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On the Cover

Ray Troll (b. 1954)
Fishes of Amazonia (2000)
Acrylic on canvas (21.13 m × 4.57 m)
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About the Cover p. 682

Research

p. 544

p. 589

Dispatches

EMERGING INFECTIOUS DISEASES
April 2009

Another Dimension

Letters

The Amazon Region

Commentary

Synopsis

Research

CME ACTIVITY

p. 635

p. 647

Dispatches

Letters

News and Notes

Experimental Infection of Potential Reservoir Hosts with Venezuelan Equine Encephalitis Virus, Mexico

Eleanor R. Deardorff, Naomi L. Forrester, Amelia P. Travassos da Rosa, Jose G. Estrada-Franco, Roberto Navarro-Lopez, Robert B. Tesh, and Scott C. Weaver

In 1993, an outbreak of encephalitis among 125 affected equids in coastal Chiapas, Mexico, resulted in a 50% case-fatality rate. The outbreak was attributed to Venezuelan equine encephalitis virus (VEEV) subtype IE, not previously associated with equine disease and death. To better understand the ecology of this VEEV strain in Chiapas, we experimentally infected 5 species of wild rodents and evaluated their competence as reservoir and amplifying hosts. Rodents from 1 species (*Baiomys musculus*) showed signs of disease and died by day 8 postinoculation. Rodents from the 4 other species (*Liomys salvini*, *Oligoryzomys fulvescens*, *Oryzomys couesi,* and *Sigmodon hispidus*) became viremic but survived and developed neutralizing antibodies, indicating that multiple species may contribute to VEEV maintenance. By infecting numerous rodent species and producing adequate viremia, VEEV may increase its chances of long-term persistence in nature and could increase risk for establishment in disease-endemic areas and amplification outside the disease-endemic range.

Venezuelan equine encephalitis (VEE) is a potentially fatal, reemerging disease in tropical America (the portions of North, South, and Central America between the tropics of Cancer and Capricorn) that can cause outbreaks involving hundreds of thousands of humans and equids. VEE virus (VEEV; *Togaviridae*: *Alphavirus*) strains are categorized as either epizootic (associated with equine disease and major epidemics of human disease through equine amplification), or enzootic (not known to cause equine disease). Most VEEV strains, both epizootic and enzootic, have been associated with human disease (*1*). VEEV is also of biodefense importance; it has been developed as a biological weapon, mainly because it is highly infectious by aerosol transmission and can infect humans with a relatively low dose (*2*).

During the mid-1990s, 2 epizootic equine outbreaks occurred in coastal Oaxaca and Chiapas states in Mexico; the causative agent was determined to be VEEV subtype IE (VEEV-IE), which was previously considered to be not virulent for equids (*1*). On the basis of the spread of a VEEV subtype IAB epizootic/epidemic through Mexico and into Texas in 1971 (*3*), the 1993 and 1996 outbreaks were considered to have the potential to spread to other regions of Mexico or the United States. To prevent, detect, and evaluate potential reemergence of this virus in the United States, we need to understand the factors that govern circulation and persistence of this virus in its enzootic foci and epizootic cycles.

Enzootic strains of VEEV are maintained naturally by transmission between mosquitoes of the subgenus *Culex (Melanoconion)* and wild rodents (*4*). These viruses are thought to circulate continuously among mosquitoes and their principal vertebrate amplifying hosts, whereas horses and humans are considered spillover, dead-end hosts not required for maintenance of the natural cycle. Several studies have shown that terrestrial mammals of 5 genera (*Didelphis*, *Oryzomys*, *Proechimys*, *Sigmodon*, and *Zygodontomys*) are susceptible to VEEV-IE infection; they develop viremia sufficient to infect mosquito vectors, yet they usually survive infection (*5*–*10*).

Several species of wild rodents captured in coastal Chiapas have VEEV-specific antibodies (*11*). To address which of these species are likely to play a role as reser-

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voir and/or amplification hosts, we captured rodents from 5 genera (*Baiomys*, *Liomys*, *Oligoryzomys*, *Oryzomys*, and *Sigmodon*) and transported them to the laboratory for experimental infection studies. Our goals were to evaluate the role of various vertebrate species in VEEV-IE maintenance and to help interpret seroprevalence data gathered in the field.

Materials and Methods

Animals

During October 2007, wild rodents of 5 species were collected from coastal Chiapas, Mexico: *Baiomys musculus* (southern pygmy mouse), *Liomys salvini* (Salvins spiny pocket mouse), *Oligoryzomys fulvescens* (fulvus pygmy rice rat), *Oryzomys couesi* (Coues' rice rat) and *Sigmodon hispidus* (hispid cotton rat). All animals were captured from an overgrown field surrounding a stream in Mapastepec municipality, ≈2 km from the Pacific coast (15.413°N and 093.070°W) by using live-capture Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA). Species identification was based initially on morphologic features (*12*) and later confirmed genetically by using cytochrome-B gene sequences (*13*). Animals were housed individually and transported in Taconic Transit Cages (Taconic Farms, Inc., Hudson, NY, USA) to the Animal Biosafety Level 3 Facility at the University of Texas Medical Branch in Galveston, Texas, USA. Animals were captured under permit number SGPA/DGVS/03858/07 Julio 2 de 2007, issued to J.G.E.-F.; all studies were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Virus and Infection

Immediately before rodents were inoculated with virus, a baseline serum sample was taken from each rodent for subsequent antibody assays. For inoculation we used VEEV strain MX01-22 (subtype IE). This strain had been isolated in 2001 from a sentinel hamster in coastal Chiapas, Mexico, and passaged once in Vero cells to generate a sufficient volume of high-titer virus for experimentation. We chose this strain because it is the most recent low-passage isolate of VEEV from the outbreak area and because transmission of this strain by VEEV mosquito vector species from this area has been studied (*14*,*15*). Additionally, this strain is genetically highly similar to the equine-virulent strains that were isolated during the 1993 outbreak (*11*) and caused encephalitis in horses (R. Bowen, pers. comm.).

All animals were inoculated subcutaneously in the right thigh with 3.2 $\log_{10}$ PFU of virus, a dose that approximates the maximum amount of VEEV transmitted by a mosquito bite (*16*). After inoculation, all animals were weighed daily for 1 week and observed for signs of illness for 2 weeks.

Viremia Assays

Blood was collected daily for the first 7 days after inoculation, then on days 10, 14, 28, 42, and 66. After the animals were anesthetized with inhaled isoflurane, retroorbital sinus blood was collected in heparinized glass capillary tubes and transferred to 5 volumes of phosphate-buffered saline (PBS). Erythrocytes were removed by centrifugation to yield an ≈1:10 dilution of serum, which was stored at –80ºC. Viremia titers were determined by plaque assay on Vero cells (*17*).

Necropsy was performed on all animals, and tissues were frozen at –80ºC. Using a TissueLyser (QIAGEN Inc, Valencia, CA, USA), we homogenized ≈2–10 mg of tissue in minimal essential medium (Eagle) supplemented with 20% fetal bovine serum, L-glutamine, penicillin, streptomycin, gentamicin, and fungizone. Tissue virus titers were determined by plaque assay on Vero cells.

Antibody Assays

To detect VEEV-IE–specific antibodies, we performed hemagglutination inhibition assays (*17*) using antigen derived from the same VEEV strain used for infection (MX01-22) as well as from 3 other arboviruses: Eastern equine encephalitis virus (TenBroeck strain), West Nile virus (strain 385-99), and St. Louis encephalitis virus (strain TBH28). Briefly, 4–8 units of hemagglutinin antigen were reacted with heat-inactivated test serum in various concentrations in PBS. Failure to hemagglutinate goose erythrocytes was considered a positive result. Antibody titers were confirmed by plaque reduction neutralization tests (*17*). Test serum samples were serially diluted in PBS and heat inactivated at 56°C for 1 h, then mixed with ≈100 PFU of virus and incubated at 37°C for 1 h. The mixture was inoculated onto Vero cells. Dilutions resulting in ≥80% reduction in virus titer were considered positive; titers were reported as the reciprocal of the endpoint dilution.

Results

Clinical Responses and Survival Rates

Of the 5 rodent species examined, only those of species *B. musculus* showed signs of disease with neurologic manifestations. These animals began to exhibit tremor, lethargy, dehydration, hunching, and staggering during days 4–6 postinoculation. By day 8, all 4 (100%) of these *B. musculus* rodents had died or were euthanized after becoming moribund (Figure 1, panel A). Rodents of this species were the only ones that lost body weight after inoculation (average 22% loss; Figure 1, panel B).

No animal from the other 4 species exhibited weight loss or outward signs of illness after inoculation. Most of these rodents survived until the end of the experiment, day 66 postinoculation. However, during the first 2 weeks af-

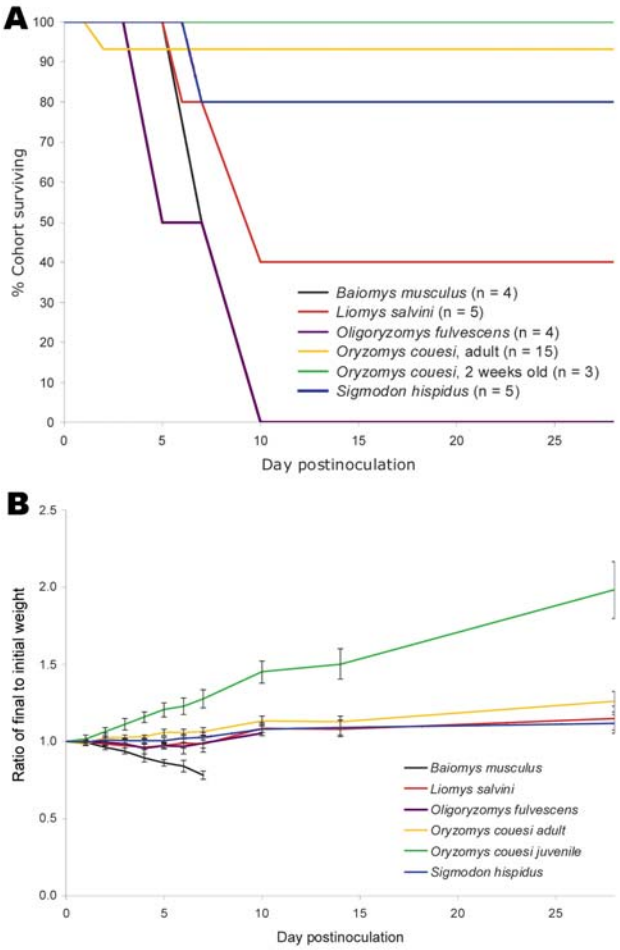

Figure 1. Survival rates and weight change of wild rodents from Chiapas, Mexico, after experimental infection with 3 $\log_{10}$ PFU of Venezuelan equine encephalitis virus subtype IE, strain MX01-22. A) Survival rates. Black and yellow lines represent animals whose brains yielded live virus after necropsy. Red, green, blue, and purple lines indicate animals whose death was attributed to manipulation and/or stress, not to VEEV infection. B) Weight change. Mean cohort weight (grams) divided by mean cohort starting weight (day 0). Weight gain or loss was used as an indicator of disease. Only *Baiomys musculus* rodents showed weight loss during acute infection. Data for days 42 and 66 (not shown) did not differ significantly from that for day 28. Error bars indicate SEM.

ter inoculation, 9 animals died without weight loss or signs of illness. These animals did not have high levels of virus in their tissues (Table) and are considered to have died of stress from daily manipulations rather than of VEEV infection. To address this possibility, a subcohort of 2 *L. salvini* and 3 *O. fulvescens* rodents, the 2 species that had had the most manipulation-related deaths, were inoculated and observed for 15 days without daily blood sampling. All 5 animals survived with little to no illness; they were found to have seroconverted by day 15 (reciprocal mean titer = 2.7 ± 2.3 $\log_{10}$, standard error) and remained seropositive through day 42 (3.0 ± 2.9 $\log_{10}$). Similar deaths of wild rodents in the absence of an infectious cause have been encountered previously (*10*).

Virus Titers

Of 35 animals tested, 22 (comprising all 5 species) had measurable virus levels during the first week after inoculation (limit of detection was 1.5 $\log_{10}$ PFU/mL) (Figure 2). Viremia (≥2.7 $\log_{10}$ PFU/mL) developed in all (100%) *O. fulvescens, L. salvini*, and *B. musculus* rodents and lasted as long as 4, 5, and 8 days, respectively. Conversely, detectable viremia developed in only 60% of the cohort of *S. hispidus* rodents (3/5 animals), lasting as long as 4 days, and in only 39% of the *O. couesi* cohort (7/18 animals), lasting as long as 2 days.

In the cohorts of *L. salvini*, *O. fulvescens,* and *O. couesi* rodents, maximum viremia occurred on day 1 postinoculation; mean titers were 3.4 ± 0.6 (SEM), 3.3 ± 0.2, and 2.5 ± 0.6 $\log_{10}$ PFU/mL, respectively (Figure 2). In *S. hispidus* rodents, the cohort peak viremia occurred on day 2 postinoculation; mean was 2.9 $\log_{10}$ ± 0.9. In the cohort of *B. musculus* rodents, peak viremia occurred on day 3; mean was 5.5 ± 0.4 PFU/mL (Figure 2).

Antibody Responses

Of the 40 animals used in this study, only 1 (*S. hispidus*) was found to have preexisting VEEV antibodies. This animal had a hemagglutination inhibition reciprocal antibody titer of 2.8 $\log_{10}$ on day 0 and 2.2 $\log_{10}$ on day 6, when it died during anesthesia and blood collection. For rodents of all 4 surviving species, antibodies were detectable by day 5 and lasted through the end of the experiment (Figure 2).

Age Dependence

An unanticipated cohort of 3 juvenile rodents (*O. couesi*) provided an opportunity to examine whether age affected outcome of VEEV infection. The species of these 3 animals was initially identified as *O. fulvescens* but later determined, based on cytochrome-B gene sequencing, to be juvenile *O. couesi* (*13*). Age at infection was ≈2 weeks, determined on the basis of growth of 3 litters of *O. couesi* rodents born in captivity.

No differences were found between the juvenile and the adult *O. couesi* rodents in terms of survival rates, viremia levels, or antibody responses (Figures 1, 2). Viremia was detected in 1 (33%) of 3 juvenile and 6 (40%) of 15 adult *O. couesi* rodents. Mean maximum viremia was 2.3 $\log_{10}$ PFU/mL for the juveniles and 2.6 ± 0.6 $\log_{10}$ PFU/mL for the adults. No viremia was detected after day 1 for either juveniles or adults, except for 1 adult that had a titer of 2.6 $\log_{10}$ on day 2. Antibody responses were inconsistent among animals from both groups. Several animals from each group

Table. Viremia in rodents that died 1–14 days after inoculation with 3.2 $\log_{10}$ PFU of Venezuelan equine encephalitis virus subtype IE strain MX01-22*

Rodent genus	dpi†	Tissue virus content ($\log_{10}$ PFU/g)†					
		Brain	Heart	Spleen	Kidney	Liver	Lung
Oryzomys	2	1.8	2.7	3.3	2.0	3.2	0
Oligoryzomys	4	0	0	4.0	0	0	0
	6	0	0	3.4	0	0	0
Baiomys	6	3.2	5.0	5.0	4.7	4.9	3.9
	7	4.6	3.2	4.2	0	4.3	4.3
	7	2.0	2.0	3.0	3.4	2.6	4.1
	8	5.0	3.0	5.7	5.0	1.9	5.0

*Not shown are 3 *Liomys salvini*, 2 *Oligoryzomys fulvescens*, and 1 *Sigmodon hispidus* rodents. These animals died on days 5–10 postinoculation and showed no detectable virus in any organs tested. dpi, days postinoculation.
†Limit of detection was 1 plaque in150 µL homogenate. Tissue sample weight varied between 0.002 and 0.01 grams.

showed weak antibody responses of short duration, delayed onset, or both, after having no detectable viremia.

Discussion

Reservoir Status and Potential

Of the 5 species of rodents evaluated in this study, only *S. hispidus* rodents have been included in previous experimental VEEV infection studies. In Panama (*10*) and Florida (*5*,*7*), *S. hispidus* rodents are considered to be competent, mostly disease-resistant reservoir hosts for disease caused by sympatric VEE complex alphaviruses. In 2007, Carrara et al. (*7*) infected 3 geographically distinct populations of *S. hispidus* rodents with 2 enzootic VEEV strains and found that only the population from a VEE complex alphavirus–endemic region (Florida) survived infection; cohorts from the 2 non–virus-endemic populations succumbed to disease. For this reason, we used a sympatric VEEV strain for our studies.

In addition to *S. hispidus* rodents, 3 other species (*Proechimys semispinosus*, *Zygodontomys microtinus*, and *Oryzomys capito*) had viremia sufficient to infect at least some mosquito vectors and survive after inoculation with sympatric strains of VEEV (*8*–*10*). Our results support the hypothesis that enzootic VEEV selects for resistance to disease in its sympatric reservoir host populations (*10*).

Several field studies in Mexico have reported VEEV-specific antibodies in a variety of wild vertebrate species.

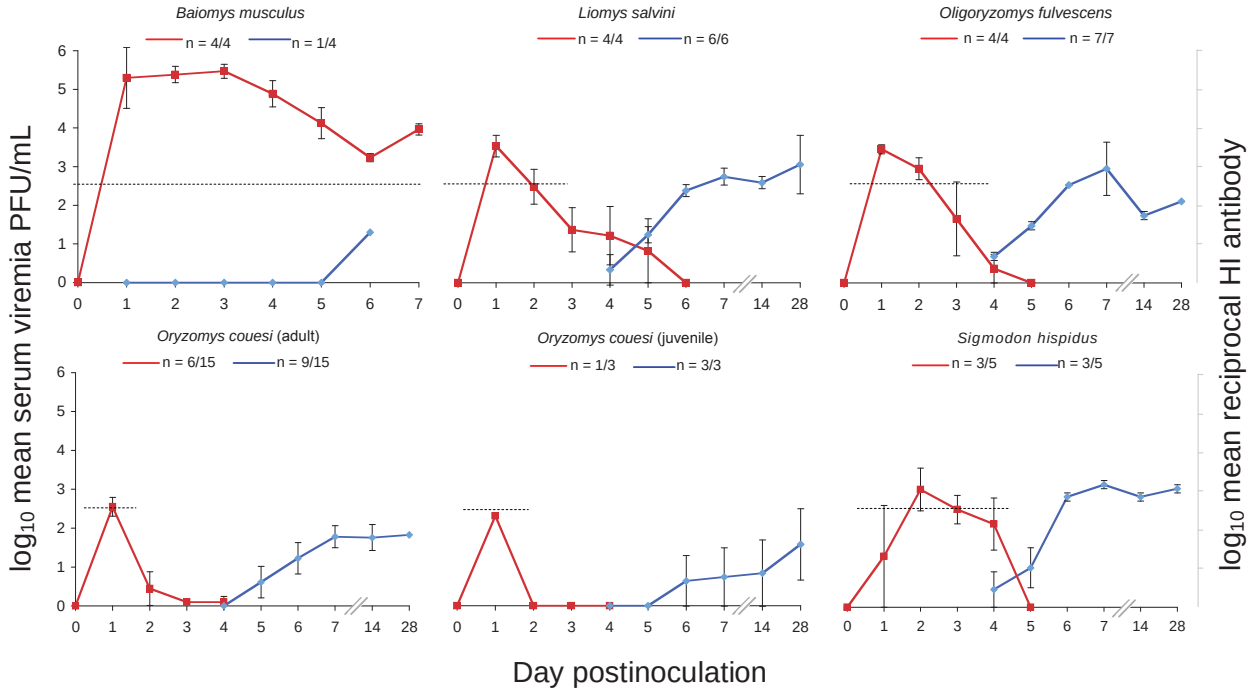

Figure 2. Mean viremia profile (red lines) and mean hemagglutination inhibition (HI) antibody profile (blue lines) of 5 species of wild rodents after experimental infection with 3 $\log_{10}$ PFU of Venezuelan equine encephalitis virus type-IE, strain MX01-22. Black dashed lines indicate approximate mosquito infection viremia threshold for the enzootic vector *Culex (Melanoconion) taeniopus*. Fractions represent proportion of total cohort that had measurable response. Data for days 42 and 66 (not shown) did not differ significantly from data for day 28. Error bars indicate SEM.

Aguirre et al. (*18*) found 7 species of wild mammals and 17 species of wild birds that were seropositive against VEEV-IE in 1992. In the same area from which the animals for our study were captured, VEEV-neutralizing antibodies were detected in wild *S. hispidus, Oryzomys alfaroi*, and *Didelphis marsupialis* rodents (*11*). In an extensive field study in southern Mexico during the 1960s, Scherer et al. (*6*) found 29 species of wild birds, 10 genera of terrestrial mammals, and 3 genera of bats with serologic evidence of natural VEEV infection. Evidence of similar broad host ranges of VEEV has been found in coastal Guatemala, where 7 genera of terrestrial mammals and 11 species of birds had VEEV-specific antibodies (*19*). After the 1971 epidemic of VEEV-IAB that started in Central America and reached southern Texas, extensive field studies were conducted to determine whether the virus would or could establish a new enzootic focus (*20*). In that study, mammals of 10 genera had VEEV-specific antibodies. In 2 follow-up studies in which wild mammals and wild birds were infected with a strain of VEEV-IB isolated during the outbreak, viremia and mortality rates for rodents were high (*21*,*22*). In a longitudinal field study performed concurrent with the study reported here, seroprevalance for wild rodents was found to be much lower than previously found for this area (*11*).

Viremia and Immunologic Response

All 5 of the species tested produced viremia titers sufficient to infect the proven enzootic mosquito vector *Cx. (Mel.) taeniopus.* Of these 5 species, the lowest and shortest lasting viremia was found in *O. couesi* rodents; however, even these reached levels that are considered adequate to infect a proportion of *Cx. (Mel.) taeniopus* (*23*). The other 4 species all exhibited viremia titers well above the minimum infection threshold for this vector. Therefore, assuming that they are bitten by *Cx. (Mel.) taeniopus* mosquitoes, which are known to be universal feeders and have been recently found in higher numbers than previously reported in the area where these animals were captured, all 5 species we studied should be able to infect this mosquito (*11*,*24*).

The uniform susceptibility of *B. musculus* rodents to VEE disease was an unexpected result and appeared to contradict the hypothesis that VEEV circulation selects for resistance to disease in wild rodents. This difference is evidently not reflective of the taxonomic relatedness of these 5 species (Figure 3). A different potential explanation is the lack of temporal overlap of activity between *B. musculus* rodents and the enzootic vector, *Cx. (Mel.) taeniopus. Baiomys* spp. rodents are diurnally active (*12*), but *Cx. (Mel.) taeniopus* mosquitoes are nocturnal feeders (*24*,*25*). Although the rodents and mosquito vectors coexist spatially, they are not active at the same time of day, which may limit their contact. This lack of contact time may preclude the selection for resistance to VEE that is manifested in the other 4 rodent species, which are nocturnal and presumably regularly exposed to bites from this vector. Experimental infection of other diurnal species from the study area, or similar studies in another VEE-endemic area, could be used to test this hypothesis. Of the 5 species, *B. musculus* rodents were the only species not encountered in previous capture-and-release studies; however, because of the severity of disease in this species, seropositive individuals would be unlikely to survive (and thereby be caught) in the wild.

We ended our study at 66 days postinoculation for the original cohort and 42 days postinoculation for the subcohorts of *L. salvini* and *O. fulvescens* that survived. The antibody responses for all animals that developed measurable viremia persisted through the end of the experiment. The only exception was several *O. couesi* animals that did not develop viremia but did demonstrate brief, low-titer ($\leq$1.6 $\log_{10}$) antibody responses. Wild rodents have been shown to remain seropositive for as many as 6 months postinoculation with VEEV (*7*). For some species with short life spans in the wild, this antibody response is tantamount to lifelong immunity offering protection against reinfection and affording more opportunity for the animal to reproduce.

