like symptoms and exposure history, we prescribed oral doxycycline (100 mg every 12 hours) for 5 days as empirical therapy on the second day at the hospital, suspecting a zoonosis such as rickettsioses, Q fever, or leptospirosis. Headache and glove-and-stocking numbness subsided. Her blood sugar level and thyroid function were within normal limits. Chest radiograph, liver function, renal function, and levels of electrolytes were all normal.

Whole blood counts were normal, although differential counts demonstrated a left shift (polymorphonuclear leukocytes 80.7%, reference range 37%–75%). C-reactive protein level was 66.7 μg/mL, (reference <5 μg/mL) upon admission. Blood culture and urine culture were negative for bacteria.

Additionally, painful vesicles on the external genitalia appeared on the fourth day postadmission, and valacyclovir was administered for 5 days because of suspected infection with herpes simplex virus. The lesion subsided after valacyclovir treatment and the patient was discharged in good condition.

Patient whole blood specimens were collected on days 4 and 16 after the onset of fever and sent to the Taiwan Centers for Disease Control for laboratory diagnosis of rickettsial infection. For molecular diagnosis, DNA from the acute-phase blood sample (day 4) was analyzed by using the SYBR green-based real-time PCR specific for 17-kDa antigen, 60-kDa heat-shock protein (*groEL*) gene, and outer membrane protein B (*ompB*) gene for typhus group and SFG rickettsiae and primers listed in the Table. Nucleotide sequences of real-time PCR products demonstrated 100% identity with 17-kDa antigen, *groEL*, and *ompB* genes of *R. felis* URRWXCal2. Real-time PCR results were negative for *Orientia tsutsugamushi* and *Coxiella burnetii* (*8*).

For serologic diagnosis, serum samples were tested for rickettsial-specific antibodies by IFA using whole cell antigens of *R. felis* isolated from the cat flea. The patient's serum (days 4 and 16) had immunoglobulin (Ig) G, IgA, and IgM titers of 160 and 2,560, respectively. The serum sample collected from *R. felis*–infected cat served as the positive control. Test results were negative for *R. typhi*, *R. conorii*, *R. rickettsii*, *R. japonica*, *O. tsutsugamushi*, and *C. burnetii*.

Absence of rash, eschar, and unawareness of arthropod bite may be easily overlooked in some patients with rickettsial infections. In this case, suspicion of rickettsial infection was based on exposure history and acute polyneuropathy, which responded quickly to doxycycline treatment. There are limited reports of rickettsioses with polyneuropathy, and none for cases of *R. felis* infection (*9*,*10*). It was hard to tell whether the urinary tract and herpes simplex virus infections were associated with an *R. felis* infection, but it is quite rare for 3 different infections to occur in a patient at the same time as isolated entities. The finding of a human case of infection and the existence of *R. felis* in cat fleas highlights the need for further studies on flea-borne rickettsioses in Taiwan.

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Table. Primer sequences for the SYBR-based real-time PCR assay*

Primer	Genomic region	Sequence (5′ → 3′)	Amplicon size, bp
RR-F8	17-kDa (*Rickettsia* spp.)	GGC GGY GCA TTA CTT GGT TCT CAA TTC GC	304
RR-R12		GTT TTC CSC CTA TTA CAA CTG TTT GAG T	
RR-F1	*groEL* gene (*Rickettsia* spp.)	AAA ATG GTT GCT GAG CTT GAA AAT CCT TT	191
RR-R2		ACT TTC AAA CCA CCA CGT AAT CTA TTG AC	
RR-F22	*ompB* gene (*Rickettsia* spp.)	ATG GTR TAT GGG CWA AAC CTT TCT ATA	330
RR-R25		TAG MMT CGA AGA AGT AAC GCT GAC TTT	
RST-14F	56-kDa gene (*Orientia tsutsugamushi*)	CCA TTT GGT GGT ACA TTA GCT GCA GGT	233
RST-6R		TCA CGA TCA GCT ATA CTT ATA GGC A	
OMP3	*com-1* (*Coxiella burnetii*)	GAA GCG CAA CAA GAA GAA CAC	438
OMP4		TTG GAA GTT ATC ACG CAG TTG	