Ecological Implications

Although the ability of laboratory experimentation to elucidate natural processes is limited, data gathered in the laboratory are sometimes more complete and detailed than field data. In this study, 5 of the most commonly captured rodent species in coastal Chiapas, Mexico, were evaluated for their ability to participate in the natural transmission cycle of enzootic VEEV-IE. *S. hispidus* and *O. capito* rodents have previously been implicated in amplification of other VEE subtypes, ID, IE, and II (*7*–*9*), but the other 3 species (*B. musculus, L. salvini*, and *O. fulvescens*) had been studied little or not at all. Rodents of all 5 species developed viremia titers sufficient to infect the enzootic mosquito vector, *Cx. (Mel.) taeniopus*. However, only 4 of the 5 species

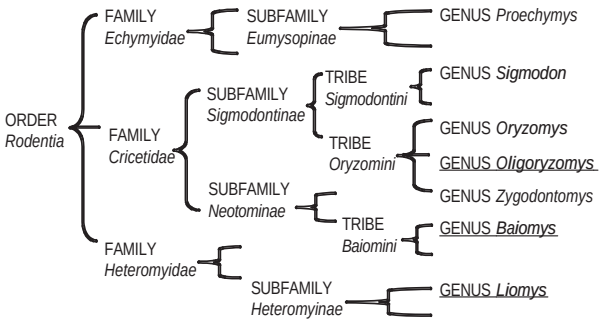

Figure 3. Relatedness of 7 wild rodent genera that have been experimentally evaluated for suitability as amplifying hosts in enzootic transmission cycles of Venezuelan equine encephalitis virus. The 5 genera included in this study are presented in **boldface;** the 3 novel genera are underlined**.**

survived infection with the potential to reproduce, a trait considered critical for true reservoir status in that it avoids population declines that might jeopardize long-term virus circulation.

History has shown that an outbreak of highly virulent VEEV in southern Mexico can easily and rapidly spread into the United States, as it did in 1971. Therefore, a better understanding of VEEV ecology in Mexico is essential for assessing the risk for widespread disease. Our results support the conclusions of Scherer et al. (*6*) that VEEV has a wide range of mammalian hosts that may participate in the natural transmission cycle. This strategy may be an adaptive one that affords greater population stability than does specialization for 1 amplifying host species. By being able to infect numerous rodent species and produce adequate viremia for mosquito transmission, VEEV may increase its chances of long-term persistence in nature when weather or environmental conditions affect some but not all reservoir host populations. This ability could also increase the risk for endemic establishment as well as amplification when outbreaks spread outside their disease-endemic range.

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Ms Deardorff is a doctoral student in the Experimental Pathology Department at the University of Texas Medical Branch. Her research interests include zoonotic disease, viral evolution and ecology, and wildlife conservation biology.

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Exotic Small Mammals as Potential Reservoirs of Zoonotic *Bartonella* spp.

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To evaluate the risk for emerging human infections caused by zoonotic *Bartonella* spp. from exotic small mammals, we investigated the prevalence of *Bartonella* spp. in 546 small mammals (28 species) that had been imported into Japan as pets from Asia, North America, Europe, and the Middle and Near East. We obtained 407 *Bartonella* isolates and characterized them by molecular phylogenetic analysis of the citrate synthase gene, *gltA*. The animals examined carried 4 zoonotic *Bartonella* spp. that cause human endocarditis and neuroretinitis and 6 novel *Bartonella* spp. at a high prevalence (26.0%, 142/546). We conclude that exotic small mammals potentially serve as reservoirs of several zoonotic *Bartonella* spp.

The genus *Bartonella* includes a variety of gram-negative, fastidious, hemotrophic bacteria that are transmitted by blood-sucking arthropod vectors. The genus consists of 20 species and 3 subspecies; at least 11 of these species are known or suspected to be pathogenic for humans as causative agents of emerging zoonoses (*1*).

The following *Bartonella* spp. have been isolated from wild mice: *B. birtlesii* (*2*), *B. doshiae*, *B. grahamii*, *B. taylorii* (*3*), and *B. vinsonii* subsp. *arupensis* and subsp. *vinsonii* (*4*). In several countries, the following species have been carried by rats of the genus *Rattus*: *B. elizabethae* (*5*), *B. tribocorum* (*6*), *B. phoceensis*, and *B. rattimassiliensis* (*7*). In South Africa, strains genetically related to *B. elizabethae* also have been isolated from mice of the genera *Aethomys* and *Tatera* (*8*). The main reservoir of *B. washoensis* is considered to be wild squirrels (*9*). Of these rodent-associated *Bartonella* spp., *B. elizabethae*, *B. grahamii*, *B. vinsonii* subsp. *arupensis*, and *B. washoensis* have been implicated in the human infections endocarditis (*10*), neuroretinitis (*11*), pyrexia and endocarditis (*4*,*12*), and myocarditis (*9*), respectively.

Previous studies have demonstrated high prevalence of infection with *Bartonella* spp. in wild and peridomestic small animals in Europe (*7*,*13*–*15*), North and South America (*5*,*16*–*19*), Asia (*20*–*23*), and Africa (*8*). Thus, these animals are thought to be reservoirs of several *Bartonella* spp. and sources of infection for humans.

Many exotic animals are traded as pets around the world and have been imported into Japan without quarantine. However, no data exist on the prevalence of infection with *Bartonella* spp., especially in exotic pet animals. Our study objectives were to 1) examine the prevalence of *Bartonella* spp. infection in exotic small mammals imported into Japan from various countries, 2) compare the diversity of these *Bartonella* strains by analyzing the partial sequence of the citrate synthase gene (*gltA*), and 3) evaluate the possibility that these mammals may serve as potential reservoirs of zoonotic *Bartonella* spp.

Materials and Methods

Animals and Samples

For this study, 546 exotic small mammals were purchased from trading companies. The animals represented 3 orders and included 6 families, 23 genera, and 28 species (Table 1). They had been imported into Japan as pets from June 2004 through October 2007 from 8 countries in 4 geographic regions: Asia (China, Thailand, and Indonesia), Europe (the Netherlands and Czech Republic), North America (United States), and the Middle and Near East (Egypt and Pakistan). Of the 546 animals, 367 had been captured in their natural environment and 179 had been

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bred in the exporting countries. Heparinized blood samples were aseptically collected from each animal (anesthetized with chloroform) and centrifuged at 3,000 rpm for 15 min. Plasma was removed and the blood sample pellets were kept at –80ºC until examination.

Isolation of Bacteria

The blood sample pellets were thawed at room temperature, 100-µL supplemented Medium 199 (*24*) was added to each pellet, and each sample was mixed well. A 100-µL sample of each mixture was plated on 2 heart infusion agar

Table 1. Prevalence of *Bartonella* spp. among exotic small mammals imported into Japan as pets, June 2004–October 2007

Origin	Animal, taxonomic species	No. positive/no. tested (%)	Subtotal (%)
Wild-captive			
Asia			
China	*Spermophilus dauricus**	4/10 (40.0)	42/89 (47.2)
	Sciurus vulgaris subsp. *orientis**	2/10 (20.0)	
	*Tamias sibiricus**	12/29 (41.4)	
	*Pteromys volans**	5/10 (50.0)	
Thailand	*Callosciurus notatus**	19/30 (63.3)	
North America			
USA	*Tamiasciurus hudosonicus**	3/18 (16.7)	27/68 (39.7)
	*Glaucomys volans**	6/10 (60.0)	
Unknown	*Sp. columbianus**	6/20 (30.0)	
	*Sp. richardsonii**	12/20 (60.0)	
Europe			
The Netherlands	*Pachyuromys duprasi*†	13/18 (72.2)	13/47 (27.7)
The Netherlands, Czech Republic	*Octodon degus*‡§	0/29 (0.0)	
Middle and Near East			
Egypt	*Mus minutoides*†	0/20 (0.0)	55/163 (33.7)
	Acomys cahirinus†	3/31 (9.7)	
	A. russatus†	8/13 (61.5)	
	Lemniscomys barbarus†	0/11 (0.0)	
	Psammomys obesus†	6/10 (60.0)	
	Meriones tristrami†	0/4 (0.0)	
	Sekeetamys calurus†	10/10 (100)	
	Gerbillus pyramidum†	9/10 (90.0)	
	Jaculus orientalis¶	13/16 (81.3)	
	J. jaculus¶	6/8 (75.0)	
	Hemiechinus auritus#	0/10 (0.0)	
Pakistan	*Salpingotulus michaelis*¶	0/20 (0.0)	
	Subtotal	137/367 (37.3)	
Breeder facility			
Asia			
China	*Tamias sibiricus**	5/30 (16.7)	5/60 (8.3)
Indonesia	*Petaurus breviceps***	0/20 (0.0)	
Thailand	*Pe. breviceps***	0/10 (0.0)	
Europe			
The Netherlands	*Lagurus lagurus*†	0/9 (0.0)	0/99 (0.0)
	Pa. duprasi†	0/10 (0.0)	
	Mesocricetus auratus†	0/20 (0.0)	
	Phodopus roborovskii†	0/10 (0.0)	
The Netherlands, Czech Republic	*Ph. sungorus*†‡	0/30 (0.0)	
	O. degus‡§	0/20 (0.0)	
Middle and Near East			
Pakistan	*Sa. michaelis*¶	0/20 (0.0)	0/20 (0.0)
	Subtotal	5/179 (2.8)	
	Total	142/546 (26.0)	

*Member of the order Rodentia, family Sciuridae.
†Member of the order Rodentia, family Muridae.
‡Data for the Netherlands and Czech Republic are pooled because number of animals from these 2 countries was unknown.
§Member of the order Rodentia, family Octodontidae.
¶Member of the order Rodentia, family Dipodidae.
#Member of the order Insectivora, family Erinaceidae.
**Member of the order Diprotodonia, family Petauridae.

(DIFCO, Sparks Glencoe, MI, USA) plates containing 5% defibrinated rabbit blood. The plates were incubated at 35ºC under 5% CO_2. After 2 weeks of incubation, 2 or 3 colonies with genus *Bartonella* morphologic characteristics (small, gray or cream-yellow, round colonies) were picked from each plate, confirmed to be gram-negative pleomorphic bacteria, and subcultured using the same conditions used for the original cultures.

DNA Extraction and PCR

The genomic DNA of each isolate was extracted by using InstaGene Matrix (Bio-Rad, Hercules, CA, USA). The extracted DNA was used for PCR analysis of a 312-bp part of the *gltA* gene to confirm that the bacteria were from the genus *Bartonella*. PCR was performed by using an iCycler (Bio-Rad) with a 20-μL mixture containing 20 ng extracted DNA, 200 μM of each deoxynucleoside triphosphate, 1.5 mmol/L $MgCl_2$, 0.5 U Taq DNA polymerase (Promega, Madison, WI, USA), and 1 pmol of each primer. The specific primer pair and PCR conditions for *gltA* were as previously reported (*25*).

DNA Sequencing and Phylogenetic Analysis

The PCR products were purified by using a commercial kit (Spin Column PCR Products Purification Kit; Bio Basic, Markham, Ontario, Canada). Direct DNA sequencing of the purified PCR products was carried out by using dye terminator chemistry with specific primers (*25*) and a Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The 312-bp *gltA* sequences from the isolates and type strains of established *Bartonella* spp. in GenBank/EMBL/DDBJ were aligned with the Clustal X program (*26*), and a phylogenetic tree was drawn, based on the sequence data and using the neighbor-joining method (*27*) with the Kimura 2-parameter distance method (*28*) in MEGA 4 (*29*). Bootstrap analysis was carried out with 1,000 replications (*30*).

Statistical Analysis

The results were analyzed in 2×2 tables. Chi-square tests were used to examine the statistical significance; $p<0.05$ was considered significant.

Results

Prevalence of Bartonellae

The prevalence of bartonellae in the exotic small mammals examined was 26.0% (142/546). A total of 407 isolates were obtained from the 142 bacteremic animals (Table 1). The prevalence by animal origin was 37.3% (137/367) in captive animals and 2.8% (5/179) in animals from breeder facilities. A significantly higher prevalence of bartonellae was observed in captive animals than in animals from breeder facilities ($p<0.001$). In the captive animals, the prevalence by region varied up to 47.2% in Asia, which is higher than the 39.7% prevalence in North America. The prevalence of bartonellae by corresponding taxonomic family of host animal ranged from 38.6% (49/127) in the family Muridae to 43.9% (69/157) in the family Sciuridae. No bartonellae were detected in animals in the families Octodontidae and Erinaceidae. Among animals from breeders, only 5 chipmunks (*Tamias sibiricus*) from China were found to be infected with bartonellae; no bartonellae were isolated from animals in the families Petauridae, Muridae, Octodontidae, or Dipodidae.

Bartonellae were isolated from 17 of the 28 animal species studied (Table 1). The prevalence by animal species varied from 9.7% (3/31) in the Cairo spiny mouse (*Acomys cahirinus*) to 100% (10/10) in the bushy-tailed jird (*Sekeetamys calurus*). Prevelances were considerably higher for the bushy-tailed jird, large Egyptian gerbil (*Gerbillus pyramidum*), greater Egyptian jerboa (*Jaculus orientalis*), and lesser Egyptian jerboa (*J. jaculus*) at 100% (10/10), 90.0% (9/10), 81.3% (13/16), and 75.0% (6/8), respectively.

DNA Sequences and Phylogeny of Isolates

The 407 isolates in this study were classified into 53 genotypes on the basis of DNA sequence analysis of a 312-bp fragment of their *gltA* genes. The sequence of a genotype from a Cairo spiny mouse was identical to that of the *B. elizabethae* type strain (GenBank accession no. Z70009) isolated from a human patient with endocarditis (*10*). The other 52 genotypes were found to be novel genotypes after comparison with known *Bartonella* spp. The phylogenetic tree of the *gltA* sequences shows that the 52 novel genotypes are clearly clustered in 10 genogroups, designated A to J (Figure).

Of the 52 novel genotypes, genogroup A, which consisted of 21 genotypes (AB444954 to AB444974) isolated from 7 squirrel species, was related to *B. washoensis* strain Sb944nv (AF470616), which was isolated from a California ground squirrel (*Spermophilus beecheyi*) and was genetically identical to an isolate from a human patient with myocarditis (*9*). The sequence similarities of these genotypes and *B. washoensis* strain Sb944nv ranged from 94.2% to 97.4%. Genogroup A contained *B. washoensis*–like genotypes; the genotypes from each squirrel species formed a separate clade, except for the genotypes from Richardson's ground squirrels (*Sp. richardsonii*) and Columbian ground squirrels (*Sp. columbianus*), which formed a mixed clade (Figure).

In this study, 18 genotypes formed the 6 unique genogroups B to G. The DNA sequences of the genotypes in each genogroup showed relatively low similarity (82.4%–94.6%) to the type strains of known *Bartonella* spp., and

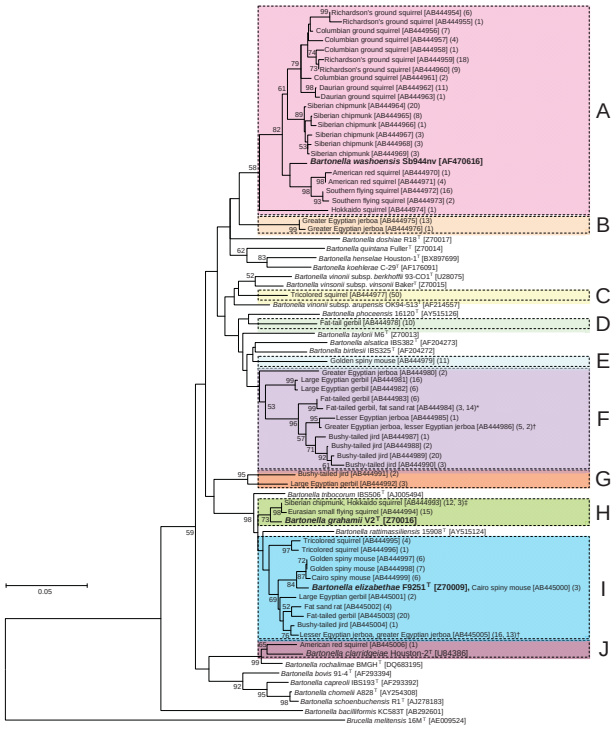

Figure. Phylogenetic tree based on a 312-bp region of the citrate synthase (*gltA*) gene sequence, constructed from *Bartonella* spp. isolates from 142 exotic small mammals imported into Japan as pets, June 2004–October 2007.Isolates from imported animals were compared with the type strains of known *Bartonella* spp. The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were obtained with 1,000 replicates if values >50% were noted. The *Brucella melitensis* strain 16M sequence was used as an out-group. The GenBank accession number and the number of isolates are indicated in brackets and parentheses, respectively. The scale bar indicates 0.05 estimated nucleotide substitutions per site. Each colored column corresponds to genogroup A to J. Isolates showing identical genotypes were obtained from fat-tailed gerbils and fat sand rats (*), greater Egyptian jerboas and lesser Egyptian jerboas (†), and Siberian chipmunks and Hokkaido squirrels (‡).

sequence similarities between genogroups B to G were also low (87.5%–93.6%). The novel *Bartonella* genogroups B, C, D, and E were isolated from greater Egyptian jerboas, tricolored squirrels (*Callosciurus notatus*), fat-tailed gerbils (*Pachyuromys duprasi*), and golden spiny mice (*A. russatus*), respectively. The genotypes in group F were isolated from 6 animal species: large Egyptian gerbils, fat-tailed gerbils, fat sand rats (*Psammomys obesus*), lesser Egyptian jerboas, greater Egyptian jerboas, and bushy-tailed jirds; those in genogroup G were isolated from a bushy-tailed jird and a large Egyptian gerbil (Figure). In genogroup F, 3 of the 9 isolates from fat-tailed gerbils and the 14 isolates from fat sand rats had identical *gltA* DNA sequences. Furthermore, 5 of the 7 isolates from greater Egyptian jerboas and 2 of the 3 isolates from a lesser Egyptian jerboa also had identical sequences.

The 2 novel genotypes (AB444993 and AB444994) in genogroup H were also isolated from Siberian chipmunks, a Hokkaido squirrel (*Sciurus vulgaris* subsp. *orientis*), and Eurasian small flying squirrels (*Pteromys volans*). Their sequences showed high similarity (98.4%–98.7%) to *B. grahami* type strain (V2) (Figure).

The 10 novel genotypes in genogroup I were isolated from 9 animal species, and the sequence similarities between the genotypes (AB444995 to AB445005) and *B. elizabethae* type strain (F9251) ranged from 95.5% to 98.7%. The DNA sequences of *gltA* of the 3 isolates from a Cairo spiny mouse (AB445000) were identical to that of *B. elizabethae* (F9251). The sequences of the 13 isolates from lesser Egyptian jerboas were identical to those of the 16 isolates from greater Egyptian jerboas.

In genogroup J, a unique genotype (AB445006) was isolated from an American red squirrel (*Tamiasciurus hudosonicus*); it had 96.2% sequence similarity to *B. clarridgeiae* type strain (Houston-2), whose natural reservoir is cats (Figure).

Multiple Infections with Different *Bartonella* Genogroups and Genotypes

Of the 142 *Bartonella*-positive animals, 25 (17.6%) were found to be infected with different *Bartonella* genogroups or genotypes (Table 2). A lesser Egyptian jerboa carried 3 different genotypes in 2 genogroups; the other 24 animals carried 2 different genogroups or genotypes. Of these 24 animals, an American red squirrel carried a *B. washoensis*–like strain in genogroup A and *B. clarridgeiae*–like strains in genogroup J; 11 animals were infected with *B. elizabethae*–like strains in genogroup I and strains in genogroups B, C, D, E, F, or G, and the remaining 12 carried different genotypes in the same genogroup (Table 2).

Discussion

We report prevalence of bartonellae in exotic small mammals imported into Japan as pets. We found that 26.0% (142/546) of the animals examined had bartonellae in their blood. Prevalence among wild captive animal species was high (37.3%), significantly higher ($p<0.001$) than that among animals from breeder facilities. Of the 179 animals (representing 9 species) from breeder facilities, only 5 Siberian chipmunks imported from a Chinese breeder were found to carry bartonellae, and these were of the same genotype as bartonellae from wild captive animals. These results suggest that animals in breeder facilities may be maintained under hygienic conditions from birth to export, so they rarely have contact with wild animals or blood-sucking arthropod vectors.

Table 2. Multiple infection of different *Bartonella* genotypes in exotic small mammals imported into Japan as pets, June 2004–October 2007

Host	No. animals	GenBank accession nos. of the isolates in 9 genogroups*								
		A	B	C	D	E	F	G	I	J
Daurian ground squirrel	1	4962 4963								
Siberian chipmunk	1	4965 4966								
	1	4964 4965								
Tricolored squirrel	2			4977					4995	
	1			4977					4996	
American red squirrel	1	4971								5006
Southern flying squirrel	1	4972 4973								
Columbian ground squirrel	1	4957 4958								
Richardson's ground squirrel	2	4959 4960								
	1	4954 4959								
	1	4954 4955								
Fat-tailed gerbil	3				4978				5003	
Golden spiny mouse	1					4979			4998	
Fat sand rat	1						4984		5002	
Bushy-tailed jird	1						4988 4989			
	1							4991	5004	
	1						4987 4989			
Large Egyptian gerbil	1						4981		5001	
Greater Egyptian jerboa	1		4975 4976							
	1						4986		5005	
Lesser Egyptian jerboa	1						4986 4985		5005	

*GenBank accession numbers all begin with AB44 and are abbreviated to the last 4 digits; e.g., AB444962 appears as 4962.

Most isolates from animals in the family Sciuridae (58.7%; 122/208) were in genogroup A and showed high sequence similarity to *B. washoensis*. Kosoy et al. (*9*) have reported that *B. washoensis* is widely distributed in ground squirrels in the western part of the United States and that it was isolated from a human with myocarditis in Nevada, USA. Thus, captive squirrels carrying *B. washoensis*–like organisms could serve as a source of infection for humans.

Animals in the family Sciuridae were also found to be carrying several genotypes of bartonellae in genogroups C, *B. grahamii*–like strains in genogroup H, *B. elizabethae*–like strains in genogroup I, and *B. clarridgeiae*–like strains in genogroup J. The sequence similarities between the genotypes and the related *Bartonella* spp. type strains ranged from 98.4% to 98.7% for *B. grahamii*, from 95.5% to 95.8% for *B. elizabethae*, and were 96.2% for *B. clarridgeiae*. In humans, *B. grahamii*, *B. elizabethae*, and *B. clarridgeiae* have been reported to cause neuroretinitis (*11*), endocarditis (*10*), and cat-scratch disease (*31*), respectively. These findings suggest that exotic squirrels also might be a potential source of *Bartonella* infections in humans. Although *B. clarridgeiae* has mainly been isolated from cats (*1*), *B. clarridgeiae*–like strains were isolated from an American red squirrel in this study. *B. clarridgeiae*–like organisms have also been isolated from yellow-necked mice (*Apodemus flavicollis*) in Sweden (*14*) and Greece (*15*).

The sequence similarity of the *gltA* sequence (312 bp) of the *B. clarridgeiae*–like genotype isolated in our study to that of the strain isolated from the yellow-necked mouse (AF391788) was relatively high (97.7%). Recently, *B. rochalimae*, a *B. clarridgeiae*–like organism, was isolated from a human patient with bacteremia, fever, and splenomegaly (*32*). The *B. clarridgeiae*–like strain from the American red squirrel in this study also showed high *gltA* sequence similarity (96.8%) with that of *B. rochalimae* strain BMGH. Studies will be required to clarify the pathogenicity of *B. clarridgeiae*–like organisms for humans. Such studies would include 1) evaluation of the organisms' ability to invade human erythrocytes and/or endothelial cells, 2) demonstration of the presence and expression of the genes of type 4 secretion systems (VirB/VirD4 or Vbh) and Trw, and 3) comparisons of the entire genome sequences of the organisms and with those of other human pathogenic *Bartonella* spp.

In this study, *Bartonella* genogroups D, E, and G were isolated from animals in the family Muridae, and *Bartonella* genogroup B was isolated from animals in the family Dipodidae. These findings suggest strict host specificity between the strains in these genogroups and the host animal family. However, findings also showed wide host species diversity; strains in genogroup F were isolated from 6 animal species, and strains from genogroup I (*B. elizabethae*–like) were isolated from 9 animal species. *Bartonella* strains in genogroup F were isolated from animals in the families Muridae and Dipodidae. Genogroup I (*B. elizabethae*–like) strains were also isolated from animals in the family Sciuridae. *B. elizabethae* has been isolated from different animal species, e.g., a human patient and genus *Rattus* rats (*5*,*10*), and *B. elizabethae* DNA has been isolated from a dog (*33*). In our study, 3 *Bartonella* isolates from a Cairo spiny mouse imported from Egypt had an identical *gltA* sequence to that of the *B. elizabethae* type strain. Thus, some *Bartonella* spp., such as *B. elizabethae* and *B. washoensis*, infect host animals in diverse families and may have zoonotic potential.

In the present study, 17.6% (25/142) of exotic animals were infected with different *Bartonella* genotypes or genogroups. In particular, 3 isolates from a greater Egyptian jerboa were classified in 3 different genotypes. Of the 25 *Bartonella*-positive animals, 13 showed co-infection with different *Bartonella* genogroups. Of these 13 animals, 12 carried *B. elizabethae*–like strains in genogroup I. In contrast, strains with identical *gltA* sequences were isolated from 2 different animal species, such as greater Egyptian jerboas and lesser Egyptian jerboas, Siberian chipmunks and Hokkaido squirrels, and fat-tailed gerbils and fat sand rats. These findings suggest that some *Bartonella* species have a wide host range and may be transmitted horizontally by some blood-sucking arthropod vectors with low host specificity.

In summary, we examined the possibility that exotic small mammals may be reservoirs of zoonotic *Bartonella* spp. around the world. The animals in this study carried, at high prevalence, several *Bartonella* spp. that are human pathogens. Novel species were suggested by the fact that some of the genotypes in 6 genogroups (B to G) showed relatively low similarity (<94.6%) to known *Bartonella* spp. and formed independent clusters according to phylogenetic analysis based on partial *gltA* sequences. More taxonomic studies should sequence other housekeeping genes, such as *rpoB*, 16S rRNA, *ftsZ*, *groEL*, and *ribC,* to confirm whether these isolates are novel *Bartonella* spp. (*34*). To prevent human infections by *Bartonella* spp. carried by exotic small mammals, a quarantine system for these animals should be established as early as possible. Further studies will be necessary to clarify the route of transmission among exotic small mammals and to evaluate the pathogenicity for humans and animals of the isolates belonging to novel *Bartonella* genotypes found in this study.

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Enhancing Time-Series Detection Algorithms for Automated Biosurveillance

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BioSense is a US national system that uses data from health information systems for automated disease surveillance. We studied 4 time-series algorithm modifications designed to improve sensitivity for detecting artificially added data. To test these modified algorithms, we used reports of daily syndrome visits from 308 Department of Defense (DoD) facilities and 340 hospital emergency departments (EDs). At a constant alert rate of 1%, sensitivity was improved for both datasets by using a minimum standard deviation (SD) of 1.0, a 14–28 day baseline duration for calculating mean and SD, and an adjustment for total clinic visits as a surrogate denominator. Stratifying baseline days into weekdays versus weekends to account for day-of-week effects increased sensitivity for the DoD data but not for the ED data. These enhanced methods may increase sensitivity without increasing the alert rate and may improve the ability to detect outbreaks by using automated surveillance system data.

Since the late 1990s, the threats of bioterrorist attacks, the potential for outbreaks of natural disease such as severe acute respiratory syndrome and pandemic influenza, and the availability of computerized data have prompted the use of automated disease surveillance systems (*1*). Sources of information include clinical data, such as records of hospital emergency department visits, and nonclinical information, such as sales of over-the-counter remedies (*2*). However, human resources are limited for interpreting the large volume of available information. Thus, statistical algorithms are needed to filter large volumes of data, focus attention on potential public health problems, and provide an objective measure of increases in disease activity.

BioSense is a US national automated surveillance system that receives data from various sources and makes them available for public health use. The data may be viewed simultaneously by local, state, and federal public health officials through the Internet-based BioSense Application, which may be accessed on a jurisdiction-specific basis through the Centers for Disease Control and Prevention (CDC) Secure Data Network (*3*). Data received include coded final diagnoses and free-text chief complaints, which are assigned as appropriate to ≥1 of 11 syndrome groupings representing general illness categories such as respiratory and gastrointestinal illnesses (*4*) and to ≥1 of 78 subsyndromes representing more specific categories such as asthma or cough (*5*). To identify days when disease indicator activity is higher than expected, BioSense uses a modified version of the C2 algorithm, 1 of 3 algorithms (C1, C2, and C3) developed for the Early Aberration Reporting System (EARS) (*6,7*).

The C2 algorithm uses a sliding baseline of 7 consecutive recent days' counts to calculate a mean (μ) and SD (s_t). The test statistic is $(x_t - \mu)/s_t$, the number of SDs by which the current value x_t exceeds μ, or 0 if x_t does not exceed μ. EARS uses a test statistic ≥3 to signal an alert (*6,7*). Owing to their simplicity, ease of implementation, and implicit correction for seasonal trends (only data from the prior 9 days are used), the EARS algorithms are widely used (*8–10*). However, the algorithms do not perform optimally under all circumstances. First, because daily counts often vary by day of week, many alerts may be produced on high-count days such as Mondays and Tuesdays, and few

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may be produced on low-count days such as weekend days. Second, the short (7-day) baseline period may produce unstable values for the mean and SD; thus, the minimum daily count that triggers an alert may vary widely over a short period. Third, using simple count data does not account for the population at risk, which is generally unknown in these systems and which may vary, especially during crisis situations. Although C2 can be used on rates rather than counts, prior evaluations have not shown that using rates improves performance (L. Hutwagner, pers. comm.). Finally, occurrences of many disease indicators are rare, resulting in calculations for both expected values and SDs of 0; the EARS methods are not recommended in such instances. A minimum SD may be used to avoid division by zero, but if this minimum value is set to 0.2, a count of 1 will be 5 SDs above the mean and trigger a high-level alert.

This article describes and evaluates modifications of C2 that retain its inherent advantages, address its potential limitations, and improve its performance. We used real daily syndrome counts from 2 sources as baseline data and assessed the ability of various algorithms to detect additional counts artificially added to the data. Because all analyses were conducted at a constant alert rate of 1%, improvements in sensitivity were not accompanied by an increase in alerts.

Methods

Four algorithm modifications, designed to address shortcomings in the C2 algorithm, were tested. The first modification tested was stratification by weekdays versus weekend days. Although many methods have been used to adjust for differing counts by day of week (*11*), these methods may require customization to specific datasets and a long data history (up to several years). Our simple method is to stratify the baseline days used to calculate μ and s_t into weekdays versus weekend days. This stratification is denoted the W2 algorithm. For example, a 7-day W2 baseline for weekdays contains the most recent 7 weekdays. For unstratified and stratified analyses, the 2 days immediately before the index day were excluded from the baseline, a standard practice for C2, to avoid contamination with the upswing of an outbreak.

The second modification tested was lengthening the baseline period. Because a 7-day period may provide insufficient data for an accurate and stable calculation of μ and s_t, we tested baseline periods of 7, 14, and 28 days. However, because we used data from $\leq$56 days before the index day, the stratified 28-day baseline will include only $\approx$16 days for weekend days.

The third modification tested was adjustment for total daily visits. For the adjustment procedure, we used a formula in which n_0 = count of visits on the index day for the chosen syndrome (e.g., visits for the respiratory syndrome), and d_0 = the total number of facility visits on the index day, including visits that were both assigned and unassigned to any of the 11 syndromes. Σn_i = total syndrome visits summed for all i baseline days. Σd_i = total facility visits summed for all i baseline days. The formula for the adjusted expected value was $e_0 = d_0 \times \Sigma n_i/\Sigma d_i$, which differed considerably from the mean of the n_i if d_0 was high or low. Fewer visits for a given syndrome were thus expected on a day when the facility had fewer total visits. The estimated adjusted SD, s_0, was taken as the mean absolute value of $(n_i - d_i \times \Sigma n_i/\Sigma d_i)$ over i baseline days; that is, $s_0 = \Sigma$ (abs $(n_i - d_i \times \Sigma n_i/\Sigma d_i))/i$. The test statistic adjusted for total visits was $(n_0 - e_0)/s_0$, analogous to the C2 statistic $(n_0 - \mu)/s_t$, where μ and s_t are the mean and SD of n_i, the counts on baseline days. In the discussion below, we refer to this adjustment as the rate algorithm.

The fourth modification tested was increased minimum value for SD. We studied minimum values of 0.2 and 1.0.

To test these modifications, 2 datasets were used: records of Department of Defense (DoD) facility final diagnoses for September 2004–November 2007 and records of hospital emergency department (ED) chief complaints for March 2006–November 2007. The DoD data consisted primarily of data from outpatient clinics; however, $\approx$15% of the visits in this evaluation were from patients seen in emergency facilities and cannot currently be differentiated in the BioSense System. We studied the 11 syndrome groups designed to be indicative of infections resulting from exposure to pathogens plausibly used in a bioterrorist attack (*4*). The DoD data consisted of daily counts of patient visits with International Classification of Diseases, 9th Revision (ICD-9)–coded diagnoses categorized into the 11 syndrome groups. The hospital ED data consisted of free-text chief complaints, which were first parsed for a specified set of keywords, abbreviations, and misspellings and then categorized into 10 of the syndrome groups (1 syndrome, specific infection, was used for diagnosis but not for chief complaint data). Some ICD-9 codes and chief complaints may be included in $\geq$2 syndromes. However, counts of different syndromes were analyzed separately, not added together, and therefore are not double-counted in the analyses. For both datasets, we analyzed counts aggregated by facility. We included facility-syndrome combinations that had mean counts $\geq$0.5 over all facility–syndrome days in the study period. Many DoD clinics are closed on holidays. Therefore, for the DoD data, 11 days (days on which federal holidays are observed and the day after Thanksgiving) were recoded as weekend days for purposes of stratified algorithm calculations (*5*). Because hospital EDs typically are open on these holidays, no recoding for holidays was performed for this dataset.

The mean count for each facility syndrome was calculated and categorized as follows: 0.5 to <2, 2 to <4, 4 to <6,

6 to <8, 8 to <10, 10 to <20, 20 to <40, and ≥40. Empirical distributions of the test statistic (e.g., number of SDs by which the observed count exceeds the expected value) were conducted separately for each dataset, algorithm, and mean count category; the 99th percentile value for each of these distributions was used as the cutoff value to define an alert rate of 1%. For example, for the standard C2 algorithm in DoD data with mean count 4 to <6, a cutoff value of 3.9 was used because 1% of the facility-syndrome days had a test statistic ≥3.9. Because no attempt was made to find and exclude real outbreaks from the data, these cutoff values define an alert rate rather than a false alert rate, the latter being equivalent to 1-specificity (*12*).

At a constant alert rate of 1% for all methods, the sensitivity for detecting additional counts was calculated by performing the following steps: 1) running the algorithm to determine expected values and SDs for each facility-syndrome-day; 2) finding the 99th percentile cutoff value for the test statistic for each dataset-algorithm-mean count category as explained above; 3) for each facility-syndrome day, determining whether the observed count plus additional counts is greater than or equal to the threshold value (threshold value = expected value + SD × 99th percentile cutoff value); and 4) calculating sensitivity as the percentage of days on which the additional counts would exceed the threshold value and therefore be detected. Using this method, a single computer run can calculate sensitivity for detecting single-day additional counts on all days in the dataset; if the additional counts are spread over multiple days, separate computer runs would be needed (*7*).

Results

The DoD diagnosis data contained 1,939,993 facility–syndrome days from 308 facilities in 48 states with an overall mean of 7.7 counts per facility per day; of the 11 syndromes, respiratory visits comprised the highest percentage (16% of total facility–syndrome days) and had the highest mean count (26.0 visits per facility per day) (Table 1). The hospital ED data contained 768,195 facility–syndrome days from 340 facilities in 21 states and had an overall mean of 7.8 counts per facility per day; no visits for lymphadenitis and severe injury and death were included because no facilities had a mean count >0.5 per day for these syndromes.

The DoD data had a strong day-of-week effect; 16%–21% of total weekly visits occurred per day on weekdays, and only 3%–4% of visits occurred per day on weekend days and holidays (Figure 1). The hospital ED data had a minimal day-of-week effect: 14%–16% of visits occurred per day on weekdays, and 14%–15% of visits occurred per day on weekend days.

The accuracy of expected value calculation was evaluated by using mean absolute residuals. For lower residuals, expected values are closer to observed values than they are for higher residuals. Similarly, the expected value calculation is more accurate for lower residuals than for higher residuals. For the DoD data, lower residuals were seen with stratification (W2) and the rate algorithm: mean residual 4.2 for unstratified count algorithm versus 2.2 for stratified rate algorithm (Table 2). For the hospital ED data, residuals were lower for the rate algorithm, and stratification had a minimal effect. Varying the baseline duration and minimum SD had no effect on the accuracy of expected value calculation (data not shown).

The effect of modifications of the initial algorithm on the sensitivity for detecting additional counts was examined; each modification was added consecutively (Table 3). For the DoD data, sensitivity was 40.6% for the initial algorithm and increased to 43.9% when the rate method was used; 70.8% when the minimum SD was increased to 1.0; 79.4% when the baseline duration was increased to 28 days; and 82.0% when a stratified baseline was used. Comparing the initial algoithm to the best algorithm showed a

Table 1. Distribution of hospital emergency department visits and mean count per day, by syndrome and dataset, for selected BioSense data used in algorithm modification study*

	Department of Defense clinic diagnosis		Hospital emergency department chief complaint	
Syndrome	Mean count/d	% Facility–syndrome days	Mean count/d	% Facility–syndrome days
Botulism-like	2.5	3.8	0.9	1.8
Fever	4.4	10.1	6.3	14.3
Gastrointestinal	8.9	13.7	14.5	14.7
Hemorrhagic	2.2	5.7	2.6	13.6
Localized cutaneous lesion	3.0	10.8	2.6	13.2
Lymphadenitits	1.1	4.8	NA	0†
Neurologic	3.6	10.6	5.2	14.4
Rash	4.3	11.2	2.2	13.1
Respiratory	26.0	16.0	20.0	14.7
Severe injury and death	2.2	2.6	NA	0†
Specific infection	3.2	10.7	NA‡	0‡
All	7.7	100	7.8	100

*NA, not applicable.
†Facilities were not included because none had mean counts ≥0.5 for syndromes.
‡Chief complaint data are not assigned to this syndrome.

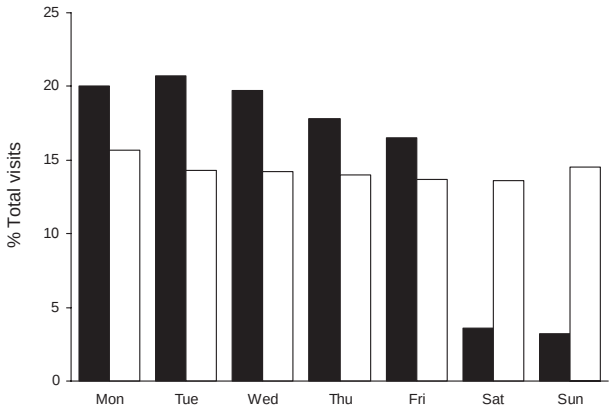

Figure 1. Distribution of syndrome counts, by day of week and data source, for selected BioSense data used in algorithm modification study. Black bars show Department of Defense data, and white bars show hospital emergency department data.

41.4% increase in sensitivity. For the hospital ED data, sensitivity was 40.2% for the initial algorithm and increased to 64.8% for the best method (minimum SD = 1, 28-day baseline, rate method, unstratified baseline); however, when the stratified baseline was used, sensitivity decreased to 62.1%; the initial algorithm compared with the best algorithm showed a 24.6% increase in sensitivity. When these sensitivity calculations were stratified by mean count for each facility-syndrome (data not shown), we found that the modifications increased sensitivity in all strata of the DoD data; for the hospital ED data, the rate method reduced sensitivity by 1.0% in the 8 to <10 count category and by 0.5% in the 10 to <20 count category, but increased sensitivity in other categories and overall.

When we limited analysis to ED data with a mean count of 4 to <6 per day and explored sensitivity for detecting varying numbers of additional counts (Figure 2), we found, as expected, that as the number of additional counts increased, sensitivity increased. The difference between the initial and best algorithms was highest when sensitivity was ≈50% for the initial algorithm. That is, for 10 additional counts, sensitivity was 49.8% for the initial algorithm and 85.3% for the best algorithm, an improvement of 35.5%. However, if the initial C2 algorithm had either low or high sensitivity, the modifications had little effect.

As an example, we analyzed fever syndrome data from 1 ED. The mean count was 4.9 per day, and the 99th percentile threshold values were 3.86 SDs for the initial and 3.55 for the best algorithm. Over 632 days, the sensitivity for detecting 8 additional counts was 47.2% for the initial and 70.9% for the best algorithm (23.7% difference). Data for a 2-month period showed that the calculated SD (Figure 3, panel A) and the threshold value (i.e., count needed to trigger an alert; Figure 3, panel B) varied substantially for the initial algorithm but were comparatively stable for the best algorithm. During the 2-month period, 8 additional counts would be detected by initial and best algorithms on 30 days, by only the initial algorithm on 2 days, and by only the best algorithm on 19 days; neither algorithm detected the additional counts on 10 days (Figure 3, panel C).

Discussion

Our results demonstrate that simple modifications of the widely used C2 algorithm can substantially improve the ability to accurately recognize 1-day increases in disease syndrome activity. Depending on the dataset, mean count in the data, and the number of additional counts added, the enhanced methods may increase sensitivity by 20%–40%. These improvements were achieved without an increase in the alert rate, which was held constant at 1% for all methods. Although we chose a 1% alert rate for testing purposes, in practice, it is useful to vary the alert rate to fit the circumstances, and the BioSense application enables the alert rate to be varied between 0.1% and 2%. Regardless of the alert rate used, the modified methods have higher sensitivity. For the DoD and hospital ED datasets, sensitivity was improved by using a higher minimum SD of 1.0, a longer baseline duration of 28 days, and adjusting for total visits. Stratifying baseline days into weekdays versus weekends/holidays increased sensitivity in the DoD data, which has a strong day-of-week effect, but modestly decreased sensitivity in the hospital ED data, which does not have such an effect. Thus, the best analytic methods depend on dataset characteristics, especially the day-of-week effect, and could be varied by manual or automated selection. These findings can be used to improve both early event detection and situation awareness because accurate recognition of unusually high counts is needed for both uses.

These modifications were apparently effective for the following reasons. Accounting for total visits to the facility

Table 2. Mean absolute residual, by method and dataset, for selected BioSense data used in algorithm modification study*

Stratification of baseline by weekday vs. weekend	Mean absolute residual			
	Department of Defense		Hospital emergency department	
	Count	Rate	Count	Rate
Unstratified	4.2	2.4	2.2	2.0
Stratified	2.4	2.2	2.3	2.0

*The count method uses only numerator data; the rate method uses numerator and denominator data. Because varying the baseline duration did not affect residuals (data not shown), all calculations shown here use a baseline duration of 7 days.

Table 3. Sensitivity for detection of additional counts, by method and dataset, for selected BioSense data used in algorithm modification study*

Minimum SD	Stratified baseline	Baseline duration, d	Sensitivity			
			Department of Defense		Hospital emergency department	
			Count	Rate	Count	Rate
0.2	No	7	40.6†	43.9	40.2†	39.1
1.0	No	7	52.3	70.8	50.4	53.6
1.0	No	14	58.6	76.8	58.7	60.9
1.0	No	28	62.0	79.4	62.8	64.8‡
1.0	Yes	7	64.9	75.7	50.2	53.8
1.0	Yes	14	75.1	80.4	57.6	60.1
1.0	Yes	28	77.0	82.0‡	60.5	62.1

*All facility–syndrome days were included in calculations. The number of additional counts varied according to categories of average count for each facility–syndrome (0.5–<2, 2–<4, 4–<6, 6–<8, 8–<10, 10–<20, 20–<40, and ≥40) to produce 40% sensitivity for the initial method. For the Department of Defense, the additional counts were 5.0, 9.1, 11.7, 13.6, 16.0, 20.9, 30.4, and 40.0 for the average count categories, respectively. For the hospital emergency departments, the additional counts were 4.3, 6.3, 8.2, 9.5, 10.4, 12.9, 18.7, and 28.2, respectively.
†Initial method.
‡Best method for the dataset.

(i.e., rate method) produces a more accurate expected value and lower residuals (Table 2). Although number of total visits is not the ideal denominator, in general it is better than no denominator at all. An advantage of the rate method is that calculations may be made when only partial data for a given day are available. However, adjusting for total visits may reduce sensitivity slightly in some subgroups, as we found for the hospital ED data when the mean count was 8 to <20. Stratification by weekday versus weekend day improves expected value calculations when a substantial day-of-week effect exists, such as in the DoD data. When such an effect is not present, stratification causes days further from the index day to be used in the baseline period, therefore producing slightly less accurate expected values. Longer baseline durations have no effect on the accuracy of expected value calculation and improve sensitivity by producing more accurate and stable SD values. Using a higher minimum SD avoids nuisance alerts that may be prompted by small fluctuations in the daily visit count. This method also changes the distribution of test statistic values, which results in a lower 99th percentile cutoff value, which increases sensitivity for detecting moderate-to-high numbers of added counts. Using a higher minimum SD is beneficial if disease indicators with low and high counts are analyzed; an alternate approach is to use different methods for low- versus high-count data.

The issues focused on by our suggested modifications may alternately be addressed by various sophisticated mathematical modeling approaches. However, health departments, which are generally limited in resources and in analysis expertise, may resist use of decision-support methods that are expensive, difficult to implement, or not transparent to human data monitors. For example, sophisticated Serfling-type regression models have long been used by CDC for tracking the progress of influenza season (*13*,*14*) and have been used to analyze selected data in the BioSense system. However, these models have both strengths and weaknesses and have not been widely embraced for daily disease surveillance. Even if the expertise and hardware capability for applying them were made available to local health departments, many time series are unsuitable for this approach. We present simple and easily understood and implemented enhancements to C2 to extend its applicability and improve its performance. These enhancements may be applicable to other control chart-based algorithms as well.

Automated surveillance systems based on chief complaints and diagnoses have a number of uses: providing assistance in data collection; monitoring seasonal influenza (*15*); monitoring total ED visits during a crisis; and monitoring simple surrogates of infectious diseases, injuries, and chronic diseases during large outbreaks or disasters (*16*). The utility of these systems has not been demonstrated for

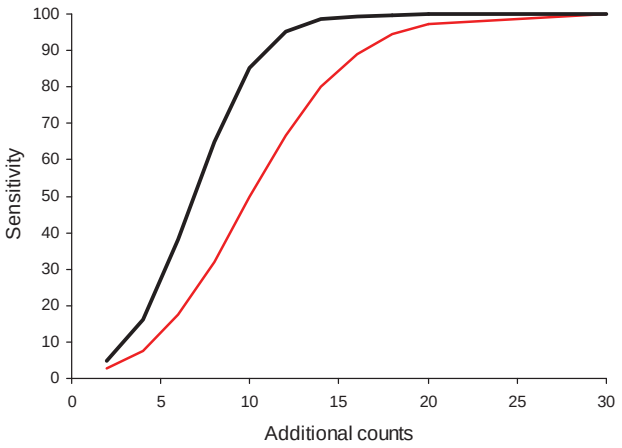

Figure 2. Sensitivity of detecting various numbers of additional counts, by using initial versus best algorithms for hospital emergency department chief complaint data, for selected BioSense data. Red line shows the initial algorithm (minimum SD = 0.2, 7-day baseline, count method, unstratified baseline), and black line shows the best algorithm (minimum SD = 1.0, 28-day baseline, rate method, unstratified baseline).

monitoring small- or intermediate-sized outbreaks or illnesses defined primarily by laboratory testing. Even when using these suggested modifications, sensitivity for detecting additional counts at the facility level remains modest. However, the utility of automated biosurveillance will be expanded with the availability of better population coverage and more specific data, the use of multiple data types in combination, and improved detection algorithms, such as those proposed here.