**groEL*, 60-kDa heat-shock protein; *ompB*, outer membrane protein B; *com-1*, 27-kDa outer membrane protein.

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Bartonella spp. and *Rickettsia felis* in Fleas, Democratic Republic of Congo

To the Editor: *Bartonella* and *Rickettsia* species are pathogens of humans and domestic mammals that may be transmitted by fleas and other arthropods. *Rickettsia felis* causes flea-borne spotted fever in humans who come into contact with flea-infested domestic and peridomestic animals; worldwide distribution of this pathogen in ectoparasites and mammals makes it an emerging threat to human health (*1*,*2*). Likewise, species of the genus *Bartonella* are associated with an increasing array of human diseases, including trench fever, cat-scratch disease, and endocarditis in immunocompetent patients, and bacillary angiomatosis and peliosis hepatitis in immunocompromised patients (*3*–*5*). Although *Bartonella* spp. and *R. felis* appear to be globally distributed, their presence in the Democratic Republic of Congo (DRC) has not been previously documented.

Off-host *Pulex irritans*, *Tunga penetrans*, *Ctenocephalides felis strongylus*, *Echidnophaga gallinacea*, and *Xenopsylla brasiliensis* were collected in the Ituri district of northeastern DRC from March through April 2007, during an investigation of a plague outbreak. Our investigation area was limited to 4 villages: Djalusene and Kpandruma, which had confirmed plague patients, and Wanyale and Zaa, which had several suspect cases (*6*).

We collected fleas by using a kerosene lamp hung above a 45-cm diameter tray containing water (*7*). Captured fleas were identified using a dissecting microscope and standard morphologic keys, sorted into vials by species and locality, and preserved in 70% ethanol (*7*). Fleas were separated into 193 pools (2–5 fleas per pool), triturated for 10 minutes; the resultant flea triturate was centrifuged at 3,000 rpm for 10 minutes to collect flea tissue. DNA was then obtained by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA).

Bartonella DNA was detected by PCR amplifying a 379-bp fragment of the citrate synthase gene (*gltA*) (*8*). For *Rickettsia typhi* and *R. felis*, a real-time multiplex PCR assay targeting a conserved fragment of *glt*A was used (unpub. data). All assays were run in duplicate, and positive and negative controls were included in all assays. Amplicons were purified with the QIAquick PCR purification kit (QIAGEN) and sequenced in both directions by using a BigDye sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. Resultant sequences of *Bartonella* spp. were aligned with MegAlign by using the Clustal algorithm (DNASTAR, Inc., Madison, WI, USA), and compared with reference sequences obtained from GenBank.

Although *Yersinia* DNA and *R. typhi* were not detected, 89 of the 193 pools were PCR positive for either *Bartonella* spp. or *R. felis* (Table). Using the Microsoft Excel Add-In PooledInfRate software (Redmond, WA, USA; www.cdc.gov/ncidod/dvbid/westnile/software.htm), we calculated an estimated infection rate of 10.72% (95% confidence interval [CI] 8.52–13.31) for *R. felis*, 3.66% for *Bartonella* species, and 0.91% (95% CI 0.40–1.78) for both *Bartonella* spp. and R. *felis* (Table).

Phylogenetic analysis indicated several *Bartonella* spp. in fleas that were closely aligned with pathogenic *Bartonella* spp., including *B. vinsonii*, *Candidatus B. rochalimaea*, and *B. clarridgeiae* (data not shown). Moreover, *Bartonella* from 3 pools of *P. irritans* demonstrated only 1.8% to 2.4% divergence to *B. vinsonii* subspecies *arupensis* isolated from a human patient in Wyoming, USA. Likewise, sequences of *Bartonella* from

Table. Detection of *Bartonella* spp. and *Rickettsia* spp. DNA in fleas collected in the Democratic Republic of Congo*

Village	Flea species†	Sample no.	*Bartonella* spp.‡	*Rickettsia* spp.‡
Kpandruma	*Pulex irritans*	1–2	*B. clarridgeiae*	
	P. irritans	3–5	*B. vinsonii*	
	P. irritans	**6**	***B. vinsonii***	***R. felis***
	P. irritans	7–8	Unique	
	P. irritans	9–24		*R. felis*
	Xenopsylla brasiliensis	25		*R. felis*
	Tunga penetrans	26		*R. felis*
Djalusene	*P. irritans*	27	*B. vinsonii*	
	T. penetrans	28	Identical to EU549693	
	P. irritans	29	*B. clarridgeiae*	
	P. irritans	30–34	*Candidatus B. rochalimae*	
	Ctenocephalides felis strongylus	**35**	***B. clarridgeiae***	***R. felis***
	T. penetrans	**36**	***Candidatus B. rochalimae***	***R. felis***
	P. irritans	**37**	***Candidatus B. rochalimae***	***R. felis***
	T. penetrans	38–39		*R. felis*
	P. irritans	40–45		*R. felis*
	C. felis strongylus/P.irritans	46		*R. felis*
	T. penetrans/Echidnophaga gallinacea	47		*R. felis*
	E. gallinacea	48		*R. felis*
Wanyele	***P. irritans***	**49**	***B. vinsonii***	***R. felis***
	C. felis strongylus	**50**	***Candidatus B. rochalimae***	***R. felis***
	E. gallinacea	51		*R. felis*
	C. felis strongylus	52–57		*R. felis*
	T. penetrans	58–59		*R. felis*
	P. irritans	60–63		*R. felis*
Zaa	*P. irritans*	64	*B. vinsonii*	
	P. irritans	65–66	*Candidatus B. rochalimae*	
	P. irritans	**67**	***Candidatus B. rochalimae***	***R. felis***
	C. felis strongylus	68	*B. clarridgeiae*	
	P. irritans	69–87		*R. felis*
	E. gallinacea	88		*R. felis*
	C. felis strongylus/P. irritans	89		*R. felis*

*__Boldface__ indicates dual infection. An expanded version of this table showing all species detected is available online (www.cdc.gov/EID/content/14/12/1972-T.htm).
†Identified by using standard taxonomic keys.
‡Detected by PCR as described in Methods.

1 pool of *T. penetrans* and 1 pool of *P. irritans* were 100% identical to *Bartonella* isolated from a *Neotoma mexicana* wood rat (GenBank accession no. AF110312); a sequence obtained from 1 flea pool of *T. penetrans* was 100% identical to the *glt*A *Bartonella* sequence found in *Orchopeas sexdentatus,* collected from *Neotoma micropus* in New Mexico, USA (data not shown). This finding indicates a new *Bartonella* species with multiple rodent origins and a more ubiquitous global dissemination than previously determined. Our results also demonstrate the previously unreported detection of *R. felis* in *P. irritans*, *E. gallinacea*, *X. brasiliensis*, and *T. penetrans* flea species.

This report suggests that *Bartonella* spp. and *R. felis* exists in fleas within the DRC. In addition, we report *Bartonella* spp. and *R. felis* DNA in *T. penetrans* fleas and *R. felis* DNA in *E. gallinacea* fleas, vectors not previously associated with these pathogens. Co-infections were also observed in *T. penetrans*, *P. irritans*, and *C. felis* fleas, suggesting a common vector or mammalian host shared by *R. felis* and *Bartonella* spp. Flea feedings occur intermittently and on potentially different hosts, thus the vectors described here may acquire multiple bacterial strains for transmission to humans (*8*). Moreover, PCR assays targeting the cytochrome B gene indicated human blood in the flea pools, demonstrating dual infection (data not shown); this finding shows that the flea species recovered are capable of feeding on humans, have a broad host range, and are capable of transmitting disease to humans (*9*).

Bartonella spp. and *R. felis* have been detected previously in fleas within northern and sub-Saharan Africa (*10*). The presence of *Bartonella* spp. and *R. felis* in the fleas is important because they were collected in close contact with humans at risk for multiple exposures within households. Our results suggest that both *R. felis* and *Bartonella* spp. are prevalent in this region of the DRC and should be included in the differential diagnosis of potential flea-borne infections in this region of sub-Saharan Africa**.**

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Antibodies to Nipah or Nipah-like Viruses in Bats, China

To the Editor: *Hendra virus* (HeV) and *Nipah virus* (NiV), the only known members of the genus *Henipavirus,* are 2 emerging paramyxoviruses that are highly pathogenic in a variety of vertebrate animals, including humans (*1*). Since the initial discovery of the viruses in Australia and Malaysia (*2*,*3*), sporadic HeV outbreaks have been reported from 1995 to 2007 in Australia (*4*), and regular NiV outbreaks have occurred in Bangladesh (*5*) and India (*6*). Numerous frugivorous bat species (genus *Pteropus*), and some insectivous bat species have been found to be reservoir hosts of henipaviruses in Australia and Asian countries (*7*–*9*).