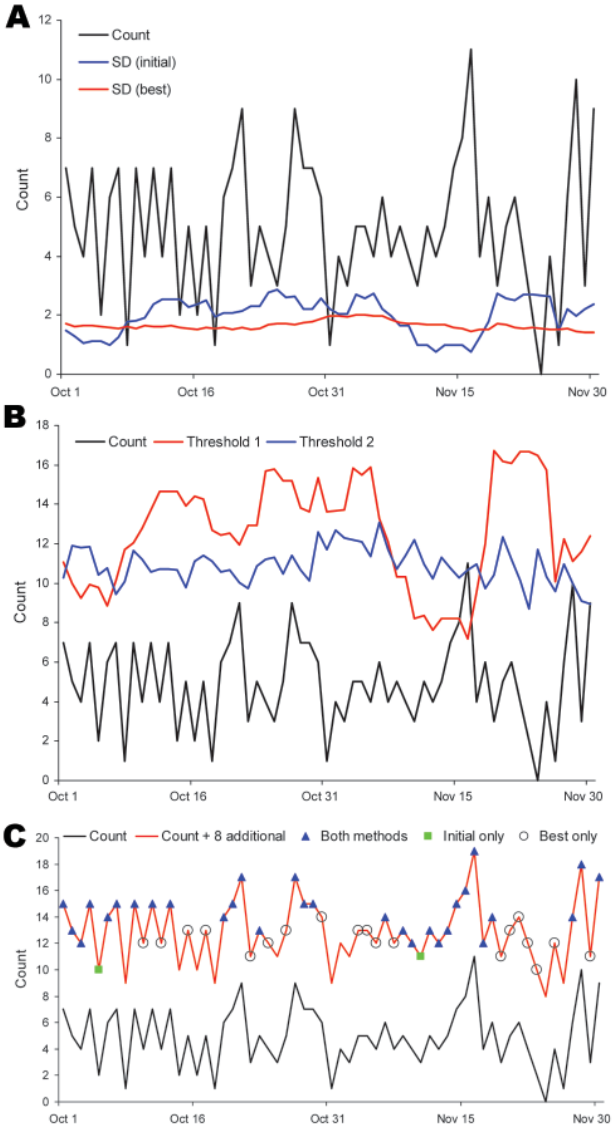

Figure 3. Comparison of initial versus best algorithms for analysis of fever syndrome data at an example emergency department, October–November 2006. A) SD comparison. Count, fever syndrome counts; SD (initial), SD by using initial algorithm (minimum SD = 0.2, 7-day baseline, count method, unstratified baseline); SD (best), SD by using best algorithm (minimum SD = 1.0, 28-day baseline, rate method, unstratified baseline). B) Count threshold comparison. Count, fever syndrome counts; threshold 1, minimum count needed to trigger an alert by using initial method; threshold 2, minimum count needed to trigger an alert by using best method (for the best algorithm, which accounts for rate, 8 counts were added to total visits for calculating the threshold). C) Detection of 8 additional counts. Count, daily fever syndrome counts; count + 8, daily count plus 8 counts; both methods, 30 days with the additional counts detected by both the initial and best methods; initial only, 2 days with the additional counts detected by using initial method only; and best only, 19 days with additional counts detected by using best method only.

The limitations of this study include using only data with a mean count ≥0.5 per day; analyses of sparser data might show different results. We studied only facility-level aggregation of data, selected patient types (e.g., hospital inpatients were not studied), selected data types (e.g., ED diagnoses were not studied), and broadly defined syndromes (the more granular subsyndromes, which are likely to yield lower counts, were not studied). Although we evaluated only a simple time-series detection method, optimizing performance of simple methods is useful before they can be meaningfully compared with more sophisticated methods, such as regression. Also, we studied effects of additional counts on single days rather than multiday outbreak effects; however, because the C2 algorithm considers data from only 1 day at a time, this is a reasonable initial approach. These results must be confirmed by trials of multiday signal injection and performance evaluated for multiple subgroups (e.g., syndrome, day of week, season). We adopted the approach of evaluating sensitivity at a fixed 1% alert rate defined empirically for each algorithm and dataset, as used by Jackson et al. (*12*). Our approach is in accord with a recent review that recommended basing alert thresholds on empirical data rather than on classical statistical theory (*17*). A major strength of the study is that BioSense is a national system that provided access to 2 major datasets with differing characteristics and to data from hundreds of facilities in many states. The length, geographic spread, and syndrome variation of the study datasets lend weight to the results.

The field of electronic biosurveillance is in its infancy and is rapidly changing. Early work focused on attempts to detect outbreaks (early event detection) by using broadly defined syndromes (e.g., respiratory syndrome) based on chief complaints and diagnoses. Emphasis has recently shifted to monitoring for ongoing outbreaks (situational awareness) and for specific disease indicators (e.g., cough, dyspnea) called subsyndromes. The field is now beginning to develop methods for case-based surveillance (i.e., automated application of a formal case definition using computerized data) (*18*). Each data type and disease indicator may have unique characteristics that require modifications of standard data analysis methods. However, because the adaptation of time-series methods to recognize outbreaks will be an ongoing need, the enhanced methods identified by this study are likely to have lasting usefulness.

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Animal Reservoir Hosts and Fish-borne Zoonotic Trematode Infections on Fish Farms, Vietnam

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Fish-borne zoonotic trematodes (FZT) pose a risk to human food safety and health and may cause substantial economic losses in the aquaculture industry. In Nghe An Province, Vietnam, low prevalence of FZT for fish farmers but high prevalence for fish indicate that reservoir hosts other than humans may play a role in sustaining transmission. To determine whether domestic animals may be reservoir hosts, we assessed prevalence and species composition of FZT infections in dogs, cats, and pigs in a fish-farming community in Vietnam. Feces from 35 cats, 80 dogs, and 114 pigs contained small trematode eggs at 48.6%, 35.0%, and 14.4%, respectively; 7 species of adult FZT were recovered from these hosts. These results, combined with data from previous investigations in this community, imply that domestic animals serve as reservoir hosts for FZT and therefore must be included in any control programs to prevent FZT infection in humans.

In Asia, fish-borne zoonotic trematodes (FZT), including liver and intestinal flukes, are widely reported (*1*–*3*). FZT not only pose risks to food safety and human health but also may cause substantial economic losses in the aquaculture industry, resulting from to restrictions on exports and reduced consumer demand because of food safety concerns (*4*). A range of mammals and birds serve as definitive hosts for FZT (*2*). Although information on infection levels and species distribution in humans and fish is becoming increasingly available, similar information for reservoir hosts such as wild and domestic animals and fish-eating birds is scarce (*1*).

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Recent studies conducted in Nghe An Province, a major area for freshwater aquaculture in Vietnam, found prevalence of FZT in humans to be low (0.6%) and prevalence in fish from farms to be high (>35%) (*5*,*6*). These findings suggest that reservoir hosts other than humans play a major role in sustaining transmission of FZT in this community. We therefore investigated the role of the domestic animals on these fish farms. We determined prevalence and species composition of FZT infections in dogs, cats, and pigs in the community and analyzed potential risk factors for the transmission of FZT to animals and animals' role in sustaining FZT infections in cultured fish.

Materials and Methods

Study Design, Sampling, and Laboratory Analysis

The study was conducted in November 2005 in Nghe An Province, northern Vietnam (Figure 1), in fish-farming households previously investigated for human and fish FZT infections (*5*,*6*). From a total of 1,281 households, 50 were randomly selected in proportion to farm numbers in 5 districts: Hung Nguyen (n = 8), Nam Dan (n = 15), Yen Thanh (n = 9), Thanh Chuong (n = 10), and Tan Ky (n = 8). Another fish farm previously found to have cases of FZT in humans (*5*) was included, yielding a total of 51 fish-farming households in the study. Before the study, all farmers were informed about the study (objectives, risks, rights, and benefits) and asked for consent. Permission to conduct the study was obtained from National Institute of Veterinary Research.

Fecal samples were collected from every animal in the selected households: 80 dogs, 35 cats, and 114 pigs. Animals that were <2 months of age or pregnant were excluded. Fecal samples were collected from the rectums of dogs and pigs and from the cages of cats that had been confined

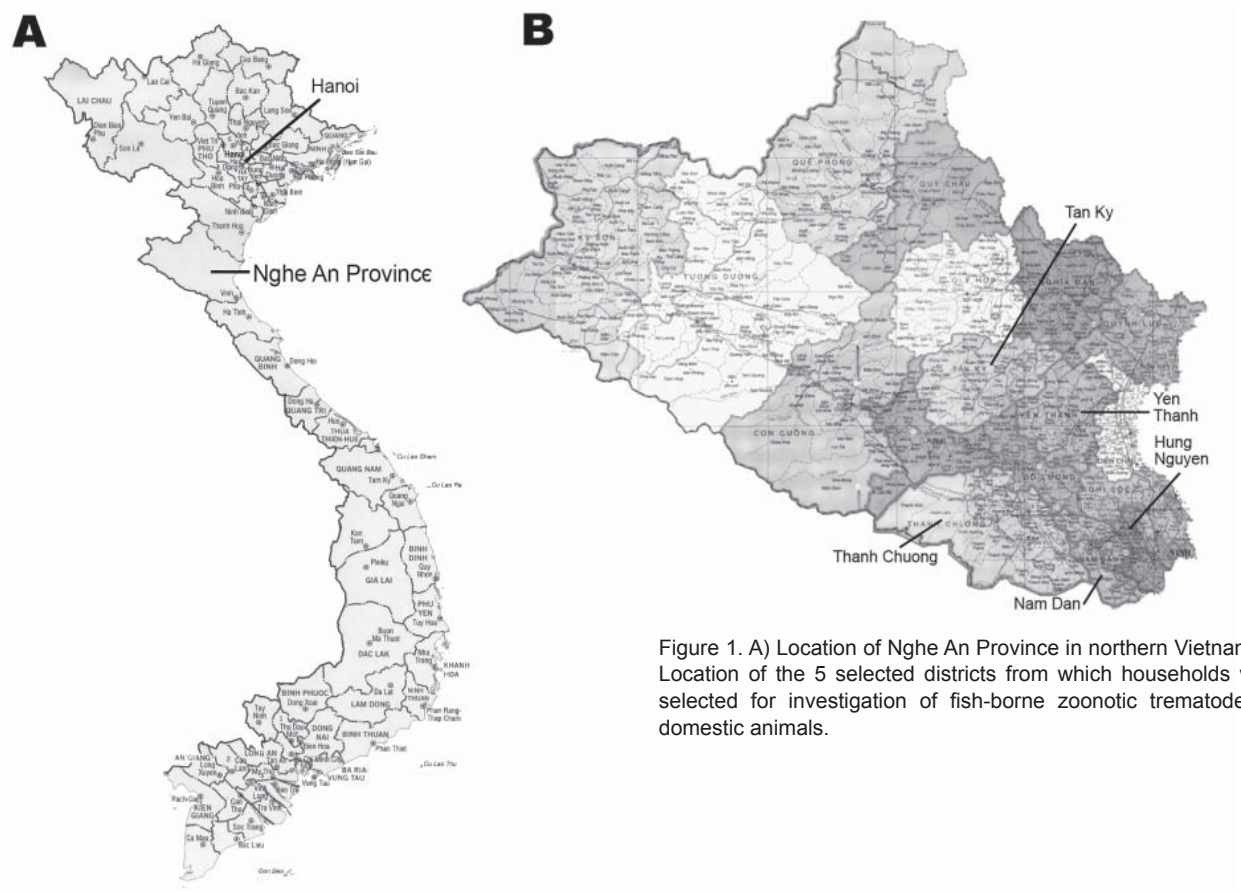

Figure 1. A) Location of Nghe An Province in northern Vietnam. B) Location of the 5 selected districts from which households were selected for investigation of fish-borne zoonotic trematodes in domestic animals.

overnight. Samples were stored in coolers and transferred to the laboratory, where 1–2 mL of 10% formalin was added and the samples were kept refrigerated until examination within 6 weeks. A standard questionnaire was used to interview each animal's owner about the behavior of the animals and about animal husbandry practices relevant to transmission of FZT.

Fecal samples (5 g each) were examined by a combined filtration, sedimentation, and centrifugation method described by Willingham et al. (*7*) and Anh et al. (*8*). For each sample, trematode eggs were counted 3 times, the sum of which was equivalent to eggs per gram (epg). All eggs <50 μm were designated "small trematode eggs" (*3*). Data on prevalence and species of metacercaria in fish were obtained from Chi et al. (*6*). Human prevalence data, collected according to the Kato-Katz method, were obtained from Olsen et al. (*5*).

Worm Recovery and Identification

Among the small trematode egg–positive animals, 27 dogs, 18 cats, and 5 pigs were randomly selected for necropsy and adult worm recovery from the liver and small intestine. The animals were housed and handled in accordance with national standards of experimental animal care. Dogs and pigs were anesthetized by intramuscular injection of xylazine and subsequently killed by an intravenous overdose of ketamine (Troy Laboratories Pty.Td, Smithfield, New South Wales, Australia). Cats were killed by an intramuscular overdose of ketamine. The livers were removed and cut open along the main tributaries of the biliary duct, and trematodes were collected and placed in a Petri dish with saline. The liver was subsequently cut into small, thin pieces and placed in saline solution for 10 min, after which the liver tissue was crushed and worms were isolated by filtration of the solution through a tea strainer. The contents of the small intestines were flushed into a bucket by tap water, then filtered through a tea strainer and sieve (mesh size 400 μm). The sediment remaining on the sieve was washed into a Petri dish and examined for intestinal flukes under a stereomicroscope. After 30 min, the sediment in the wash water was also examined for flukes. To recover the remaining flukes, we cut the intestines into small pieces and placed them in a bucket with warm saline (90°C) for 1 h. The bucket fluid was poured into conical flasks and allowed to settle for 30 min before the final sediment was examined in a Petri dish under a stereomicroscope. All isolated flukes were collected by pipette and preserved in saline (90°C) before being pooled in 1 flask and counted.

Worms were preserved with 5% formalin in Eppendorf tubes; when high worm loads were isolated, a subsample was preserved in 70% ethanol for later analysis by PCR. As many as 40 formalin-preserved flukes per animal were stained, mounted on slides, and identified to species level according to published taxonomic references (*9*,*10*).

Relative Transmission Index

We determined total daily eggs excreted (TDEE) for each animal species and for humans by multiplying 4 factors: number of animals and humans in the districts, FZT prevalence for animals and humans, mean epg in feces, and amount of feces excreted per day. A relative transmission index (RTI) was used to assess the potential contribution of animals and humans to FZT transmission. RTI was defined as the proportion of the total daily trematode egg excretion produced by each species and calculated by using the following formula:

RTI = TDEE for each species × 100/TDEE for all species

The estimated amounts of feces defecated daily (mean ± SD) were obtained from Wang et al.: humans 160 ± 58 g, dogs 99 ± 19 g, cats 20 ± 19 g, and pigs 1,516 ± 196 g (*11*). Data on number of persons in the 5 districts were collected from the Vietnam Administrative Atlas (*12*), and data on numbers of domestic animals were collected from local veterinary centers in the districts. Data on prevalence and intensity of the eggs from humans in the same study areas were collected from Olsen at al. (*5*) and from Annette Olsen (pers. comm.), respectively.

Data Analysis

Data from parasitologic examinations were combined with information collected from questionnaires administered to the animals' owners, recorded on an Excel spreadsheet (Microsoft, Redmond, WA, USA), and transferred to SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) for statistical analysis. The outcome variable was FZT infection status of the animals (yes/no). Explanatory variables obtained from the questionnaire were sex of animal (male/female); age group (≤12 months, >12 months); district; animal species (dogs, cats, pigs); free roaming of the animal (never, sometimes, always); farmer fed raw fish to animal (never, sometimes); farmer observed the animal eating raw fish (never, sometimes); farmer observed the animal catching fish from canal or river (yes/no); farmer's practice of feeding dead fish from pond to the animals (yes/no); deworming of the animals (never, one time when animals were young, sometimes if animal was ill, regularly [2 times/year]); place where animals defecate (garden, stable, wherever, on the fish pond bank, in the kitchen with straw); farmer's use of animal feces (rice field, vegetable garden, fish pond, wherever, rice field and fish pond); and composting of feces (never, sometime, always).

For risk factor analysis, univariable and multivariable logistic regression analyses were conducted for 2 subsets of data: all animals and each animal species. Univariable analysis was performed to assess possible risk factors for FZT infection; multivariable logistic regression was used to evaluate the effect of risk factors when adjusting for the effect of other risk factors. Risk factors with $p<0.25$ in univariable analysis were included in the initial multivariable models. Backward elimination was used to include only risk factors with $p<0.05$ in the resulting model. Interaction and possible confounding were checked. Where interaction was found, it was included in the resulting model. Goodness-of-fit was checked by the Hosmer-Lemeshow strategy. Comparisons of prevalence of the infections between animal species were performed by using the Fisher exact test.

Data from parasitologic examination of fish in the same households (*6*) were used to assign codes to households: code 1 (positive) if any fish had FZT metacercariae and code 0 if all fish examined were negative. The Fisher exact test was used to analyze relationships at the household level between the infections in fish and in domestic animals together or between infections in fish and each animal species.

Results

Prevalence and Species Composition of Small Trematodes

The prevalence of small trematode infections, determined by egg counts in fecal samples from 229 animals of 51 households, was 35.0% for dogs, 48.6% for cats, and 14.4% for pigs (Table 1). Prevalence of infection was higher for cats and dogs than for pigs ($p<0.0001$), but no significant difference was found between prevalence of infections for dogs and cats ($p = 0.21$). Intensity (small trematode egg counts; mean ± SD) was highest for cats (66 ± 129 epg), intermediate for dogs (25 ± 73 epg), and lowest for pigs (4 ± 18 epg).

All trematodes recovered from necropsy samples were fishborne-zoonotic intestinal flukes (Table 2). *Haplorchis pumilio* was the most prevalent species in fecal-positive dogs, cats, and pigs, followed by *H. taichui* and *H. yokogawai* (Figure 2).

Association between Risk Factors and Infections

Distributions of dogs, cats, and pigs were comparable in the 5 districts. According to the questionnaire, 100% of cats and 95.0% of dogs were free roaming, whereas 94.7% of pigs were confined. Feeding raw fish to dogs, cats, and pigs was reported by 41.3%, 45.7%, and 36.8% of farm-

Table 1. Small trematode infections in domestic animals, Nghe An Province, Vietnam, November 2005*

Sample source	No. animals sampled	Infection prevalence, %†	Egg intensity, epg	
			Mean ± SD	Maximum
All animals	229	26.9	21 ± 70	518
Dogs	80	35.0	25 ± 73	508
Cats	35	48.6	66 ± 129	518
Pigs	114	14.4	4 ± 18	160

*epg, eggs per gram feces.
†Based on fecal examination.

ers, respectively. Raw fish consumption was observed for 61.3% of dogs and 62.8% of cats. Univariable analysis showed the following to be significantly associated with FZT infections: animal species, free roaming, being fed raw fish, eating raw fish, catching fish from canals, and farmers giving dead fish from fish ponds to animals (Table 3).

Goodness-of-fit test for all observations was 1.013, which suggests that the model fits the data. Multivariable logistic analysis showed that, overall, animal species was strongly associated with FZT infections; however, an interaction was found between animal species and being fed raw fish. The risk of being infected was 4.75× higher for pigs fed raw fish than for pigs not fed raw fish. Risk for infection was higher for dogs and cats, regardless of whether they were fed raw fish, than for pigs that were not fed raw fish. Factors not significantly associated with infection in dogs, cats, or pigs were sex, ability to roam freely, district, age, access to fish from canals, being fed dead fish from ponds, deworming, composting of feces, and place of defecation.

Dogs that regularly ate raw fish had a 3.4× higher risk (odds ratio) of being infected than dogs that did not ($p = 0.017$, 95% confidence interval 1.18–9.74). In contrast, no such significant difference was observed for cats and pigs (data not shown).

Association between Infections in Domestic Animals and in Cultured Fish

From 48 of the 51 fish-farming households, 89.6% of these households had FZT-infected fish and 68.8% had FZT-infected domestic animals. Fisher exact test showed significant associations between the infections in fish and domestic animals ($p = 0.028$) and, at the animal species level, between infections in fish and cats ($p = 0.042$).

Table 2. Trematode infections in domestic animals, by trematode species, Nghe An Province, Vietnam, November 2005

Animal species	Trematode species	Prevalence, %*	Intensity of adult worms	
			Mean ± SD†	Maximum
Dogs (n = 27)	*Haplorchis pumilio*	92.6	47 ± 83	348
	H. taichui	62.9	28 ± 47	160
	H. yokogawai	25.9	13 ± 25	69
	Haplorchis spp.	62.9	30 ± 41	123
	Echinochasmus japonicus	29.6	38 ± 79	231
	E. perfoliatus	18.5	3 ± 3	9
	Echinostoma cinetorchis	3.7	2	2
	Centrocestus formosanus	11.1	16 ± 24	44
	Not identified	11.1	10 ± 8	15
	All	100	99 ± 201	924
Cats (n = 18)	*H. pumilio*	100	33 ± 62	268
	H. taichui	77.8	33 ± 94	357
	H. yokogawai	11.1	105 ± 136	201
	Haplorchis spp.	77.8	51 ± 135	514
	E. perfoliatus	5.6	3	3
	C. formosanus	5.6	4	4
	Not identified	22.2	8 ± 4	10
	All	100	112 ± 309	1,340
Pigs (n = 5)	*H. pumilio*	100	5 ± 4	13
	H. taichui	60	2	2
	H. yokogawai	40	2	2
	Haplorchis spp.	100	12 ± 13	29
	E. japonicus	60	3 ± 3	6
	Not identified	60	13 ± 16	32
	All	100	29 ± 32	84

*Based on estimation of worm burdens in animals with positive fecal egg counts.
†SD provided only for trematode species in >1 animal.

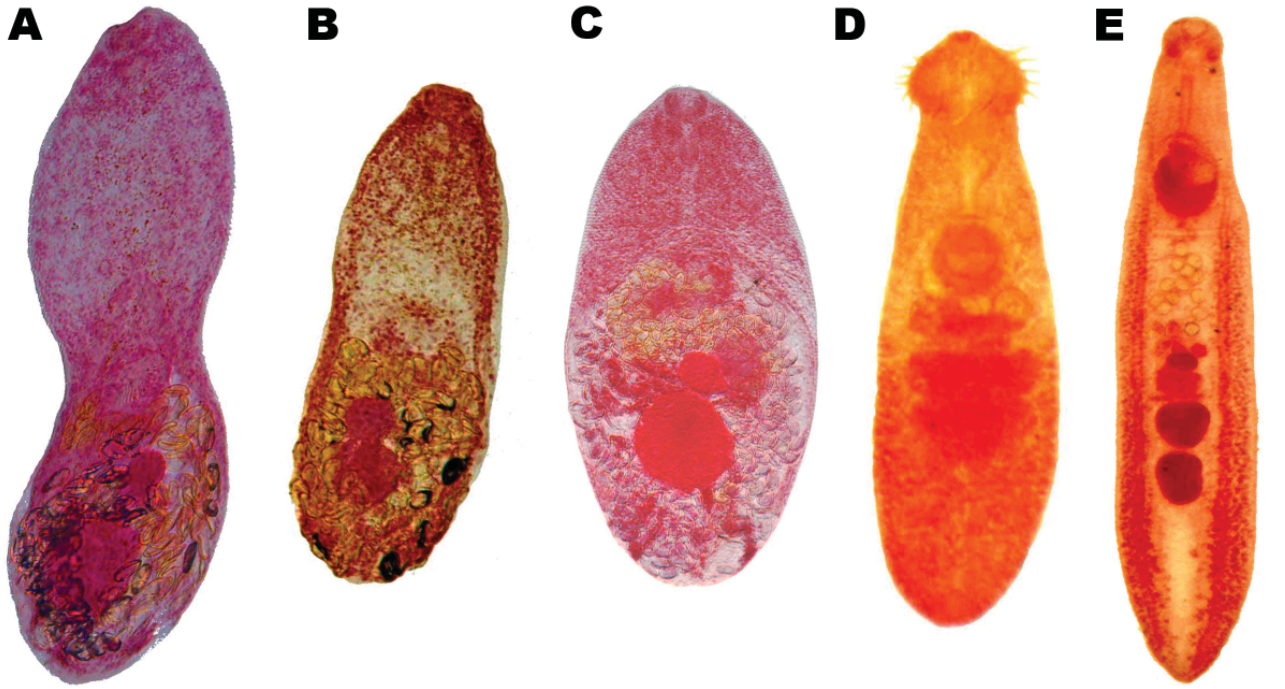

Figure 2. Adult trematodes recovered from domestic animals in Nghe An Province, Vietnam. A) *Haplorchis taichui*; B) *H. pumilio*; C) *H. yokogawai*; D) *Echinochasmus japonicus*; E) *Echinostoma cinetorchis.*

Relative Transmission Index

TDEE from humans and domestic animals in the 5 districts in Nghe An Province was 933×10^6, of which 371×10^6 eggs were from pigs (Table 4). RTI was highest in pigs, followed by dogs and humans, and lowest in cats. Although FZT prevalence was lowest for pigs, their contribution to egg contamination in the community was the greatest.

Discussion

The likelihood that these reservoir hosts have a major role in sustaining transmission of FZT to cultured fish, regardless of prevalence in humans, is high on the basis of the following: relatively high FZT prevalence, intensity of egg excretion and RTI in domestic animals, and the similar FZT species composition found in infected domestic

Table 3. Risk factors for fish-borne zoonotic trematodes infection in domestic animals, Nghe An Province, Vietnam, November 2005*

Variable	No. samples	Univariable analysis		
		Crude OR	95% CI	p value
Animal species†				<0.001
Dogs	80	3.3	1.6–6.6	
Cats	35	5.7	2.5–13.5	
Pigs	114	1	–	
Age, mo				
≤12	174	1.2	0.6–2.3	0.64
>12	55	1	–	
Free-roaming				0.0001
Always	117	0.6	0.4–0.8	
Sometimes	4	Inf		
Never	108	1	–	
Fed raw fish				
Sometimes	91	1.9	1.0–3.4	0.04
Never	138	1		
Eats raw fish				
Sometimes	158	4.0	2.2–7.5	<0.0001
Never	71	1		

*Data from all dogs, cats, and pigs were analyzed. OR, odds ratio; CI, confidence interval; Inf, infinity.
†Multivariable analysis showed an interaction only between animal species and feeding raw fish; p = 0.02.