In this study conducted during 2004–2007, bats were trapped within their natural habitat from 10 provinces in mainland People's Republic of China. Serum, pharyngeal, and fecal swab samples were collected and stored as described previously (*10*). An ELISA was developed to detect antibodies to the NiV nucleocapsid (N) and attachment glycoprotein (G) proteins. For confirmation, ELISA-positive samples were tested by using Western blot against a recombinant NiV G fragment (aa 71–193) fused with the maltose-binding protein. Virus neutralization tests were conducted with live NiV and HeV under Biosaftey Level 4 containment in Australia. In addition, a surrogate neutralization test was developed by using recombinant *env*$^-$ HIV-1, pseudotyped with NiV G and F. RNA was extracted by using the QIA amp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription–PCR (RT-PCR) was performed by using primers against the NiV N gene as described previously (*3*).

In total, 692 bat serum specimens were screened for antibody to NiV N or G protein (or both) by ELISA, and 33 were positive (online Appendix Table, available from www.cdc.gov/EID/content/14/12/1974-appT.htm). These specimens were from 9 of the 23 bat species examined in this study. Of the 33 serum samples reactive in ELISA, 25 with sufficient quantity left were further tested by Western blot, and 17 of 25 serum samples were reactive with MBP-NiV G fusion fragment, but not with the control MBP. None of the samples inhibited entry of NiV F/G-pseudotyped virus or neutralized either HeV or NiV. No NiV-specific RNA was detected by RT-PCR among 479 fecal swab samples and 67 throat swab samples tested; therefore, virus isolation was not attempted.

This study systematically investigated NiV presence among bats in China. The detection of henipavirus antibody suggests that several bat species have been exposed to NiV or a closely related virus. The prevalence of antibody was especially prominent among *Myotis* species from Yunnan

Province. Antibody was detected in samples from 3 of 4 *Myotis* species captured in the same location in 2006 and 2007. A relatively high prevalence of henipavirus antibody was also found among *Rousettus leschenaultia* samples from Hainan Province in 2007. Notably, Yunnan and Hainan are both located in southern China. Although pteropid bats are not found in China, these data suggest henipaviruses could be introduced into China by other susceptible bat species that overlap their habitat with pteropid bats in neighboring countries.

Several possibilities may explain the failure to detect neutralizing antibodies. One might be the unique immune response among those nonpteropid bats, which results in a low level of neutralizing antibodies that are difficult to detect in the current assay systems. Alternatively, and perhaps more likely, ≥1 Nipah-like viruses could be circulating among the bat populations sampled in this study, producing antibodies that cross-react with, but do not neutralize, the prototype Malaysian NiV virus isolate. This phenomenon has been observed previously by our group for severe acute respiratory syndrome (SARS)–like viruses in horseshoe bats, whose sera cross-reacted with, but did not neutralize, the SARS virus in humans (*10*).

Obtaining serologic evidence of viruses in bat populations is typically more successful as a screening tool than either nucleic acid based assays or virus isolation; this is likely attributable to the often low-level of virus replication, the transient nature of the infection in bats, or both. The inability to amplify NiV sequences may have been attributable to the viral RNA present among these samples being below the threshold of detection in our assay or to the absence of infection in the population at the time of sampling. In addition, the primers used in the PCR may target regions of the NiV N protein that exhibit substantial sequence divergence in a Nipah-like virus.

Bat species in the genera *Rousettus, Myotis*, *Miniopterus,* and *Hipposideros* naturally reside in trees, buildings, and caves that can be in close proximity to human residential areas, which increases the potential of transmission of zoonotic pathogens from bats to humans. The increased risk for these zoonotic infections to spread from bats to humans in areas of cohabitation is best illustrated by the repeated spillover events involving NiV in Bangladesh (*5*). Given the present initial evidence of exposure among bats in mainland China shown here, there is an urgent need to continue and expand surveillance studies for henipaviruses in China and elsewhere on the Asian continent.

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Erratum—Vol. 14, No. 9

The article Obligations to Report Outbreaks of Foodborne Disease under the International Health Regulations (2005) (M.D. Kirk et al.) contained incorrect figures in the abstract and conclusion. The text stated that 7 (50%) of 14 outbreaks would have required notification to the World Health Organization (WHO). The correct proportion is 6 (43%) of 14 outbreaks that would have required notification to the WHO. The article has been corrected online (www.cdc.gov/eid/content/14/9/1440.htm).

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Campylobacter, 3rd Edition

Irving Nachamkin, Christine M. Szymanski, Martin J. Blaser, editors

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This valuable new edition documents advances in *Campylobacter* spp. molecular biology, epidemiology, immunology, and food safety interventions over the past quarter century. It is an easy-to-read resource for both lay readers and scientists who are active in these research fields.

The book contains 6 complementary sections. The first section provides an overview of the taxonomy of *Campylobacter* and related species, followed by in-depth chapters on population biology and molecular techniques. The second section reviews clinical and epidemiologic aspects of human infections. The next 3 sections describe advances in pathogenesis, immunity, glycobiology, and gene expression. Food safety interventions are discussed in the final section.

Throughout the book, current references are cited, such as one that describes a recent finding of a possible link between *C. rectus* and *C. concisus* infections and Barrett's esophagitis, a precursor of esophageal adenocarcinoma. One chapter provides an up-to-date summary of risk factors associated with human campylobacteriosis. Other chapters describe current methods for examining antibiotic resistance, mechanisms of resistance, and subtyping infectious strains. The center of the book is dedicated to technical research areas that may eventually lead to new prevention technologies. The final chapters relate to policies for enhancing food safety.

The third edition of this book is a valuable reference. It provides a history of the science for this field, a review of current understanding, and expert guidance on avenues for future research. Its theme can be summarized by paraphrasing Stephen On and colleagues: With the integration of statistics, epidemiology, biology, and a risk-based paradigm *Campylobacter,* science is coming of age. Future discoveries are likely to be as thrilling, challenging, and surprising as the developments of the past 25 years (On et al., Chapter 10, p. 207).

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Marc Chagall (1887–1985) I and the Village (1911) Oil on canvas (192.1 cm × 151.4 cm) Copyright ARS, NY. Mrs. Simon Guggenheim Fund (146.1945) Digital Image Copyright the Museum of Modern Art/Licensed by SCALA/Art Resource, NY. The Museum of Modern Art, New York, NY, USA

Eye to Eye in the Village

Polyxeni Potter

"One fine day as my mother was putting the bread in the oven," recounted Marc Chagall, "I went up to her and taking her by her flour-smeared elbow I said to her, 'Mama I want to be a painter.'" This wish was out of line with the circumstances. The oldest of nine children in a family of modest means, he was aware of the constraints and his father's toil in the fish-curing business, "My heart used to twist like a Turkish bagel as I watched him lift those weights and stir the herring with his frozen hands."

Chagall had little exposure to art during childhood, until one day, he saw a schoolmate draw a picture from a magazine. Ridiculed for his astonishment, he revolted, "It roused a hyena in me." He started copying from magazines himself and showed early talent. Later, Russian icons, which he found "magical and unreal," also provided inspiration. The family lived in Vitebsk, Byelorussia (now Belarus), then part of the Russian Empire. This small village community fueled his life's work with honesty and charm, poetry and humor, lyricism and pure joy.

Chagall's studies began in 1906 under local artist Yehuda Pen. "I learned about Pen when I was riding on a tram. It was crossing the Cathedral Square and I saw a signboard—white letters on blue: 'Artist Pen's School.' 'What a cultured city is our Vitebsk' I thought." Soon he moved to St. Petersburg to join the Society of Art Supporters School, where he studied briefly under Nikolas Roerich. At the time, Jewish residents needed a permit to live in the city, so he was briefly jailed for violating this restriction.

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He continued under Léon Bakst at the Zvantseva School of Drawing and Painting.