Table 4. Total daily egg excretions and relative transmission index of domestic animals and humans, Nghe An Province, Vietnam, November 2005*

Species	Total no. animals	Prevalence, %	Intensity, epg	TDEE, 10^6	RTI, %
Humans	886,700	0.6†	215†	183	19
Dogs	332,039	35.0	25	288	31
Cats	141,254	48.6	66	91	10
Pigs	425,306	14.4	4	371	40

*Epg, eggs per gram; TDEE, total daily egg excretions; RTI, relative transmission index.
†Data obtained from Olsen et al. (*5*) and Annette Olsen (pers. comm.).

animals and infected fish. Although prevalence and intensity were markedly lower for pigs, their relatively large amount of feces makes them a major source of FZT eggs that can contaminate local bodies of water and infect snails (Figure 3). Among the other animal species, dogs excreted about 3× more FZT eggs than did cats. We conclude that these domestic animals, especially dogs and pigs, play a major role in the epidemiology of FZT in aquaculture. Although reports from other Southeast Asian countries have suggested that nonhuman reservoir hosts play only a minor role in the epidemiology of liver flukes, specific studies on the role of reservoir hosts, other than ours, have not been carried out (*1*,*13*,*14*). One limitation of our study is that the use of 2 different methods to obtain prevalence data from humans (*5*) and domestic animals may have introduced a bias when comparing the relative contributions of the eggs to the environment. However, the Kato-Katz method and our method were evaluated as reliable and the most suitable methods for detection of eggs in human and domestic animal studies, respectively (*8*,*15*).

The prevalence and species diversity of FZT in dogs, cats, and pigs in Vietnam (*H. pumilio, H. taichui, H. yokogawai, Echinochasmus japonicus, E. perfoliatus, Echinostoma cinetorchis*, and *Centrocestus formosanus*) may represent an emerging problem. Each of these species has been linked to health problems in humans (*1*,*16*). Intestinal

Figure 3. Typical pig pen built on bank of fish pond in Nghe An Province, Vietnam. The design allows fecal waste to drain into the pond.

trematodes in Thailand and Korea are also often reported (*17*,*18*), and recently, mixed FZT infections in humans in Vietnam and Laos were reported (*3*,*19*). Although the liver fluke *Clonorchis sinensis* was recently reported to have been found in humans in northern Vietnam (*3*), it was not detected in humans or fish in this fish farming area (*5*,*6*). Liver fluke distribution may thus be limited compared with intestinal fluke distribution; however, more geographically comprehensive surveys are needed.

The generally higher prevalence and intensity of infections for dogs and cats than for pigs can be explained by the free roaming of dogs and cats, which allows them greater access to fish from the ponds or pond banks; pigs are normally confined and are only exposed to infection if farmers feed them raw fish or fish waste. This explanation is supported by risk-factor analysis showing that the risk for FZT infections in dogs was related to their behavior of eating raw fish whereas infections in pigs were closely related to their being fed raw fish or fish waste. This finding suggests that educating farmers about preventive animal husbandry practices could affect FZT transmission.

Fish-eating birds and ducks are also known definitive hosts for some FZT species (*20*,*21*) and may contaminate fish ponds with eggs; however, they were excluded from this study because of an avian influenza epidemic in the study areas during sampling. To our knowledge, prevalence of FZT in fish-eating birds and ducks has not been systematically investigated in Southeast Asia, although avian infections are documented (*20*,*21*). The role of fish-eating birds and ducks in the epidemiology of FZT should be assessed.

In conclusion, prevention of FZT infections in domestic animals must be included in any public health strategy to control FZT in humans in fish-farming communities. This can be accomplished by including these hosts in drug-treatment programs aimed at their human owners, proper disposal or inactivation of eggs in feces that may contaminate water, and education of farmers about the dangers of risky feeding practices.

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Novel Type of *Streptococcus pneumoniae* Causing Multidrug-Resistant Acute Otitis Media in Children

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After our recent discovery of a *Streptococcus pneumoniae* 19A "superbug" (Legacy strain) that is resistant to all Food and Drug Administration–approved antimicrobial drugs for treatment of acute otitis media (AOM) in children, other *S. pneumoniae* isolates from children with AOM were characterized by multilocus sequence typing (MLST). Among 40 isolates studied, 16 (40%) were serotype 19A, and 9 (23%) were resistant to multiple antimicrobial drugs. Two others had unreported sequence types (STs) that expressed the 19A capsule, and 8 (88%) of the 9 multidrug-resistant strains were serotype 19A, including the Legacy strain with the new ST-2722. In genetic relatedness, ST-2722 belonged to a cluster of reported strains of *S. pneumoniae* in which all strains had 6 of the same alleles as ST-156. The multidrug-resistant strains related to ST-156 expressed different capsular serotypes: 9V, 14, 11A, 15C, and 19F.

The increasing global emergence and rapid spread of multidrug-resistant *Streptococcus pneumoniae* is a serious concern (*1*,*2*). Clonal dissemination of problematic pneumococcal strains have created clinically important treatment problems (*3*,*4*). Pneumococcal resistance to antimicrobial drugs was first reported in the mid-1960s (*5*,*6*). Since 1990, drug-resistant isolates of *S. pneumoniae* have spread rapidly throughout the United States (*7*). In the early 1990s, high-level resistance to penicillin and other antimicrobial drugs appeared in the United States with a low prevalence (*8*). Over the past decade, multidrug-resistant clones of *S. pneumoniae* have rapidly emerged (*8*–*11*). Of 90 serotypes, 19A is one of the most common types found in children. It can cause severe disease and easily develop antimicrobial drug resistance (*9*). The first 19A strain of *S. pneumoniae* with penicillin resistance was reported in the United States in 1986 (*12*). Introduction in 2000 of a pneumococcal 7-valent pneumococcal conjugate vaccine (PCV7) in the United States has substantially curtailed pneumococcal infections caused by 7 vaccine strains in children (*13*–*15*). However, nonvaccine strains, including drug-resistant strains, are increasingly being identified in patients. Among multidrug-resistant strains, a high proportion is serotype 19A, a strain not included in the vaccine; however, the proportion of invasive pneumococcal diseases caused by serotype 19A has substantially increased (*8*–*11*).

We recently discovered and reported a "superbug" strain of *S. pneumoniae* (Legacy strain) that is resistant to all Food and Drug Administration (FDA)–approved antimicrobial drugs and to 8 non-FDA–approved antimicrobial drugs used to treat acute otitis media (AOM) in children (*11*). Using molecular epidemiologic methods, particularly multilocus sequence typing (MLST), we characterized the molecular type of multidrug-resistant strains of *S. pneumoniae* recently isolated from children with AOM, compared the Legacy strain sequence type (ST) 2722 with 67 strains with the closest types in the MLST database, and reported the likely evolution and spread of the ancestor strains of the Legacy strain.

Methods

Pneumococcal Isolates, Serotype Analysis, and Antimicrobial Drug–Susceptibility Testing

All pneumococcal bacteria were isolated from children seen at a private pediatric group in suburban Rochester,

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New York (Legacy Pediatrics). Isolates were obtained from middle ear fluid of children with AOM at 6–36 months of age during 2004–2006. The children received age-appropriate inoculations with PCV7 at 2, 4, 6, and 12–15 months of age. The University of Rochester Institutional Review Board approved the study, and written informed consent was obtained from parents or guardians for the study and for all tympanocentesis procedures. Serotypes of pneumococci were determined by latex agglutination (Pneumotest-Latex; Statens Serum Institute, Copenhagen, Denmark) according to the manufacturer's instructions and confirmed by Quelling reaction. The antimicrobial drug susceptibility of pneumococci was determined as described previously by Etest or microbroth dilution (*11*).

Multilocus Sequence Typing

The internal fragments of 7 housekeeping genes (*aroE* [shikimate dehydrogenase], *gdh* [glucose-6-phosphate dehydrogenase], *gki* [glucose kinase], *recP* [transketolase], *spi* [signal peptidase I], *xpt* [xanthine phosphoribosyltransferase], and *ddl* [D-alanine-D-alanine ligase]) were amplified from chromosomal DNA by PCR. Chromosomal DNA was extracted from subculture of *S. pneumoniae* isolations recovered from middle ear fluid, nasal wash, or nasal swabs. PCR amplification was performed using primer pairs *aroE*-up, 5′-GCCTTTGAGGCGACAGC-3′ and *aroE*-dn, 5′-TGCAGTTCA(G/A)AAACAT(A/T)TTCTAA-3′; *gdh*-up, 5′-ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT-3′ and *gdh*-dn, 5′-GCTTGAGGTCCCAT(G/A)CT(G/A/T/C) CC-3′; *gki*-up, 5′-GGCATTGGAATGGGATCACC-3′ and *gki*-dn, 5′-TCTCCCGCAGCTGACAC-3′; *recP*-up, 5′-GCCAACTCAGGTCATCCAGG-3′ and *rec*P-dn, 5′-TGCAACCGTAGCATTGTAAC-3′; and *spi*-up, 5′-TTATTCCTCCTGATTCTGTC-3′ and *spi*-dn, 5′-GTGATTGGCCAGAAGCGGAA-3′. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s; annealing at 50°C–55°C for 30 s; and extension at 72°C for 30 s. The amplified DNA fragments were purified by using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and were sequenced in each direction by using the same primers used for amplification and by using the BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystems Prism 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences at each of the 7 loci were then compared with the sequences of all of the known alleles at those loci in the database at the pneumococcal MLST website (http://spneumoniae.mlst.net). The sequences identical to a known sequence were assigned the same allele number, and nonidentities to any known allele sequences were assigned new allele numbers. The allele at each of the 7 loci defines the allelic profile of each strain, as well as its ST. New allelic number or new ST number was assigned by a curator of the pneumococcal MLST database. The relatedness of isolates and known similar strains in the database was determined by constructing a neighbor-joining tree using a program online, Draw Tree Using Own MLST Data, found at the pneumococcal MLST website.

Results

Among 40 *S. pneumoniae* isolates recovered from middle ear fluid of children with AOM, 16 (40%) were serotype 19A. Serotype 19A was the most common serotype isolated in the study during 2004–2006.

Antimicrobial drug susceptibility testing was performed on the serotype 19A isolates. Eight (50%) of the 16 isolates of 19A were highly penicillin resistant (MIC ≥2.0 μg/mL), and all 8 were also multidrug resistant.

MLST of these16 *S. pneumoniae* isolates with serotype 19A showed that 6 (38%) were ST-320; 3 (19%) were ST-199; 2 (13%) were unreported STs now assigned ST-2722 and ST-2704; 2 (13%) were ST-1673; and 1 (6%) each of the remaining 3 was ST-1451, ST-2265, and ST-63. Among the 8 multidrug-resistant isolates, 5 (63%) were ST-320, 1 (13%) was ST-1451, and 1 (13%) was the newly assigned ST-2722 (Table 1, Figure 1). The genetic distance of the Legacy strain is closest to 7 other multidrug-resistant strains with ST-320 and ST-1451 in our group of 40 recent isolates.

To determine the evolutionary relationship of the Legacy strain (ST-2722) to other strains, we constructed a neighbor-joining tree. ST-2722 is assumed to have genetic relatedness to another ST in the database when an ST has ≥5 of the same loci as ST-2722 (Figure 2). ST-2722 belonged to a cluster in which all strains have 6 of 7 same loci. This cluster consisted of 59 (88%) strains with molecular type ST-156 and 1 strain each with ST-2722, ST-2128, ST-1227, ST-2616, ST-1893, ST-334, ST-1556, ST-2684, and ST-1697. The major dissimilarity of strains in this cluster was on loci *recP* (Table 2, Figure 3). The *recP* gene had 3 major variable sites at the 10th, 121st, and 368th bp among strains. For instance, the DNA at the 10th, 121st, and 368th bp are T, C, T, respectively, in ST-2722; C, T, T in ST-156; and C, T, C in ST-2128 (Figure 3).

Forty-three (64%) of the 67 strains related to ST-2722 were penicillin resistant, and 28 (42%) of these strains were resistant to at least 1 other antimicrobial drug. Thirty-four (51%) of the 67 strains related to ST-2722 serotype 19A strains were serotype 9V; 18 (27%) were serotype 14; and 5 (7%) were serotype 11A. Among the 43 antimicrobial drug–resistant strains in this cluster, 24 (56%) were serotype 9V, and 14 (33%) were serotype 14; others were serotype 11, 15C, and 19F.

Table 1. Genotypic characteristics of *Streptococcus pneumoniae* serotype 19A strains with multiple antimicrobial drug resistance*

Strain	Sequence type	MIC, μg/mL				Allele no. of housekeeping genes†						
		Pen	Cef	Ery	S.Tri	*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
P270CR	320	2.0	1.0	>256	>32	4	16	19	15	6	20	1
060110	1451	4.0	1.0	>256	4.0	10	16	19	15	6	20	1
P270CL	320	2.0	1.0	>256	>32	4	16	19	15	6	20	1
Legacy strain	2722‡	8.0	6.0	8.0	>32	7	11	10	15	6	8	1
P239CR	320	2.0	2.0	>256	8.0	4	16	19	5	6	20	1
0601034	320	2.0	1.0	>256	16.0	4	16	19	15	6	20	1
0601004V1NW	320	2.0	1.5	>256	6.0	4	16	19	15	6	20	1
0601004V1LMEF	320	3.0	1.5	>256	4.0	4	16	19	15	6	20	1

*Pen, benzylpenicillin; Cef, ceftriaxone; Ery, erythromycin; S.Tri, sulfamethoxazole-trimethoprim.
†*aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine-D-alanine ligase).
‡New assigned sequence type.

Discussion

We described the molecular and capsular types of pneumococci causing AOM in children tested in Rochester, New York, during 2004–2006. Serotype 19A, a relatively new molecular type, accounted for 40% of the isolates and has emerged as the major serotype causing ear infections in children. Eight different molecular STs expressed the 19A capsule; most of the strains were multidrug resistant. Among the 40 strains studied, 5% were new molecular STs. In addition, we analyzed the genetic relatedness of the strains to other previously described strains of serotype 19A. We traced the genetic origin of ST-2722 to a strain first identified in 1988 in Spain as ST-156, which expressed capsular serotype 9V (*16*). Over nearly 20 years before ST-2722 emerged, variants of this original ST-156 strain were identified in 18 countries, with 8 different STs and 13 different sequence/capsular type combinations (according to records in *S. pneumoniae* database).

ST-2722 (Legacy strain) appears to have resulted from a mutation identified in the *recP* gene that coincided with acquisition of multidrug resistance, including a ceftriaxone MIC of 6.0 μg/mL, and acquisition of capsular DNA associated with the 19A serotype. Because of genetic plasticity, different capsular genes of *S. pneumoniae* may transfer through DNA-mediated genetic recombination (*17–19*).

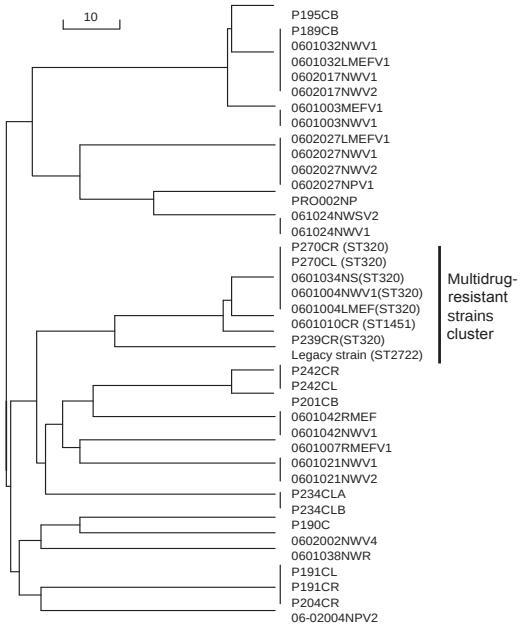

Figure 1. Neighbor-joining tree of genetic relatedness among 40 *Streptococcus pneumoniae* isolates from a private pediatric practice, Rochester, New York, USA, 2004–2006. Scale bar indicates genetic linkage distance.

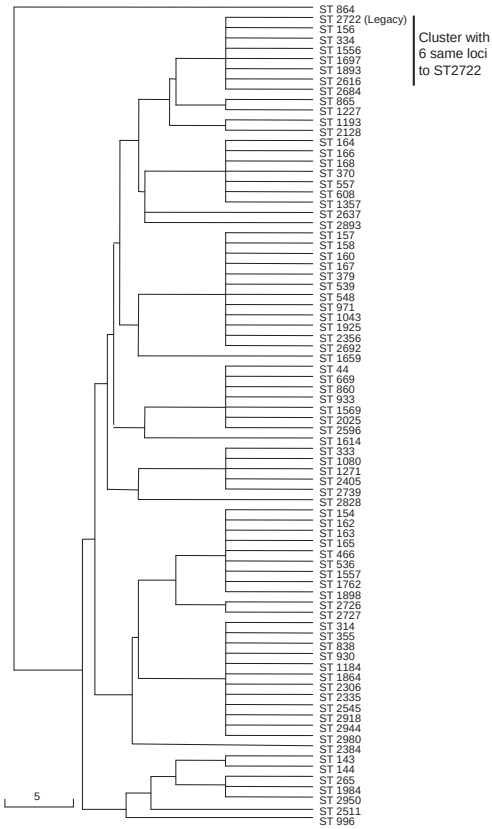

Figure 2. Genetic relatedness of *Streptococcus pneumoniae* ST-2722 (Legacy strain) to reported types that have 5 or 6 of the same loci as the Legacy strain. List of types available at the multilocus sequence typing database (http://spneumoniae.mlst.net). Scale bar indicates genetic linkage distance.

Table 2. Comparison of *Streptococcus pneumoniae* ST-2722 (Legacy strain) with the closest types that have 6 of the same alleles as ST-2722*

ST	Serotypes	Allele profile of housekeeping genes†							No. resistant/ total strains	Countries of isolation	Sources
		aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*			
156	9V/14/11A/ 15C/19F	7	11	10	1	6	8	1	38/59	BR, CA, CZ, DK, FR, DE, HU, IL, IT, LB, NL, PL, SE, PT, QA, ES, UK, UY	Nasopharynx, cerebrospinal fluid, blood, pleural, sputum, ear
1556	14	7	11	10	12	6	8	1	1/1	DE	Blood
1697	9V	7	11	10	77	6	8	1	1	UK	NA
2684	11A	7	11	10	10	6	8	1	1/1	FR	Nasopharynx
334	9	7	11	10	16	6	8	1	1/1	NO	Blood
1893	14	7	11	10	4	6	8	1	1	NO	NA
2616	14	7	11	10	2	6	8	1	1	ES	Nasopharynx
1227	14	7	11	10	29	6	8	1	1/1	SE	Nasopharynx
2128	9	7	11	10	5	6	8	1	1/1	UK	Blood
2722	19A	7	11	10	15	6	8	1	1	US	Ear

*Types reported in the multilocus sequence typing database (http://spneumoniae.mlst.net). ST, sequence type; BR, Brazil; CA, Canada; CZ, Czech Republic; DE, Germany; DK, Denmark; FR, France; HU, Hungary; IL, Israel; IT, Italy; LB, Lebanon; NL, Netherlands; NO, Norway; PL, Poland; SE, Sweden; PT, Portugal; QA, Qatar; ES, Spain; UK, United Kingdom; UY, Uruguay; US, United States; NA, not available.
†*aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine-D-alanine ligase).

The multidrug-resistant epidemic type 23F Spanish/USA clone has expressed capsular types 3, 9N, 14, and 19F. Capsular transformation may equip multidrug-resistant strains with highly virulent blood invasive phenotypes (*18*).

Many studies have documented the impact of the PCV7 on pneumococcal diseases. Introduction of PCV7 containing serotype 4, 6B, 9V, 14, 18C, 19F, and 23F has dramatically decreased the rate of carriage and disease caused by these vaccine serotypes. However, the proportion of invasive pneumococcal diseases caused by nonvaccine serotypes has increased substantially in recent years, and multiresistant strains have rapidly emerged (*16*,*20*–*23*). In addition, PCV7 offers moderate protection against AOM (*24*). The lowest level of protective activity provided by PCV7 was against serotype 19F, and 19F polysaccharide antigen provided less cross-protection for disease caused by related serotypes such as 19A (*24*,*25*). An increase in the rate of middle ear infections with 19A strains and other strains expressing capsular types not contained in PCV7 also has been reported (*11*). These developments encourage further ongoing epidemiologic surveillance. Emergence of new 19A strains may represent a successful vaccine escape mechanism used by PCV7-targeted clones, and antimicrobial drug nonsusceptibility provides an additional survival advantage that could help these organisms spread further.

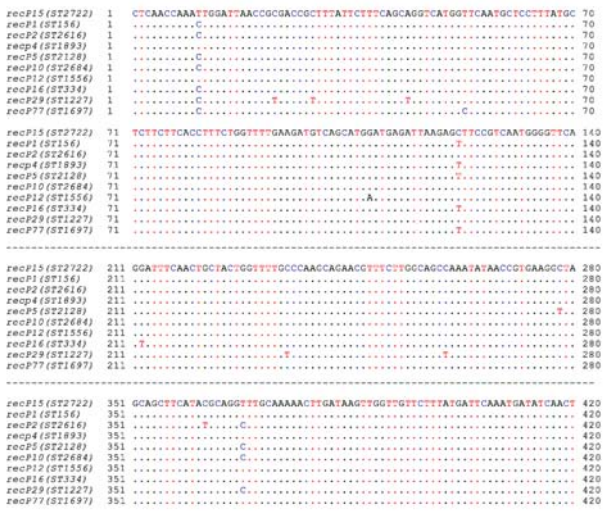

Figure 3. Variable sites (colors) in *recP* DNA sequences (allele) of ST-2722 (Legacy strain) and those in other *Streptococcus pneumoniae* that have the same 6 loci. List of types available at the multilocus sequence typing database (http://spneumoniae.mlst.net).

By MLST analysis and capsular typing, we found that multiple STs expressing capsular type 19A have emerged as the most important otopathogens of children. We also found a new strain of *S. pneumoniae* (ST-2722), expressing a 19A capsule that is resistant to all FDA-approved antimicrobial drugs. ST-2722 has a genetic relatedness close to ST-156 reported to the *S. pneumoniae* MLST database from 18 countries. ST-2722 was multiresistant to antimicrobial drugs but had an MIC to ceftriaxone of 6.0 μg/mL. A study by Pelton et al. showed that multidrug-resistant 19A strains with ST-320 had an MIC to ceftriaxone of 8.0 μg/mL (*10*). The clinical importance of such strains is potentially high because empiric treatment of suspected and even proven pneumococcal infections typically relies on the efficacy of ceftriaxone.

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Oseltamivir-Resistant Influenza Virus A (H1N1), Europe, 2007–08 Season

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In Europe, the 2007–08 winter season was dominated by influenza virus A (H1N1) circulation through week 7, followed by influenza B virus from week 8 onward. Oseltamivir-resistant influenza viruses A (H1N1) (ORVs) with H275Y mutation in the neuraminidase emerged independently of drug use. By country, the proportion of ORVs ranged from 0% to 68%, with the highest proportion in Norway. The average weighted prevalence of ORVs across Europe increased gradually over time, from near 0 in week 40 of 2007 to 56% in week 19 of 2008 (mean 20%). Neuraminidase genes of ORVs possessing the H275Y substitution formed a homogeneous subgroup closely related to, but distinguishable from, those of oseltamivir-sensitive influenza viruses A (H1N1). Minor variants of ORVs emerged independently, indicating multiclonal ORVs. Overall, the clinical effect of ORVs in Europe, measured by influenza-like illness or acute respiratory infection, was unremarkable and consistent with normal seasonal activity.

Infection with influenza viruses A (H1N1), A (H3N2), or B causes substantial human illness and excess deaths each year (*1*,*2*). Vaccination against seasonal influenza is the key control measure used in Europe to minimize illness and death. Antigenic mismatch between vaccine components and circulating viruses occurs every few years, requiring reformulation of the vaccine (*1*). In addition, suboptimal immunization in patient groups for which vaccine is recommended provides the rationale for use of antiviral drugs in the prophylaxis and treatment of influenza. M2 ion channel inhibitors (M2Is), amantadine and rimantadine, have been available since 1964, but adverse effects, rapid development of resistance, and lack of activity against influenza B have limited their usefulness (*3*). The introduction of neuraminidase inhibitors (NAIs), oral oseltamivir and inhaled zanamivir, which are active against both influenza type A and B viruses, was a major breakthrough

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in treatment and prophylaxis of influenza using antiviral drugs (*4*). However, prescription data indicate that they are not widely used in Europe (Figure 1); by contrast, in Japan during the 2003–04 season alone, ≈6 million NAI treatment courses were prescribed (*5*).