His reputation as an artist grew solid enough to earn him sponsorship to Paris in 1910, the heyday of modern art. Fauvism, with its emphasis on emotion and color, was still influential, and cubism, with its turn to representation from multiple points of view, was bursting onto the scene, led by Picasso and Braque. "I aspired to see with my own eyes what I had heard of from so far away …. The sun of Art then shone only on Paris."

He settled in the heart of the arts community, Montparnasse, and met avant-garde poets Blaise Cendrars and Guillaume Apollinaire and painters Robert Delaunay and Fernand Léger. He studied broadly and produced during these years some of his most famous village paintings. "In Paris, it seems to me I have found everything, but above all, the art of craftsmanship. I owe all that I have achieved to Paris, to France, whose nature, men, the very air, were the true school of my life and art."

His studio was in La Ruche (The Beehive), an artists' colony. Amedeo Modigliani lived on the same floor. "I used to stand alone … in front of my easel in the wretched light of a paraffin lamp …. It was between those four walls that I wiped the dew from my eyes and became a painter." The fauvists freed his use of color, and he learned from the cubists, though he never joined them: "Let them eat their fill of their square peas on their triangular tables." Cendrars, who titled some of his paintings, wrote his own poetic portrait of Chagall: "He's asleep/He's awake/Right away he's painting/He grabs a church and paints with the church/He grabs a cow and paints with the cow/With a sardine/With heads, hands, knives/He paints with an oxtail/…."

Soon he was exhibiting at the Salon des Indépendants, and though his works were not selling well at this the height of abstract art, he espoused neither impressionism nor cubism. Instead, he used their influences to articulate his own unique style, a blend of formal structure, brilliant color, and fluid form. "For the cubists," he wrote, "a painting was a surface covered with forms in a certain order. For me a painting is a surface covered with representations of things—objects, animals, human beings—in a certain order in which logic and illustration have no importance."

When Apollinaire saw the paintings at Chagall's studio in Montparnasse, he pronounced them *surnaturel* (supernatural), perhaps seeing in them the first signs of surrealism before he even coined the term. A poem he wrote about Chagall whom he mysteriously dubbed "Rotsoge," captured the painter's conflicted spirit—now avant-garde Paris, now the village: "Your scarlet face your biplane convertible into hydroplane/Your round house where a smoked herring swims."

The whimsy, unreality, and sheer exuberance of his compositions would later attract the attention of surrealists, who enthusiastically claimed him as one of their own. Despite his influence on them, however, he did not share their inspiration from the subconscious, the world of dreams. He drew from his experiences, "You see, here is my whole biography: They used to find my grandpa on the roof, he loved eating *tzimmes* there. And my uncle loved walking the street with just a nightshirt to his body." When at age 35 he finished writing his autobiography, My Life, it seemed he wrote it to explain his work.

After a very successful art show in 1914, Chagall went back to Vitebsk for a brief visit, which ended up lasting several years on account of World War I. In Vitebsk, he embraced the Bolshevik Revolution and was made commissioner of fine arts by the new Soviet government. He organized exhibitions and became Director of the Vitebsk Academy of Art. But with time, it became clear that neither his style nor his vision for society fit in. He moved to Moscow, where he painted theater murals and designed costumes and sets until he left Russia in 1922.

Back in Paris André Breton offered him the approval of surrealists, "With Chagall alone, the metaphor made its triumphant return into modern painting." But he resisted, "I want an art of the earth and not merely an art of the head." He followed his own style, traveled widely, and ventured into multiple media (ceramics, mosaics, stained glass). World War II forced him to flee France, this time to New York City. Three years after the end of the war, he returned to France, where he lived the rest of his life. His art career spanned more than 70 years. He left behind thousands of works, a legend in his own time.

Painted a year after Chagall first went to Paris, I and the Village, on this month's cover, integrates elements of cubism with his own vision of the world, "Lines, angles, triangles, squares, carried me far away to enchanting horizons." A geometric structure frames a village scene with overlapping images: a green-faced man with a cap, his hand holding a flowering sprig, stares directly at a cow's head. The cow stares back. The sun, the earth, and the moon in eclipse blend softly in the foreground. A peasant carries a scythe. A female fiddler floats upside down along with two houses from the row above. The village skyline crowns the horizon.