Before the introduction of NAIs in 1999, and until 2007, <1% of viruses tested from unselected surveillance studies in a number of countries demonstrated natural resistance to NAIs (*5*–*9*). Limited development of resistance to oseltamivir has been observed in persons treated, with little evidence of onward transmission of resistant viruses (*10*), although low-level transmission of resistant variants cannot be discounted (*11*). However, oseltamivir-resistant viruses emerged in 18% (9/50) of treated Japanese children with influenza virus A (H3N2) infection and 16% (7/43) of treated Japanese children with influenza virus A (H1N1) infection, also with no evidence that these viruses transmitted efficiently (*12*,*13*).

In late January 2008, we reported an unexpected high level and unexpected spread of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) in Europe caused by a H275Y (H274Y in N2 numbering) amino acid substitution in the neuraminidase (NA) of these viruses (*14*). Here, we analyze the distribution and transmission of ORVs in Europe during the winter of 2007–08, when influenza viruses A (H1N1) were the predominant circulating viruses in European countries (Table).

Methods

Clinical Influenza Activity

The European Influenza Surveillance Scheme (EISS) actively monitored influenza activity from week 40 (October 1–7) of 2007 through week 19 (May 5–11) of 2008. EISS covers all 27 European Union countries plus Croatia, Norway, Serbia, Switzerland, Turkey, and Ukraine. In each country each week, 1 or several networks of sentinel general practitioners (GPs) reported rates of consultation for influenza-like illness (ILI) or acute respiratory infection (ARI) (*15*–*17*). ARI includes ILI and all other acute respiratory infections. For Croatia, Finland, Turkey, and Ukraine, no consultation data were available.

Virologic Analysis

Sentinel GPs involved in clinical data recording of ILI or ARI also send nasal, pharyngeal, or nasopharyngeal specimens from a subset of their patients to the National Influenza Centers (NICs) for virus detection and characterization by using a variety of genetic or phenotypic methods (*18*–*20*). The NICs also analyzed specimens and influenza viruses obtained from other sources (e.g., from nonsentinel GPs, hospitals, or institutions). For Cyprus and Turkey, no virus detection data were available.

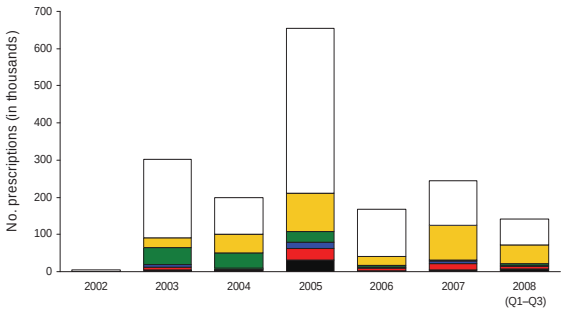
Antiviral Drug Susceptibility Monitoring

Antiviral susceptibility data were generated either through the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL) project at a single laboratory in London (UK Health Protection Agency) or directly by individual NICs by using methods described previously (*14*,*21*). Genetic analysis of virus isolates or clinical specimens was performed by using cycle-sequencing or pyrosequencing the NA gene, targeting the H275Y amino acid substitution in the N1 NA (*22*). The 50% inhibitory NAI concentration (IC_{50}) of virus isolates was determined by using fluorescent or chemiluminescent enzyme assays (*23*,*24*). ORVs were defined as influenza viruses A (H1N1) with an IC_{50} >100 nmol/L for oseltamivir. Susceptibility to zanamivir was determined by using the same enzymatic method. Susceptibility to M2Is was determined by cycle-sequencing or pyrosequencing the M2 protein gene, targeting known resistance markers. Antiviral susceptibility data were not available for Cyprus, Lithuania, and Malta.

Data Analysis

To obtain United Kingdom estimates, clinical and virologic surveillance data and antiviral susceptibility data were totaled for England, Northern Ireland, Scotland, and Wales. A single web-based European database at the EISS password-protected website (www.eiss.org) was used to collect antiviral susceptibility data and linked patient demographic and clinical data (*25*). Updates on possible resistant viruses were provided at regular intervals to EISS members, the World Health Organization, and the European Centre for Disease Prevention and Control.

The timing of the first week of continuous detection of influenza virus A and ORVs across Europe, both based on



	2002	2003	2004	2005	2006	2007	2008 (Q1–Q3)
Germany	4	211	100	442	128	121	71
France	0	27	49	104	24	93	50
Greece	0	45	41	30	4	2	4
Finland	0	9	2	15	3	7	2
Belgium	1	7	3	33	7	18	8
Austria	0	4	5	30	2	4	7

Figure 1. Prescription data of oseltamivir treatment courses for Western Europe (in thousands); 12 months of data for each year 2002–2007 and through September for 2008. Data from the United Kingdom, the Netherlands, Switzerland, and Portugal are excluded because of negligible values. Data provided by IMS Health (www.imshealth.com), London, UK.

date of specimen collection, were analyzed by linear regression analysis using center longitude and center latitude of a country as explanatory variables. A maximum interruption of 1 week with no influenza virus A or ORV detection was allowed in estimating the first week of continuous detection. The average European delay between the first week of continuous detection of influenza virus A and of ORV was calculated as the average of the differences in number of weeks between both, by country.

The analysis of temporal trends in the prevalence of ORVs in countries and for Europe was confounded by different levels of sampling in different countries (*18*), enhanced antiviral susceptibility testing in some countries, and lack of data on the proportion of ORVs for some or most weeks for several other countries. To ensure a more representative picture of temporal trends in the proportion of ORVs, a mixed effect logistic regression modeling approach (*26*,*27*) was used, which allows modeling of binomial proportions, i.e., a numerator and a denominator as a function of time, where the coefficients of this function are allowed to vary for each country around a mean value, combining data from all countries. If there are no observations or the denominator is small, the fit will shrink to its overall mean, and uncertainties increase. Three fractions were modeled: "ILI per population covered," "influenza A virus detections per specimens tested," and "A (H1N1) resistant per A (H1N1) tested." By multiplying the first 2 fractions by the total population, we obtained the number of patients with ILI who had influenza A in a country. By dividing this number by the sum of the number of patients with ILI who had influenza A for all countries, we obtained the relative weights. By multiplying the weights with the prevalences of ORVs summed over all countries, we obtained the weekly European prevalences of ORVs. The modeled weekly

Table. Peak incidence rates of ILI or ARI infection for countries for which data were available, Europe, 2000–01 through 2007–08 influenza seasons*

Country	ILI/ ARI†	Peak incidence rate/10,000 population during influenza season, 2000–01 through 2007–08: Median	Range	Consecutive no. seasons‡	Dominant virus§: 2000–01	Dominant virus§: 2007–08	Peak incidence rates¶: Incidence rate ratio	Peak incidence rates¶: p value
Austria#	ILI	168.1	108.0–263.2	4	NA	186.1 (H1)		
Belgium	ILI	51.9	30.3–95.1	8	30.3 (H1)	38.0 (H1)	1.3	0.004
Bulgaria	ARI	NA			NA	186.0 (H1)		
Czech Republic	ARI	188.1	134.5–320.0	8	310.2 (H1)	144.4 (H1)	0.5	1.000
Denmark	ILI	30.7	13.8–47.8	8	44.5 (H1)	13.8 (H1)	0.3	1.000
Estonia	ILI	2.9	0.6–4.9	3	NA	2.9 (H1)		
France	ARI	336.1	279.7–448.8	7	NA	279.7 (H1)		
Germany	ARI	185.6	136.9–256.5	8	247.3 (H1)	136.9 (H1/B)	0.6	1.000
Greece	ILI	27.7	23.1–42.1	3	NA	23.1 (H1)		
Hungary	ILI	50.1	21.0–54.6	3	NA	54.6 (H1)		
Ireland	ILI	7.5	2.9–12.1	8	12.1 (H1)	4.9 (H1/B)	0.4	1.000
Italy	ILI	79.5	27.6–428.2	8	56.7 (H1)	72.1 (H1/B)	1.3	0.0001
Latvia	ILI	45.6	25.1–93.3	5	NA	26.6 (H1)		
Lithuania	ILI	34.9	13.3–47.2	7	NA	13.3 (H1)		
Luxembourg	ILI	72.6	32.7–79.1	5	NA	67.4 (H1)		
The Netherlands	ILI	10.3	6.6–24.0	8	6.9 (H1)	7.2 (H1/B)	1.0	0.400
Norway	ILI	18.5	10.9–31.7	3	NA	10.9 (H1/B)		
Poland	ILI	23.0	6.2–66.7	7	NA	16.6 (H1)		
Portugal	ILI	8.1	3.0–17.4	8	3.8 (H1)	6.2 (H1/B)	1.6	0.016
Romania	ILI	1.2	0.4–3.7	4	NA	1.4 (H1)		
Serbia	ILI	37.8	30.6–44.9	2	NA	30.6 (H1)		
Slovakia	ILI	136.3	49.5–337.3	8	337.3 (H1)	49.5 (H1)	0.1	1.000
Slovenia	ILI	15.2	4.5–39.2	8	14.1 (H1)	20.4 (H1)	1.5	0.001
Spain	ILI	21.2	4.2–54.1	8	4.2 (H1)	20.3 (H1/B)	4.8	0.0001
Sweden#	ILI	2.0	1.6–5.8	5	5.8 (H1)	1.8 (B)	0.3	1.000
Switzerland	ILI	39.8	19.4–53.2	7	NA	29.7 (H1)		
United Kingdom	ILI	3.8	2.7–8.4	8	5.5 (H1)	2.7 (H1/B)	0.5	1.000

*ILI, influenza-like illness; ARI, acute respiratory infection; NA, data not available.
†For countries where both ILI and ARI data were available, only the ILI data are shown.
‡Leading up to 2007–08.
§Dominant virus estimated on the basis of combined sentinel and nonsentinel data. The limits for codominant virus types/subtypes were 45%:55%.
¶2007–08 compared with 2000–01. The incidence rate ratio was calculated by dividing the peak incidence rate for 2007–08 by the peak incidence rate for 2000–01. If the p value estimated using z-statistics is <0.05, the incidence rate ratio is significantly >1, and therefore the peak incidence rate for 2007–08 is significantly higher than that for 2000–01.
#Data for seasons 2002–03, 2003–04, and 2004–05 were missing.

prevalences of ORVs were subsequently used to calculate the average prevalence of ORVs by country and for Europe (online Technical Appendix, available from www.cdc.gov/EID/content/15/4/552-Techapp.pdf).

We performed all statistical analyses by using the software package R version 2.8.0 (*28*). Box-and-whisker plot analysis was used to select viruses with outlying high IC_{50} values for further analysis (*7*,*29*). For oseltamivir outlier identification, all viruses defined as resistant for oseltamivir (IC_{50} >100 nmol/L) were first removed. Minor outliers were defined as values lying between the upper quartile (UQ) + 1.5 × interquartile region (IQR) and UQ + 3 × IQR; major outliers were defined as values lying above UQ + 3 × IQR, based on analysis of all viruses in a particular subtype over a particular winter season.

Phylogenetic analysis of NA and hemagglutinin (HA) gene sequences used maximum parsimony (PAUP* version 4.0; Sinauer Associates, Sunderland, MA, USA). Sequences of ORVs and oseltamivir-sensitive influenza A (H1N1) viruses (OSVs) were chosen as representative of influenza viruses A (H1N1) isolated during the 2007–08 influenza season (i.e., weeks 40–52 of 2007 and weeks 1–19 of 2008) in different European countries and a few from other regions of the world and were compared with those of a few influenza viruses A (H1N1) isolated before the 2007–08 season, including sporadically isolated ORVs. GenBank accession numbers are listed in the online Appendix Table (available from www.cdc.gov/EID/content/15/4/552-appT.htm).

Results

Seasonal Surveillance

The 2007–08 influenza season in Europe was initially dominated by influenza viruses A (n = 10,720; 60% of all influenza virus detections). Influenza viruses B (n = 7,150; 40% of all influenza virus detections) became dominant in week 8 (Figure 2). Of the 5,984 (56%) influenza viruses A subtyped, 5,748 (96%) were H1, and 236 (4%) were H3. Overall, influenza virus detections peaked in week 6, in week 4 for influenza viruses A (H1N1), and in week 8 for influenza viruses B. Of the 2,136 influenza viruses A (H1N1) characterized antigenically, 97% were reported to be closely related to the vaccine strain A/Solomon Islands/3/2006, although half of these viruses were reported to be more closely related to A/Brisbane/59/2007, the vaccine strain recommended for the 2008–09 season (*30*).

The first countries in Europe where influenza viruses A started to circulate continuously were France, Spain, Switzerland, and the United Kingdom in week 40. Spatial analysis of the timing of the first week of continuous detection of influenza viruses A across Europe (n = 30 countries) showed a west-to-east pattern: estimated parameter for longitude was 0.261 weeks per degree longitude (95% confidence interval [CI] 0.138–0.385, p = 0.001), and for latitude –0.108 weeks per degree latitude (95% CI –0.324 through 0.108, p = 0.366), with R^2 = 0.32 for the linear regression fit.

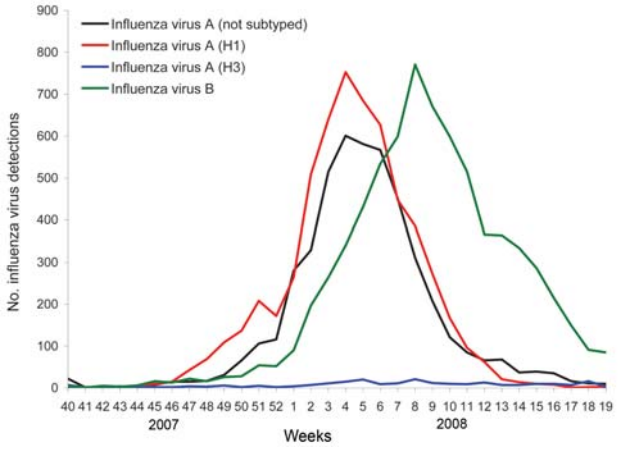


Figure 2. Total number of influenza virus detections, by type and subtype and by week, Europe, winter 2007–08.

Antiviral Drug Susceptibility

The estimated number of influenza viruses A (H1N1) among all detected influenza viruses A (n = 10,720) was 10,291 following extrapolation from the proportion of 96% influenza viruses A (H1N1) among all 5,984 subtyped influenza viruses A. Of the 10,291 influenza viruses A (H1N1), 2,949 (29%) were tested for antiviral susceptibility, 1,080 by both phenotypic assay (IC_{50}) and sequencing, 601 by phenotypic assay alone, and 1,268 by sequencing alone. Of the 2,949 viruses tested, 712 (24%) were oseltamivir resistant either by presence of the H275Y substitution (n = 548) or an IC_{50} >100 nmol/L for

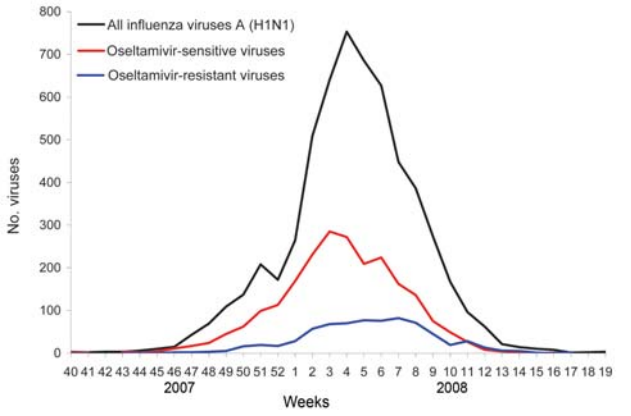


Figure 3. Total influenza A viruses subtyped as H1N1 and number of oseltamivir-resistant or oseltamivir-sensitive viruses among the subset of influenza viruses A (H1N1) for which oseltamivir susceptibility was determined, by week, Europe, winter 2007–08.

oseltamivir (n = 463) (Figure 3). Correlation was 100% between sensitive phenotype (IC_{50} <100 nmol/L) and the presence of H275 (n = 781) and between resistant phenotype (IC_{50} >100 nmol/L) and the presence of Y275 (n = 299). OSVs (n = 1,218) had a median IC_{50} of 1.7 nmol/L for oseltamivir (range 0.1 nmol/L–23.2 nmol/L) and only 9 minor outliers (thresholds IC_{50} >12.0 nmol/L and <53.1 nmol/L) were identified. ORVs (n = 463) had a median IC_{50} of 653 nmol/L (range 140 nmol/L–4,000 nmol/L). None of the 429 phenotypically characterized ORVs showed evidence of resistance to zanamivir (median IC_{50} 1.8 nmol/L, range 0.2 nmol/L–25.8 nmol/L), and only 17 minor outliers (thresholds IC_{50} >8.5 nmol/L and <27.5 nmol/L) were identified. None of 237 ORVs tested for M2I sensitivity had any of the common resistance substitutions in the M2 protein.

ORVs were detected in 22 of the 30 countries for which susceptibility data were available, with Norway having the highest proportion of ORVs (Figure 4). Modeling showed the overall average prevalence of ORVs by country ranged from 8.3% (95% CI 1.3%–21%) in Italy to 65.0% (95% CI 58.2%–71.3%) in Norway; for Europe, the average prevalence of ORVs was 20.1% (95% CI 15.2%–24.6%).

The earliest detection of ORVs was in France and the United Kingdom in week 46 and in Norway in week 47. Countries where continuous detection of ORVs first began included Norway in week 47, France in week 49, the United Kingdom in week 51, and the Netherlands in week 52. Spatial analysis of the timing of the first week of continuous ORV detection across Europe (n = 14 countries) showed a west-to-east trend pattern: estimated parameter for longitude was 0.156 weeks per degree longitude (95% CI 0.033–0.280, p = 0.031), and for latitude 0.007 weeks per degree latitude (95% CI –0.209 through 0.223, p = 0.953), with $R^2 = 0.36$ for the linear regression fit. The average delay between the first week of continuous detection of influenza virus A and continuous detection of ORV was 5.7 weeks (range 0–15, 95% CI 2.8–8.4).

Modeling showed a gradual increase for Europe in prevalence of ORVs over time, from close to 0 in week 40 to ≈56% in week 19 (Figure 5). This overall increase reflected prevalence increases in most individual countries in addition to Norway where the modeled prevalence started high at ≈60% and remained so throughout the period of virus circulation (online Appendix Figure, available from www.cdc.gov/EID/content/15/4/552-appF.htm). Outside the main influenza virus A (H1N1) outbreak period, from week 51 to week 10 (Figure 2), the CIs for the prevalence of ORVs by country and for Europe were wide (Figure 5; online Appendix Figure) because of the low numbers of influenza virus A (H1N1) detected or analyzed for antiviral resistance (online Technical Appendix).

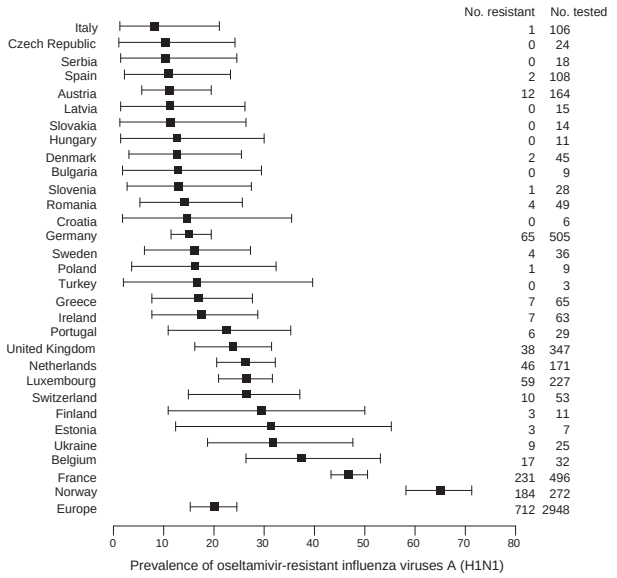


Figure 4. Modeled average prevalence of oseltamivir-resistant influenza viruses A (H1N1), with 95% confidence intervals (error bars), ranked by country, Europe, winter 2007–08. Text columns on the right list the absolute cumulative number of oseltamivir-resistant influenza viruses A (H1N1) and number of influenza viruses A (H1N1) tested for oseltamivir susceptibility per country.

Phylogenetic Analysis

Phylogenetic comparisons of HA and NA genes showed that the sequences of most recent European influenza viruses A (H1N1) fell within clade 2B, represented by A/Brisbane/59/2007, the recently recommended vaccine virus for 2008–09 (Figure 6). The NA sequences of most European ORVs form a cluster, characterized by a difference in amino acid residue 354 (D354G), as well as 275 (H275Y) compared with OSVs, including some ORVs from the United States and Japan (*30*,*31*). A degree of heterogeneity was observed, especially among ORVs from the United Kingdom; however, the NA sequences in these smaller clusters, represented by, for example, A/Scotland/5/2008 (and A/Hawaii/21/2007) or A/England/654/2007, are not distinguished from those of OSVs by any common amino acid differences other than H275Y. Some of these sequences fall close to those of ORVs recently isolated in Japan (*31*). The corresponding HA gene sequences within clade 2B, however, did not exhibit segregation complementary to that for NA gene sequences and no common amino acid changes distinguished ORVs and OSVs (Figure 6). Although the D344N substitution in NA has been associated with increases in the enzyme activity (*32*), this amino acid is common to both clades 2B and 2C, and none of the clade-specific differences between the NA (13 amino acids) or HA (6 amino acids) can readily account for the greater proportion of ORVs in clade 2B over clade 2C viruses.

Discussion

Unexpectedly, influenza viruses A (H1N1) with a single amino acid substitution H275Y in the NA, which caused a several hundred-fold selective reduction in susceptibility to oseltamivir, emerged and were sustained in circulation in Europe during 2007–08, despite low antivirual drug use (Figure 1). Before the 2007–08 season, <1% of viruses tested since the start of European antiviral surveillance in 2004 had IC_{50} values >100 nmol/L for NAI drugs (A. Lackenby et al., unpub. data), in concordance with results from worldwide surveillance (*8*,*9*). In 2007–08, influenza viruses A (H3N2) and B circulating in Europe remained sensitive to NAI drugs.

This emergence of oseltamivir-resistant influenza virus A (H1N1) in Europe coincided with the dominant circulation of this virus subtype during the 2007–08 winter in Europe and the emergence of a new drift variant, A/Brisbane/59/2007 (*30*). Of the last 12 influenza seasons, influenza viruses A (H1N1) were dominant only in 2000–01, which included a new drift variant, A/New Caledonia/20/99 (*20*). In the other 10 seasons, influenza viruses A (H1N1) played a minor role, with influenza viruses A (H3N2) dominant in 9 seasons. Compared with 2000–01, peak incidence rates for ILI or ARI in 7 of 13 countries were similar or lower in 2007–08 (Table). In 6 countries, the peak incidence rates were significantly higher in 2007–08 than in 2000–01, but with a <2-fold difference in 5 countries and, in Spain only, a 4.8-fold difference. Both the 2000–01 and 2007–08 seasons were unremarkable in the overall clinical impact of influenza, with normal seasonal activity as measured by comparison of peak incidence rates for all seasons since 2000–01.

Sporadically occurring A/New Caledonia/20/99-like ORVs with H275Y were detected during the 2006–07 season in the United Kingdom and United States but did not become epidemiologically important. Indeed, the genetic background plays a role in retaining the replication efficiency and pathogenicity of recombinant influenza viruses A (H5N1) and A (H1N1) after introduction of tyrosine at position 275 (*33*). Furthermore, other previously analyzed influenza viruses A (H1N1) with the H275Y mutation showed impaired replicative ability in cell culture and reduced infectivity and substantially compromised pathogenicity in animal models, compared with the corresponding wild-type virus (*34*,*35*). The coincidental emergence of H275Y with the circulation of the A/Brisbane/59/2007 drift variant may have favored the emergence of fit transmissible ORVs. This point is also illustrated by the emergence of A/Brisbane/59/2007-like ORVs in other parts of the Northern Hemisphere and their continued circulation during the 2008 Southern Hemisphere influenza epidemic season (*36*–*38*). Since the last quarter of 2007, ORVs have been detected in continents other than Europe, with proportions of ORVs varying from 100% in South Africa and Australia to <5% in Japan. Trend data are limited: a slight monthly increase was noted in China/Hong Kong and Japan; in Canada, the increase was similar to that in Europe, from 0% ORVs in November 2007 to 86% ORVs in April 2008 (*36*).