"Cows, milkmaids, rooster, and provincial Russian architecture … are part of the environment from which I spring," Chagall wrote, but individual parts assume roles and dimensions he created for them. "I fill up the empty space in my canvas as the structure of my picture requires with a body or an object according to my humor." He explains, "In the large cow's head…I made a small cow and woman milking visible through its muzzle because I needed that sort of form, there, for my composition."

I and the Village seems Chagall's personal notion of the community, a cosmic one (humans, animals, habitat,

stars), made intimate and poetic, jewel-colored, folksy, with music, sparkle, and humor. Two main characters, man and beast stand face to face at the entrance, deliberately linked with a line, eye to eye.

Barnyard animals, cows or goats, frequent Chagall's work, wandering freely, often airborne along with humans, unconstrained by reality. Here, they share equivalent status across the planets in the entrance of the village. In a playful standoff, each claims the "I," seeing alternatingly fences and tethers, limits and yoke; milk and honey, highways and byways. Though wide-eyed, they seem oblivious to what weighs all of us down to earth: infection and consequent illness and death, shared lavishly in the village. From the narrow perspective of "us" or "them," zoonotic infections are not well understood. Their solution requires the total picture, from the human and the animal point of view. Because each "I" sees a different village.

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Sphingomonas paucimobilis Bloodstream Infections and Intravenous Fentanyl

Selection Tool for Foodborne Norovirus Outbreaks

Enhanced Hygiene Measures and Transmission Potential during a Norovirus Outbreak

Human Infection with Highly Pathogenic Avian Influenza Virus (H5N1), Vietnam

Parapneumonic Empyema Deaths during the Past Century, Utah

Imported Case of Poliomyelitis in Melbourne, Australia Enterovirus 71 Outbreak, Brunei

Invasive Nontuberculous Mycobacteria in HIV-Infected Patients, Tanzania

Rickettsia helvetica in *Dermacentor reticulatus* Ticks

Experimental Infection of Dogs with Avian-Origin Canine Influenza Virus (H3N2)

KI and WU Polyomaviruses in Immunocompromised Respiratory Patients

Serotype G12 Rotaviruses, Lilongwe, Malawi

Novel *Bordetella* Strain from Patients with Respiratory Disease

Isolation of Candidatus *Bartonella melophagi* from Human Blood

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

Upcoming Infectious Disease Activities

February 12–13, 2009
The International Symposium on the Asian Tiger Mosquito
Rutgers University
New Brunswick, NJ, USA
http://www.rci.rutgers.edu/~vbcenter/atmsymposium.php

February 13–16, 2009
International Meeting on Emerging Diseases and Surveillance (IMED 2009)
Hotel Hilton
Vienna, Austria
http://imed.isid.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.

The Public Health Image Library (PHIL)

The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health–related images, including high-resolution (print quality) photographs, illustrations, and videos.

PHIL collections illustrate current events and articles, supply visual content for health promotion brochures, document the effects of disease, and enhance instructional media.

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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

Clinical Characteristics and Molecular Subtyping of *Vibrio vulnificus* Illnesses, Israel

CME Questions

1. Which of the following is least likely to be a predisposing factor for infections caused by *Vibrio vulnificus* biotypes 1 and 2?

A. Anemia
B. Organ transplantation
C. HIV infection
D. Chronic liver disease

2. Illness caused by *V. vulnificus* biotype 3 is best distinguished from that caused by biotypes 1 and 2 by which of the following?

A. Method of transmission
B. Genetic features
C. Geographic distribution
D. All of the above

3. Which of the following is the primary fish involved in *V. vulnificus* biotype 3 infection?

A. Gray mullet
B. Tilapia
C. Salmon
D. Sea bass

4. Which of the following best describes the mortality associated with *V. vulnificus* biotype 3 infection as reported in Israel?

A. 1.0%
B. 2.3%
C. 5.2%
D. 7.5%

5. Which of the following is least likely to be an independent predictor of mortality for *V. vulnificus* biotype 3 infection?

A. Bacteremia
B. Ischemic heart disease
C. Sex
D. Altered immune status

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

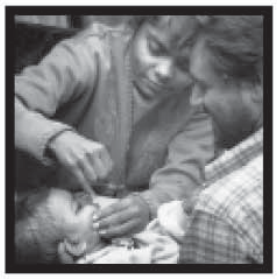
   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic 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.

Fighting the obesity epidemic

Screening Women for Breast Cancer



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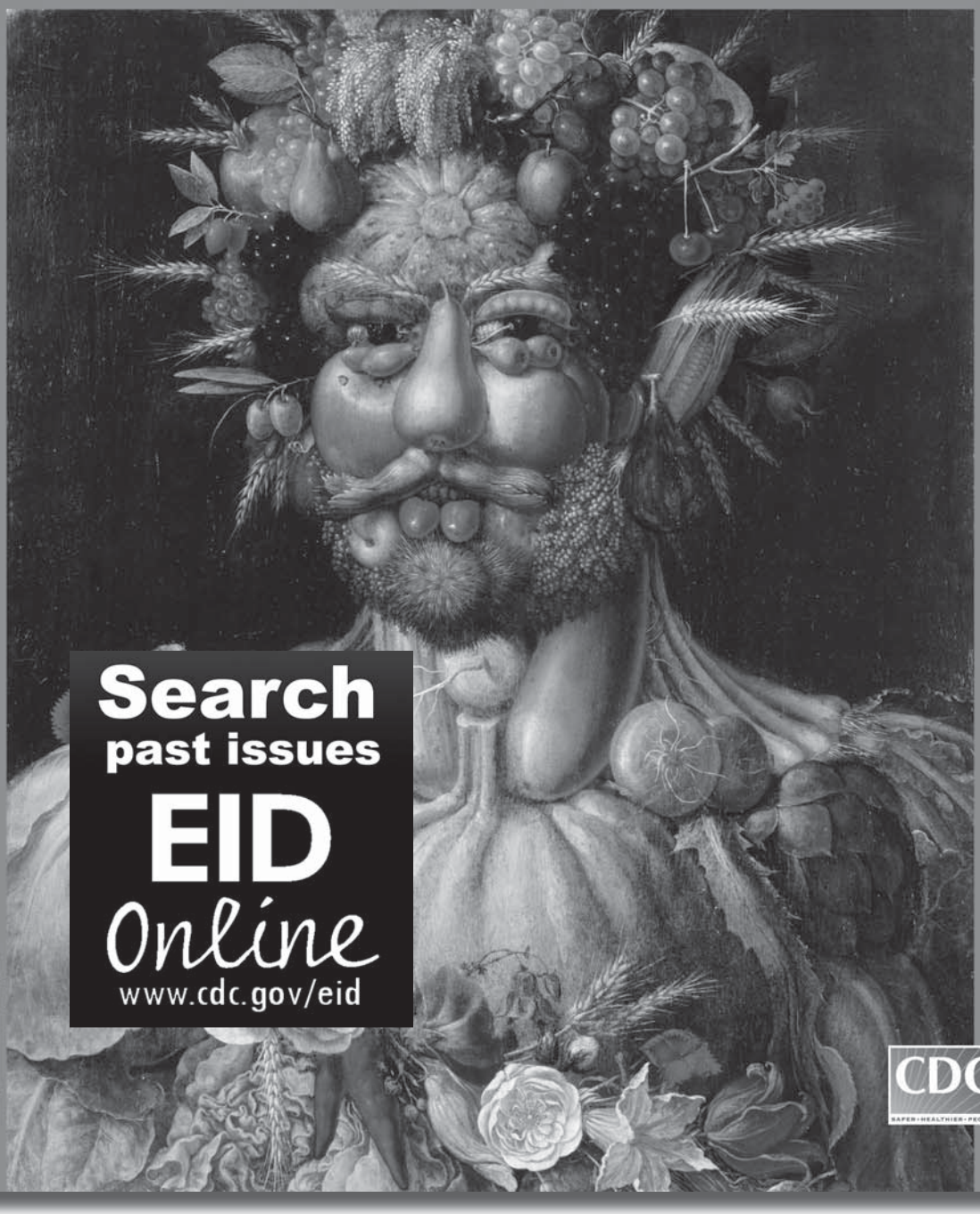
November 2008

Chimera of Infections



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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).

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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.

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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.