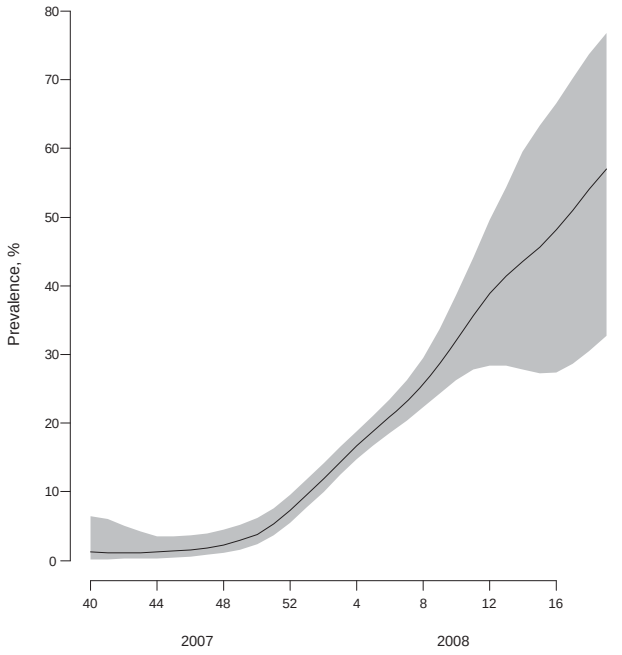


Figure 5. Weighted average prevalence of oseltamvir-resistant influenza viruses A (H1N1), Europe, winter 2007–08. The light gray region indicates the 95% confidence interval.

Using modeling, we showed that the prevalence of ORVs increased in the European region from ≈0% at the start to 56% at the end of the season. The finding of a high prevalence of ORVs in the community and the overall temporal increase in resistance demonstrates that the previously documented reduced fitness of viruses bearing the H275Y mutation, ostensibly caused by structural and functional constraints (*10*), has been overcome in currently circulating influenza viruses A (H1N1). The results of Rameix-Welti et al. (*32*) suggest that a combination of specific amino acid substitutions have increased the affinity of the NA of recent influenza viruses A (H1N1) (ORVs and OSVs) for substrate. A better balance of NA and HA activities in ORVs compared with OSVs may have contributed to the overall fitness and transmissibility of ORVs. However, growth curves conducted in tissue culture of pairs of ORVs and OSVs demonstrated no differences in growth kinetics or final virus yields. Therefore, changes in other genes also may be involved in the overall impact on the fitness of ORVs, for which whole genome sequencing is necessary.

For Europe, no focal point of initiation of spread could be identified. The spread of ORV from west to east paral-

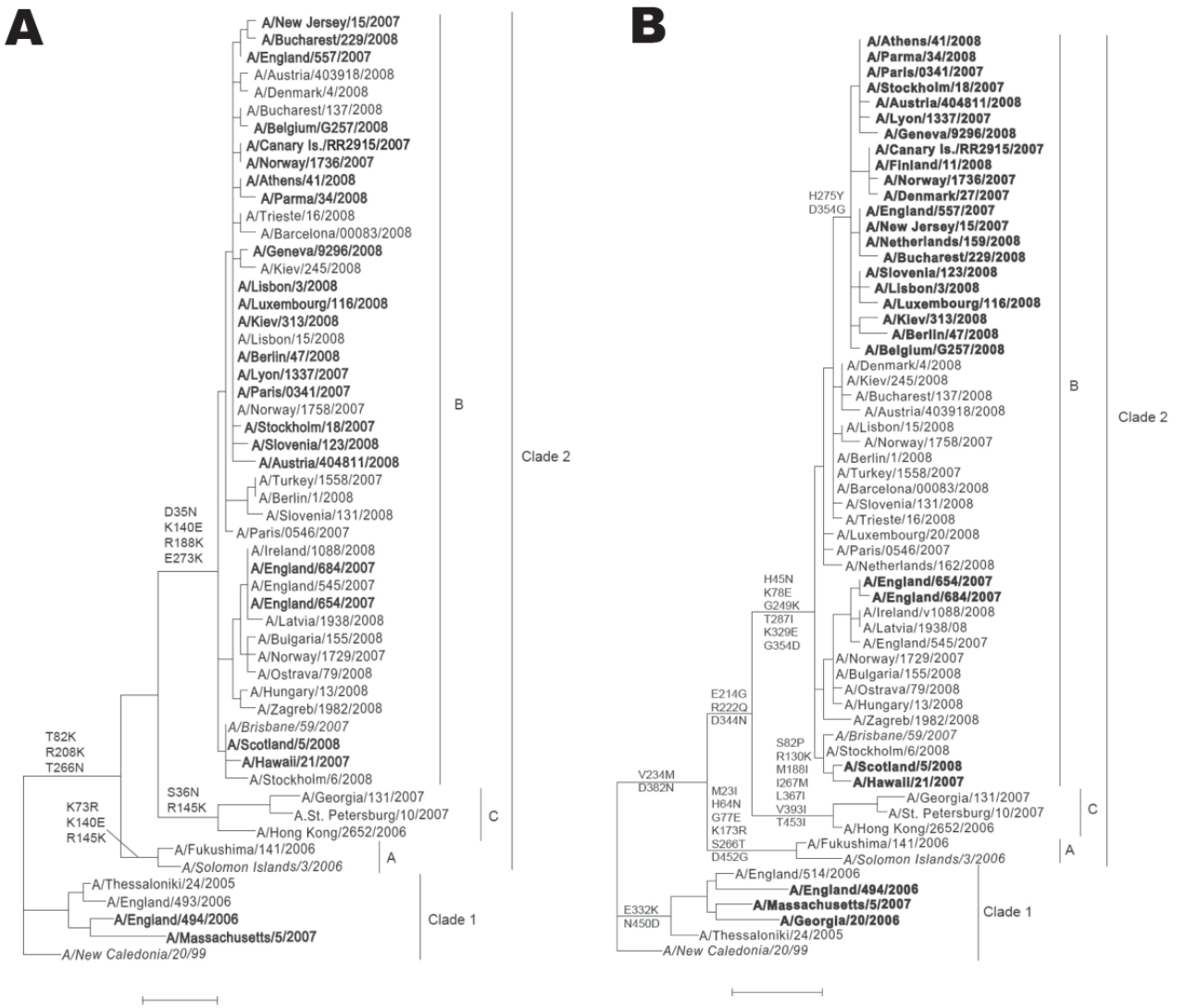


Figure 6. Phylogenetic comparisons of the hemagglutinin (A) and neuraminidase (B) genes of influenza viruses A (H1N1). Sequences of oseltamivir-resistant viruses, possessing the H275Y (H274Y in N2 numbering) mutation are in **boldface**; vaccine strains are in *italics*. Common amino acid changes that distinguish clades 1 and 2 and subgroups of clade 2 are shown. Scale bars indicate 0.01 nucleotide substitutions per site.

leled that of influenza virus A in Europe, and there was an average delay of 5.7 weeks for the appearance of ORVs after the start of influenza virus A circulation. However, the low R^2 values for both patterns make definitive conclusions difficult to draw about the spatial spread of either influenza viruses A or ORVs. Several independent introductions into European countries of a sensitive and a resistant strain might explain the low R^2 values.

Estimating whether a global focal point exists from which ORVs emerged to spread to the rest of the world is not possible, but the fact that Japan, the country with the highest per capita use of oseltamivir (5), had relatively low levels of circulating ORVs during the 2007–08 influenza season is relevant and reflects the limited circulation of the clade 2B A/Brisbane/59/2007-like viruses belonging to the European cluster in this region (*31*,*36*).

The close relationships between the NA sequences of most of the 2007–08 European ORVs and their segregation from those of OSVs suggest that resistance results in large part from the spread of a single variant. Phylogenetic analyses show that this is a property of clade 2B A/Brisbane/59/2007-like viruses and is not associated with emergence of another antigenic variant. However, identification of other resistant variants in the United Kingdom, some of which are more closely related to OSVs than to most ORVs (e.g., A/England/654/2007) indicates the independent parallel emergence of multiple resistant variants. This is emphasized by small distinct clusters of closely re-

lated ORVs in Japan that are related to European OSVs, whereas only a few of the Japanese ORVs belonged to the large European ORVs cluster (*31*). Resolution of the origin and frequency of emergence of ORVs and association with drug use clearly require substantially more intimate knowledge of the genetic relationships among OSVs and ORVs worldwide. Our observations suggest that the new genetic background of influenza viruses A (H1N1) that appeared in 2007 enabled the virus to develop oseltamivir resistance independently at several locations in the world.

The combined effect of the relatively high level of circulation of influenza viruses A (H1N1) in Europe; the introduction of a new antigenic drift variant in a susceptible population, partly related to the lack of substantial influenza virus A (H1N1) circulation since the 2000–01 season; and the uncompromised transmissibility of the ORVs contributed to the epidemiologic success of the ORVs during the 2007–08 season. This phenomenon shows clearly that continuation of antiviral susceptibility monitoring and increasing capacity for timely response are essential (*21*,*39*). In addition, the appearance of viable transmitting ORVs is a reminder that the level of resistance to oseltamivir of seasonal or pandemic virus cannot be predicted, and therefore antiviral strategies should not rely on single drugs (*40*). Although oseltamivir remains a valuable influenza antiviral agent, the emergence of natural resistance shifts attention from oseltamivir to other antiviral agents and to improved vaccination (e.g., greater vaccination coverage, more immunogenic and broadly reacting vaccines) in the fight against seasonal and pandemic influenza.

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Hantavirus Pulmonary Syndrome, Central Plateau, Southeastern, and Southern Brazil

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Hantavirus pulmonary syndrome (HPS) is an increasing health problem in Brazil because of encroachment of sprawling urban, agricultural, and cattle-raising areas into habitats of subfamily *Sigmodontinae* rodents, which serve as hantavirus reservoirs. From 1993 through June 2007, a total of 884 cases of HPS were reported in Brazil (case-fatality rate 39%). To better understand this emerging disease, we collected 89 human serum samples and 68 rodent lung samples containing antibodies to hantavirus from a 2,500-km-wide area in Brazil. RNA was isolated from human samples and rodent lung tissues and subjected to reverse transcription–PCR. Partial sequences of nucleocapsid protein and glycoprotein genes from 22 human and 16 rodent sources indicated only Araraquara virus and Juquitiba virus lineages. The case-fatality rate of HPS was higher in the area with Araraquara virus. This virus, which may be the most virulent hantavirus in Brazil, was associated with areas that have had greater anthropogenic changes.

The genus *Hantavirus* of the family *Bunyaviridae* includes a large number of rodent-borne viruses (roboviruses) that are distributed worldwide. Hantaviruses are 80–120 nm in diameter and have an envelope that contains 3 single-stranded, negative-sense segments of RNA known as small (S), medium (M), and large (L). These segments are circular because of their 5′ and 3′ complementary termini and complex with a nucleocapsid (N) protein to form individual L, M, and S nucleocapsids (*1*). The S segment encodes an N nucleoprotein, the M segment encodes a glycoprotein precursor that is processed into Gn and Gc envelope glycoproteins, and the L segment encodes a viral RNA-dependent RNA polymerase. After the first description of hantavirus pulmonary syndrome (HPS) in the United States in 1993, dozens of hantavirus genotypes have been reported in North and South America. These genotypes have been associated with wild rodent species of the subfamily *Sigmodontinae* and are transmitted to humans mostly by contact with or through aerosols of excreta and secretions of infected rodents (*2*). Nevertheless, human-to-human transmission of hantavirus has been reported in Argentina and Chile (*3*).

In South America, hantaviruses have been reported in Argentina (Andes), Chile (Andes), Uruguay (Andes), Paraguay (Laguna Negra), Bolívia (Laguna Negra and Rio Mamoré), Venezuela (Caño Delgadito), and Brazil (Araraquara and Juquitiba) (*4*). Brazil is a large country (area 8,514,215 km^2), having a predominantly tropical climate and high biodiversity that includes many important zoonoses. This biodiversity in natural ecosystems supports ≈450 of the 540 known species of *Sigmodontinae* rodents (*5*). Furthermore, a human population of ≈190 million is unevenly distributed, mostly in densely populated urban areas near the Atlantic coast of southeastern and northeastern Brazil (Brazilian Institute of Geography and Statistics, 2006, unpub. data).

HPS is an emerging health problem in Brazil because of the overlap of urban, agriculture, and cattle-raising areas with ecosystems containing several species of *Sigmodonti-*

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nae rodents that are reservoirs of hantaviruses. From 1993 through June 2007, a total of 877 HPS cases were reported in Brazil (case-fatality rate 39%): 387 in southern Brazil, 264 in southeastern Brazil, 177 in midwestern Brazil, and 49 in northern and northeastern Brazil.

Five lineages of hantavirus have been associated with most documented HPS cases: Juquitiba virus (JUQV), Araraquara virus (ARAV), Laguna Negra–like virus, Castelo dos Sonhos virus, and Anajatuba virus. HPS cases, especially those reported in northern and northeastern Brazil, were likely caused by other unknown hantaviruses (*6*–*9*; Brazilian Ministry of Health, 2007, unpub. data). Because of a lack of information about genetic diversity among hantaviruses in Brazil, we analyzed nucleotide sequences of hantaviruses infecting persons who contracted HPS and in *Sigmodontinae* rodents to better characterize genotypes and distribution of hantaviruses that cause HPS in an extensive area of Brazil. This area included the southeastern cerrado (a savanna-like ecosystem), the Central Plateau, and southern regions.

Materials and Methods

Study Area

The 2,500-km-wide study area in Brazil included the Central Plateau in the midwestern region, the southeastern and southern regions, and the Central Plateau in Goiás State and the Federal District. The Central Plateau was originally a cerrado characterized by small trees and grasses adapted to climates with long dry periods. However, during the past 3 decades the Central Plateau has been modified by farming, creation of pastures, and extensive urbanization. The southeastern region is the most densely populated region of Brazil and includes the states of São Paulo and Minas Gerais, which have cerrado in western areas and neotropical Atlantic rain forest along the coast. The rain forest is an umbrofilous tropical forest on hillsides and has high precipitation caused by an orographic effect. The southern region has Araucaria forests at higher altitudes and neotropical Atlantic rain forest along the coast. These ecosystems sustain *Sigmodontinae* rodents and have been modified, segmented, and damaged by extensive sugar cane, soybean, and coffee farming; cattle raising; and rapid and poorly planned urbanization.

Collaborative Structure

Collection and serologic analysis of hantavirus samples from humans and rodents were performed at the Adolfo Lutz Institute in São Paulo and at the Virus Research Unit of the School of Medicine, University of São Paulo in Ribeirão Preto. Initial detection of hantavirus genomic RNA was conducted at the Virus Research Unit of the University of São Paulo. Samples positive for genomic RNA were sent to the Microbiology Department of the Institute of Biomedical Sciences at the University of São Paulo for further PCR amplification, DNA sequencing, and sequence analyses.

Human and Rodent Samples

Human serum samples obtained from HPS patients during 1998–2005 were analyzed by immunoglobulin (Ig) M capture ELISA by using antigens for Andes virus and ARAV. Rodents were captured alive by using traps in rural or sylvan environments at presumed sites of HPS cases during 2002–2005 as part of routine surveillance procedures conducted by the Adolfo Lutz Institute under the mandate of the Brazilian Ministry of Health. Sampling was reviewed and approved by appropriate ethical committees on human and animal research according to Brazilian Ministry of Health Resolutions 3747/95 and 196/96. Informed consent was obtained from all patients, and information was kept confidential by the Ministry of Health. Rodent collection in the field was authorized by the Brazilian Institute of Environment and Renewable Natural Resources. Samples containing hantavirus are under control of the Ministry of Health at classified sites according to United Nations Security Council Resolution 1540 and Brazilian Ministry of Science and Technology Resolution 10. Rodent blood samples were analyzed by IgG ELISA by using antigens for Sin Nombre virus and ARAV. Rodent lungs and human serum samples positive for hantavirus were used for RNA extraction.

Extraction of RNA

RNA was extracted from 300 μL of hemolyzed whole blood or serum samples from humans and from 300 μL of a suspension of macerate of lung tissues from rodents. Samples were mixed with 1 mL of TRIzol LS Reagent (GIBCO/BRL, Gaithersburg, MD, USA) and 200 μL of chloroform–isoamyl alcohol (24:1), according to modifications described by Bowen et al. (*10*). Pellets were precipitated by centrifugation and resuspended in 10–20 μL of diethyl pyrocarbonate–treated water.

Reverse Transcription–PCR Primers, RNA Samples, and Reactions

Highly conserved regions of N and Gn genes of ARAV (GenBank accession nos. AF307325 and AF307327) were used to identify primers after aligning their nucleotide sequences to those of American hantaviruses. Two primer pairs, SAHN (amplifying 264 bp) and HANGn (amplifying 324 bp), were used (Table 1). The 2 sets of primers were used for diagnosis of hantavirus infection and DNA sequencing. Serum samples were considered positive for hantavirus if either N or Gn reverse transcription–PCR (RT-PCR) gave the expected amplicon size. Regions of hantavirus N and Gn genes were detected by RT-PCR in

Table 1. Primers used for reverse transcription–PCR of hantaviruses, Brazil, 1998–2007

Gene*/primer	Sequence (5′ → 3′)	Nucleotide annealing site
N/SAHN-C	CAAAACCAGTTGATCAACAGGG	213–236 of hantavirus small RNA segment
N/SAHN-S	GATGAATCATCCTTGAACCTTAT	454–477 of hantavirus small RNA segment
G1/HANGn-C	GGGCAGTAAGTGCTGAAAC	1301–1320 of hantavirus medium RNA segment
G1/HANGn-S	ACATTTAGCAGTTTGCCATGGG	1602–1625 of hantavirus medium RNA segment

*N, nucleocapsid; G1, glycoprotein 1.

RNA extracts of serum or hemolyzed whole blood from 19 HPS patients and RNA extracts of lung tissues from 15 rodents obtained during 2002–2005. Information on these samples is shown in Table 2.

Reverse Transcription

RT reactions were prepared in a final volume of 22 μL by mixing 5 μL of extracted RNA, 0.113 mmol/L deoxyribonucleoside triphosphates (dNTPs), 0.68 μmol/L of either SAHN-C or HANGn-C primer, and 4.4 μL of 5× RT buffer (250 mmol/L Tris-HCl, pH 8.3, 15 mmol/L $MgCl_2$, 50 mmol/L dithiothreitol). Reaction mixtures were heated at 95°C for 3 min to linearize RNA and cooled to 4°C. A total of 10 U RNase inhibitor (Pharmacia, Piscataway, NJ, USA) and 10 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia) were added to each sample, and

Table 2. Geographic origin of human and rodent sources of hantviruses, Brazil, 1999–2005

Composite taxon*	City	State	Region	Amplicon†
PR_DF_Hsp_19	Paranoá	Distrito Federal	Central Plateau	N
SS_DF_Nlas_13	São Sebastião	Distrito Federal	Central Plateau	Gn
SS_DF_Nlas_10	São Sebastião	Distrito Federal	Central Plateau	Gn, N
SS_DF_Nlas_11	São Sebastião	Distrito Federal	Central Plateau	Gn, N
SS_DF_Nlas_12	São Sebastião	Distrito Federal	Central Plateau	Gn, N
CO_GO_Hsp_20	Cocalzinho	Goiás	Central Plateau	Gn
AR_SP_Hsp_21	Araxá	Minas Gerais	Central Plateau	Gn, N
SG_MG_Nlas_8	São Gotardo	Minas Gerais	Central Plateau	Gn, N
SG_MG_Nlas_9	São Gotardo	Minas Gerais	Central Plateau	Gn, N
AG_SP_Ost_1	Aguaí	São Paulo	Southeast	Gn, N
BA_SP_Hsp_2	Batatais	São Paulo	Southeast	Gn, N
BA_SP_Hsp_1	Batatais	São Paulo	Southeast	Gn, N
CJ_SP_Hsp_3	Cajurú	São Paulo	Southeast	Gn, N
CJ_SP_Hsp_4	Cajurú	São Paulo	Southeast	N
CC_SP_Hsp_5	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Hsp_6	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Nlas_2	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Nlas_3	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Nlas_4	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Nlas_1	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CC_SP_Nlas_5	Cássia dos Coqueiros	São Paulo	Southeast	Gn, N
CV_SP_Hsp_7	Cravinhos	São Paulo	Southeast	Gn, N
GU_SP_Hsp_9	Guariba	São Paulo	Southeast	N
GU_SP_Hsp_8	Guariba-SP	São Paulo	Southeast	Gn, N
IB_SP_Nlas_6	Ibaté	São Paulo	Southeast	Gn, N
IB_SP_Nlas_7	Ibaté	São Paulo	Southeast	Gn, N
JD_SP_Hsp_10	Jardinópolis	São Paulo	Southeast	Gn, N
JU_SP_Hsp_11	Jaú	São Paulo	Southeast	Gn, N
PO_SP_Hsp_12	Pontal	São Paulo	Southeast	Gn
RB_SP_Hsp_13	Ribeirão Bonito	São Paulo	Southeast	Gn, N
RP_SP_Ako_1	Ribeirão Preto	São Paulo	Southeast	Gn, N
SC_SP_Hsp_14	São Carlos	São Paulo	Southeast	Gn, N
SE_SP_Hsp_15	Sertãozinho	São Paulo	Southeast	Gn, N
ST_SP_Hsp_17	Sertãozinho	São Paulo	Southeast	Gn, N
ST_SP_Hsp_16	Sertãozinho	São Paulo	Southeast	Gn, N
TB_SP_Hsp_18	Taubaté	São Paulo	Southeast	Gn, N
SE_SC_Oni_1	Seara	Santa Catarina	South	Gn
CX_RS_Hsp_22	AY740623	Rio Grande do Sul	South	Gn, N

*The first 2 letters indicate the city, the next 2 the state, and the next 3 the animal source (Hsp, human; Nlas, *Necromys lasiurus*; Ost, *Oligoryzomys stramineus*; Ako, *Akodon* sp.; Oni, *O. nigripis*). Numbers indicate sample number.
†N, nucleocapsid; Gn, glycoprotein.

samples were incubated for 2 h at 37°C. The cDNA was used as a template in the subsequent PCR or stored at –20°C.

PCR

PCRs were conducted in 50-μL volumes containing 0.3 μM of SAHN or HANGn primer pairs, 0.05 mmol/L dNTPs, 3 μL RT products, and 5 μL 10× buffer (100 mmol/L Tris-HCl, pH 8.5, 500 mmol/L KCl). Mixtures were heated to 80°C, 1U of thermostable Taq DNA polymerase (Pharmacia) was added, and mixtures were subjected to 35 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 240 s. Samples were then subjected to an elongation step at 70°C for 5 min to promote DNA strand extension. Eight microliters of amplified DNA was subjected to electrophoresis on 1.7% agarose gels in Tris-acetate-EDTA buffer and stained with ethidium bromide. Amplicons were visualized by using a 312-nm UV transilluminator, and their sizes were determined by comparison with a 100-bp DNA ladder.

Nucleotide Sequencing

Direct nucleotide sequencing of N (261 bp) and Gn (324 bp) PCR products was performed by using the Big Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) with 25 cycles at 95°C, 55°C for 45 s, and 72°C for 180 s. Products were analyzed in ABI PRISM 3100 and ABI 377 sequencers (Applied Biosystems). Sequences obtained were deposited in GenBank (accession nos. EU170162–EU170239).

Phylogenetic Analysis

Nucleotide sequences of N and Gn genes were aligned with those of orthologs from other South American hantaviruses (Table 3) by using ClustalX version 8 software (*11*). Alignments were edited by using SeAl version 2.0a11 software (http://tree.bio.ed.ac.uk/software/seal). Preliminary analysis of N and Gn amplicons and references (Table 2) (concatenated in a 573-member dataset that included 58 taxa for which N and Gn are available) or individual genes by using the RDP3 program (*12*) indicated no intergene recombination or reassortment that would be detected by recombination at the border between N and Gn genes in the 573-bp concatamer. This finding confirmed our previous results (*13*).

To improve the phylogenetic signal, we used the 573-bp contamer and inferred the maximum clade credibility (MCC) tree for 58 South American hantaviruses by using BEAST version 1.4.8 software (*14*) and the Bayesian Skyline model under exact conditions as described (*13*). The MCC tree was sampled from 20 million trees after Markov chain Monte Carlo algorithm sampling was stable after a preburning stage of 30 million chains. Seven of 38 taxa had neither N nor Gn sequences (Table 2), but this absence did not preclude use of tree-based Bayesian methods and maximum likelihood methods. These methods account for missing nucleotides as undefined character-states during phylogenetic reconstruction and do not use global pairwise distances. Statistical support for clustering of newly iso-

Table 3. Reference hantavirus gene sequences

Vírus	Country of origin	GenBank accession nos.	
		Glycoprotein sequences	Nucleocapsid sequences
Laguna Negra	Paraguay	AF005728	AF005727
Lechiguanas	Argentina	AF028022	AF482714
Oran	Argentina	AF028024	AF482715
Pergamino	Argentina	A028028	AF482717
Araraquara Lutz	Brazil	AY970821	None
Araraquara Johnson	Brazil	AF307327	AF307325
Andes Chile	Chile	AY228238	AY228237
Andes AH-1	Argentina	AF324901	AF324902
Choclo	Panama	DQ285047	DQ285046
Cano Delgadito	Paraguay	DQ284451	DQ285566
Juquitiba	Brazil	AY963900	EF446280
Rio Mamoré	Bolivia	AY953445	U13455
Araucária HR0271	Brazil	None	AY740624
Araucária HR0150	Brazil	None	AY740622
Araucária HR4101	Brazil	None	AY740632
Araucária HR0273	Brazil	None	AY740626
Araucária HR0272	Brazil	None	AY740625
Araucária HR4102	Brazil	None	AY740633
Araucária HR0399	Brazil	None	AY740630
Araucária HR0395	Brazil	None	AY740628
Araucária HR0397	Brazil	None	AY740629
Araucária HR3100	Brazil	None	AY740631
Araucária HR0285	Brazil	None	AY740627
Araucária HR0155	Brazil	None	AY740623

lated viruses was evaluated by running 500 nonparametric bootstraps with GARLI version 0.95 (*15*) and PhyML version 2.4.5 software (*16*). We also conducted a 5% jackknife resampling analysis of 1,000 neighbor-joining trees by using maximum likelihood distance with PAUP version 4b10 software (*17*). The best evolutionary model was estimated with GARLI software (i.e., general time reversible + Γ + I model).

Case-Fatality Rate of HPS in Brazil

The Central Plateau and southeastern region of Brazil are covered mostly by cerrado, where *Necromys lasiurus*, the reservoir of ARAV, is enzootic. The HPS case-fatality rate for this area was compared with the HPS case-fatality rate for the southern region, which is characterized by Atlantic rain forest and Araucaria subtropical forest environments. In these environments, *Oligoryzomys nigripes*, a reservoir of JUQV, is enzootic. HPS case-fatality rates for the Central Plateau and southeastern region were compared with HPS case-fatality rates for hantaviruses in other regions of Brazil. These comparisons were made by using the Fisher exact test at a 5% significance level (Brazilian Ministry of Health, 2007, unpub. data).

Results

Composite Phylogenetic Tree of N and Gn Genes

We studied 89 human samples and 68 rodent samples that had positive serologic results; N and Gn genes from 22 human and 16 rodent sources were obtained. The N/Gn composite tree (online Appendix Figure, available from www.cdc.gov/EID/content/15/4/561-appF.htm) indicates that human and rodent samples were associated with ARAV or JUQV reference strains with posterior probabilities >0.9. As expected, ARAV samples from rodents and humans in São Paulo State, the Central Plateau, and Minas Gerais State (on the border of the plateau) are mixed in the tree because human cases are almost always derived from rodents (online Appendix Figure). Nevertheless, highly supported clusters of ARAV suggest that viruses may show some geographic partitioning (e.g., clusters of isolates from *N. lasiurus* in São Sebastião in the Central Plateau differ from clusters from São Paulo in the southeastern region). Furthermore, 13 of 14 ARAV samples obtained from rodents were from *N. lasiurus* and 1 was from *Akodon sp*. All rodent and human ARAV samples were from the Central Plateau (cerrado) or southeastern region (tropical rain forests). Distribution of ARAV extends 1,000 km across the dry northern cerrado region and includes western São Paulo State to the boundary of the Atlantic rain forest in the southeastern region.

ARAV are more related to Pergamino virus than to JUQV (online Appendix Figure). Pergamono virus infects *A. azarae*, which inhabit Argentina, and JUQV infects *Oligoryzomys* spp., which inhabit southern Araucaria and eastern Atlantic forests. Two rodent samples nested with JUQV (high posterior probability 0.93) 100% of the time during jackknife resampling and 86% of the time during 500 nonparametric bootstrap iterations (online Appendix Figure). These samples were identified as AG_SP_Ost_1 isolated from *O. stramineus* in Aguaí, São Paulo, and SE_SC_Oni_1 isolated from *O. nigripis* in Seara, Santa Catarina State located in southern Brazil near the northern border with Argentina (Table 2). These data indicate that these 2 samples were divergent lineages of JUQV infecting *Oligoryzomys* spp. Detection of AG_SP_Ost_1 in Aguaí, São Paulo, was expected because Itapuã JUQV is also found in the adjacent Atlantic rain forest. Two additional samples, SG_MG_Nlas_8 from *N. lasiurus* in São Gotardo, Minas Gerais State (Central Plateau), and CX_RS_Hsp_22 from a human case in Caxias do Sul, Rio Grande do Sul (southern region), appeared to have some distant association with ARAV, JUQV, and Oran hantavirus, which are associated with *O. longicaudatus* from subtropical Oran Department in Argentina. This association was indicated by the low support of nodes connecting these 3 virus lineages to SG_MG_Nlas_8 and CX_RS_Hsp_22. Although more data are needed to better understand these associations, the most parsimonious explanation is that a hantavirus associated with *Necromys* spp./*Akodon* spp. that originated in the southern region may have been the source of the lineage that led to *Oligoryzomys* spp.–associated JUQV, which has lineages across the Araucária pine forests and along the rain forests into southeastern Brazil.

Virulence of Hantaviruses Determined by Case-Fatality Rate

The Central Plateau and southeastern and southern regions contain >80% of the HPS cases reported in Brazil in 2007. However, the case-fatality rate of HPS was distinct in these and other regions. These rates were 44.5% (149 deaths in 335 reported cases) in areas with ARAV in the Central Plateau and the southeastern region and 35.4% (192 deaths in 542 cases) in areas with ARAV in the remaining southern regions. This difference was statistically significant ($p = 0.011$, by Fisher exact test, and χ^2 3.0978, df 1, $p<0.1$). The case-fatality rate of HPS in the Central Plateau and southeastern regions with ARAV (44.5%) was significantly higher than in southern regions with JUQV (32.5%, 126 deaths in 387 cases) ($p = 0.0051$, by Fisher exact test, χ^2 4.8293, df 1, $p<0.05$) (Brazilian Ministry of Health, 2007, unpub. data). Although some geographic overlap of ARAV and JUQV occurs, these results suggest that ARAV strains may have higher virulence than JUQV or other hantaviruses in Brazil.

Discussion

The MCC composite tree (online Appendix Figure) shows that all 38 samples from HPS patients and from rodents captured near human cases were related to ARAV or JUQV groups. Moreover, ARAV lineages obtained in the Central Plateau or southeastern region grouped in a robust monophyletic group independent of human or rodent origin. This finding reinforces the probability of zoonotic origin of human cases from a rodent-borne zoonotic pool and supports the view that closely related hantavirus lineages associated with distinct rodent species may be experiencing cross-species transmission (spillover) (*13*,*18*). ARAV appears to have dispersed from the cerrado region toward the southeast throughout São Paulo, as indicated by the basal position of the SS_DF_Nlas cluster in the MCC composite tree (online Appendix Figure). JUQV dispersed across the southern temperate Araucaria pine forests and along the Atlantic rain forest, as shown by the basal position of SE_SC_Oni_1.

Several associations in the ARAV clade suggest movement of infected persons or dispersal of infected rodents. TB_SP_Hsp_18, which was isolated from a human case of HPS, was closely related to viruses from the Central Plateau. JU_SP_Hsp_11, ST_SP_Hsp_16, and AR_SP_Hsp_21 from the southeastern region were closely related to CO_GO_Hsp_20 from the Central Plateau. Data also indicated that genetic diversity of JUQV is greater than previously determined. SE_SC_Oni_1 from *O. nigripes* and AG_SP_Ost_1 from *O. stramineus* were basal taxa to JUQV with high support but may be the most divergent lineages so far detected. Levels of support in the MCC tree indicated that SG_MG_Nlas_8 and CX_RS_Hsp_22 would always branch from poorly defined nodes in the tree in a basal position relative to ARAV-Pergamino, JUQV, and Oran lineages. These results suggest that SG_MG_Nlas_8 and CX_RS_Hsp_22 may represent distinct lineages linking Argentinean Oran and Pergamino hantaviruses to lineages from which ARAV and JUQV originated.

Rooting of the tree and lineage associations suggest that ARAV and JUQV may have originated from a *Necromys* spp./*Akodon* spp.–associated hantavirus from the southern part of South America. This theory supports our finding of 1 *N. lasiurus* infected by ARAV (SG_MG_Nlas_9) and another *N. lasiurus* infected by a highly divergent lineage (SG_MG_Nlas_8) that outgroups with ARAV and JUQV. Both rodents were collected in São Gotardo in Minas Gerais State, at the edge of the central plains. The role of distinct rodent species as potential reservoirs and sources of human infection in Brazil and South America needs to be better understood. However, our data corroborate reports showing that *N. lasiurus* is a reservoir of ARAV, that *O. nigripes* is a reservoir of JUQV, and that rodents transmit hantaviruses to humans (*6*–*8*).

Rodent behavior is a factor in transmission of hantaviruses to humans, and *N. lasiurus* is an opportunistic and aggressive rodent species that is gradually being encroached upon in environments experiencing anthropogenic change in the southeastern region and the Central Plateau of Brazil. Conversely, *O. nigripes* has adapted to the Atlantic and Araucaria pine forests and has been found in lineal natural habitats bordering cultivated areas (*5*,*7*,*19*). Detection of AG_SP_Ost_1 in *O. stramineus* in the *O. nigripis*–associated JUQV group could be explained by virus spillover because *O. stramineus* is usually not infected with hantaviruses. Sampling also identified RP_SP_Ako_1 in an *Akodon* sp. rodent in Ribeirão Preto northwest of São Paulo State. This isolate branches in the tree within a well supported cluster of HPS cases reported in areas ≥50 miles of Ribeirão Preto. Distribution of *Akodon* spp. rodents includes the pampas grasslands of Argentina, and Bolivia, Paraguay, Uruguay, and southern Brazil. These rodents are known to be associated with hantaviruses in Argentina. Not yet determined is whether an *Akodon* sp. is transmitting hantavirus to humans in Ribeirão Preto (west of São Paulo State) and whether this infection was a recent cross-species transmission event from *N. lasiurus* to *Akodon* sp.

Samples we analyzed came from an extensive area that contained natural ecosystems largely degraded as a consequence of intensive agriculture and cattle raising. This region contains nearly one third of the population of Brazil in hundreds of towns and several large cities that grew during the 20th century. Other than agricultural expansion over a pristine environment, explosive and poorly planned urban expansion has also been responsible for degrading surrounding ecosystems. A recent estimate of the short-term rate of evolution of hantaviruses in South America indicates that divergence and spread of ARAV and JUQV is relatively recent, possibly within the past 200 years (*13*). Thus, increasing environmental and demographic changes during the past 100 years likely affected the ecology of wild rodent reservoirs and facilitated human infections and the emergence of HPS that we now observe.

Whether ARAV and JUQV differ in pathogenicity is unknown A possible distinction between these 2 groups is that the case-fatality rate in regions where ARAV has been isolated appears to be higher than that for JUQV. Higher case-fatality rates of HPS cases in regions with ARAV (Central Plateau and southeastern region) than in regions with JUQV (southeastern coast and southern region) suggest that ARAV is the most virulent hantavirus detected in Brazil (*6*,*7*,*19*). However, although ARAV has produced the most severe forms of HPS, many infections with ARAV are benign. This finding was observed in São Paulo State, where a serologic survey in Jardinópolis County showed that 14.3% of 32,000 local inhabitants had IgG antibodies to hantavirus (*20*), indicating that many persons were

exposed to the hantavirus but did not have severe clinical symptoms. Further study is needed to determine which factors influence the severity of disease manifestation in humans caused by infections with hantaviruses, specifically with ARAV or JUQV (*6*–*8*).

We have identified the viruses circulating in our study area as ARAV and JUQV. Based on geographic distribution of these viruses and the assumption that no other unknown lineage is causing disease in humans, we suggest that ARAV may be responsible for >50% of HPS cases reported in Brazil. ARAV was associated with areas experiencing greater anthropogenic changes and disorganized human population growth than other more stable areas. ARAV may be the most virulent hantavirus in Brazil.

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Rift Valley Fever, Mayotte, 2007–2008

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After the 2006–2007 epidemic wave of Rift Valley fever (RVF) in East Africa and its circulation in the Comoros, laboratory case-finding of RVF was conducted in Mayotte from September 2007 through May 2008. Ten recent human RVF cases were detected, which confirms the indigenous transmission of RFV virus in Mayotte.

Rift Valley fever (RVF) is a zoonotic and mosquito-borne disease that affects animals and humans (*1*). In humans, RVF infection is usually asymptomatic or characterized by an acute self-limited febrile illness. However, in ≈1%–3% of case-patients, it can cause serious manifestations that may lead to death or disabling sequelae in survivors (*2*,*3*). Identified in the 1930s in Kenya, RVF virus (RVFV) circulates now in many other African countries (*4*–*7*), as well as on the Arabian peninsula (*8*), where epizootics and associated human cases have been reported.

In early 2007, RVF outbreaks were reported in several eastern African countries (*9*). Additionally, in August 2007, autochthonous RVF transmission was found on the Indian Ocean island of Grande Comore, Republic of Comoros. A young Comorian with a 2-month history of severe encephalitis was transferred from the Republic of Comoros to Mayotte, where diagnostic and medical care facilities are more readily available. Mayotte, a French territory, is located northwest of Madagascar, in the Comoros archipelago (Figure).

Anti-RVFV immunoglobulin (Ig) M was detected by IgM antigen-capture ELISA (*10*) in the serum sample of this patient. This sentinel event had substantial public health implications for Mayotte because the Republic of Comoros and Mayotte have frequent exchanges of populations and goods, both legally and illegally.

An active laboratory-based surveillance system for RVF was implemented for livestock in Mayotte in March 2008, which has led to several recent RVF cases being detected in cattle (Bureau of Veterinary Services, Mayotte, unpub. data). Subsequently, to assess the situation of RFV among humans, we retrospectively and prospectively analyzed serum specimens from febrile patients sampled by a routine surveillance program for chikungunya and dengue viruses. We describe the clinical, biological, and epidemiologic characteristics of human RVF cases in Mayotte.

The Study

Since the 2005–2006 chikungunya outbreak (*11*), laboratory-based surveillance of chikungunya virus (CHIKV) and dengue virus (DENV) is routinely maintained in Mayotte. Specimens are collected from patients attending local primary healthcare facilities or hospitals who seek treatment for dengue-like illness, i.e., acute onset of high fever accompanied by at least 2 of the following signs: arthralgia, body pain, headache, malaise, fatigue, or rash. Stored patient samples collected from September 2007–March 2008 and prospective specimens from this system, which initially tested negative for CHIKV and DENV as well as for *Plasmodium* spp. by using the rapid diagnostic test OptiMAL (Flow Inc., Portland, OR, USA), were screened for RVFV. We defined a confirmed recent human case-patient as a person with dengue-like illness and the serum positive for anti-RVFV IgM by antigen-capture ELISA or RVFV RNA by reverse transcriptase–PCR (*12*). Any specimen positive when tested by the local reference laboratory was then forwarded for confirmation to the National Reference Centre for Arboviruses and the World Health Organization Collaborating Centre for Arboviruses, Pasteur Institute, Paris, France. Identified case-patients were investigated by using a standardized form to gather information, particularly regarding exposure data, e.g., peridomestic environment, handling of animal products, occupational exposures, and travel history. The relevant period of exposure was 3 weeks before

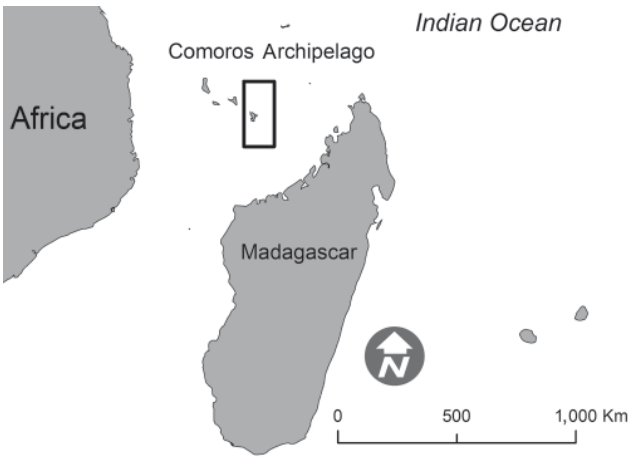

Figure. Location of Mayotte (boxed) in the Comoros Archipelago. Source: Préfecture de Mayotte.

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disease onset. Medical records of confirmed case-patients were also reviewed if needed. Investigations were conducted in accordance with the French National Institute of Public Health (Institut de Veille Sanitaire) guidelines for studies conducted in rapid response to public health threats.

From September 1, 2007, through May 31, 2008, 220 serum specimens from persons with dengue-like illness who tested negative for *Plasmodium* spp., CHIKV, and DENV were screened; recent RVFV infection (i.e., presence of viral RNA or IgM) was found in 10 samples (4.5%). The earliest recorded onset of dengue-like illness was September 27, 2007, and the latest was May 14, 2008. Seven cases (70%) occurred from January through April, during the hot, rainy season (Table). Confirmed case-patients were not clustered spatially and resided in the following counties: Mamoudzou (3), Bandraboua (2), Dembeni (1), Sada (1), Chirongui (2), and Boueni (1).

Of these confirmed-case patients, 9 (90%) were male (age range 16–53 years, median 27.5 years), 5 (50%) were French citizens native to Mayotte, and 5 (50%) were Comorian citizens. None of the cases reported recent (i.e., within 3 weeks before onset of illness) travel into countries with RVF endemic transmission.

Although 2 patients were admitted to the intensive care unit for medical management, no severe neurologic manifestations of RVF were observed, and no deaths could be attributed to RVF. Case-patient 6 (Table), with a coexisting condition of cirrhosis after hepatitis B infection, had substantial thrombopenia (< 40,000 cells/mm^3) and died as a result of gastrointestinal bleeding and hepatic encephalopathy. Case-patient 10 was admitted to the hospital in May 2008 with a 5-day history of high body temperature, body pain, joint pain (particularly in the shoulders), and fatigue, combined with symptoms consistent with right-sided heart failure (shortness of breath, hepatomegaly, and peripheral edema). The patient was discharged with a diagnosis of pericarditis. He had a follow-up visit 1 month later, and a sample obtained on this occasion tested positive for RVFV IgM and IgG.

Predictably, contact with ruminants (sheep, cattle, or goats) was the predominant means of exposure among reported case-patients; this mode was documented in 5 of 9 cases that were fully investigated. Furthermore, only the presence of numerous breeding sites for mosquitoes in the housing environment remained as a possible means of exposure for 3 other case-patients. The mode of transmission remains undetermined for the remaining case-patient, who had no admitted direct contact with ruminants and whose housing environment did not have numerous breeding sites (Table).

Table. Epidemiologic, clinical, and laboratory findings of 10 case-patients with confirmed recent Rift Valley fever virus infection, Mayotte, France, September 2007–May 2008*

Case-patient no.	Age, y/sex, occupation	Identified source of exposure(s)	Onset date; early signs and symptoms	Laboratory findings
1	47/M, gardener	Herding animals	2007 Sep 27; 1-day history of fever, arthralgia, body pain	RT-PCR positive, AST 9N, ALT 4N
2	42/F, shopkeeper	Contact with aborted animal	2008 Jan 5; 2-day history of fever, headache, malaise	RT-PCR positive
3	21/M, unknown	Unknown	2008 Jan 19; chronic hepatitis B, 3-day-history of fever, arthralpgia, and headache	RT-PCR positive, AST 4 N, ALT 3N, elevated CPK level, thrombocytopenia
4	19/M, farmer and herder of animals	Milked animal	2008 Jan 21; 2-day history of fever, joint pain, headache, malaise	RT-PCR positive
5	16/M, student	Killed and skinned sick animals	2008 Jan 21; 2-day history of fever, joint pain, headache	RT-PCR positive
6	44/M, unemployed	Mosquito breeding sites in rural household	2008 Mar 13; 2-day history of fever, body aches; hepatic encephalopathy	RT-PCR positive (threshold values), RVF IgM positive
7	19/M, student	Undetermined	2008 Mar 19; 1-day history of fever, myalgia, retro-orbital pain, joint pain, body aches, malaise	RT-PCR positive
8	24/M, handyman	Numerous mosquito breeding sites in household	2008 Mar 28; 5-day history of fever, joint pain, myalgia, nausea/vomiting	RT-PCR positive at threshold, RVF IgM positive, thrombocyopenia
9	32/M, construction worker	Mosquito breeding sites in rural household	2008 May 5; 7-day history of fever, myalgia, joint and body pain, malaise	RVF IgM positive, elevated ALT, AST, and CPK levels
10	53/M, farmer	Slaughtering and butchering animals	2008 May 14; 5-day history of fever, joint pain, body aches and symptoms of right-sided heart failure, discharge diagnosis of pericarditis; new episode of fever, fatigue, and shortness of breath 30 days later and screening for RVF	RVF IgM positive

*RT-PCR; reverse transcription–PCR; AST, aspartate aminotransferase; N, upper limit of normal titers; ALT, alanine aminotransferase; CPK, creatine phosphokinase; RVF, Rift Valley fever; Ig, immunoglobulin.

Conclusions

The laboratory case-finding initiated in response to the 2006–2007 RVFV outbreaks in eastern Africa found indigenous transmission in Mayotte. As a result, information was provided to the population, and preventive activities were undertaken, e.g., information for healthcare providers, a campaign to eliminate mosquito-breeding sites, recommendations to avoid contact with mosquitoes and sick animals, and instructions on adopting appropriate protective measures when engaging in activities related to animals, including slaughtering and butchering.

Because our investigations were mostly retrospective, we cannot exclude recall bias about exposure and incomplete documentation. Moreover, entomologic investigations in the living areas of the case-patients several months after the first signs of illness occurrence were incomplete.

RVF activity in Mayotte appears to be an expansion of the eastern Africa outbreak. As in the case of the chikungunya outbreak in 2005–2006 in Mayotte, thought to have started in Lamu, Kenya (*13*), RVFV circulation illustrates once again the risk of introduction or circulation in Mayotte or other Comorian islands of infectious agents involved in outbreaks in neighboring eastern African coastal countries (*14*). This risk is particularly important because of the favorable climatic conditions and the presence in Mayotte of replication-competent vectors of numerous arboviruses.

This epidemic is a further illustration that it is imperative for healthcare providers, epidemiologists, and policy makers in Mayotte to remain alert each time there are outbreaks in nearby countries because the risk of extension to Mayotte is always plausible. Furthermore, the documentation of RVFV circulation in Mayotte should serve as a further impetus toward the development of surveillance programs for nonspecific febrile illnesses, especially in settings where arboviruses are highly prevalent. To achieve this objective, local laboratory capabilities should be enhanced to enable biological diagnosis of a wide array of arboviruses that actively circulate in the eastern African seaboard region. Furthermore, medical education materials and feedback bulletins should regularly provide information about accessing laboratory services while encouraging patients with febrile illness to seek early medical evaluation. Ultimately, maintaining such programs is essential to promptly detecting any renewed introduction or resurgence of RVF and other pathogens, especially during the hot and rainy season.

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High Prevalence of Spirochetosis in Cholera Patients, Bangladesh

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The microbes that accompany the etiologic agent of cholera, *Vibrio cholerae*, are only now being defined. In this study, spirochetes from the genus *Brachyspira* were identified at high titers in more than one third of cholera patients in Bangladesh. Spirochetosis should now be tracked in the setting of cholera outbreaks.

Cholera in humans results in profuse, watery diarrhea that can lead to severe dehydration and death (*1*). The infection begins with ingestion of *Vibrio cholerae* from contaminated water, after which expression of cholera toxin induces a fluid loss that may reach 1 liter/hour. Cholera is an underreported disease in the developing world, and the true incidence may reach 2 million cases/year (*2*).

Because anecdotal evidence has indicated frequent cholera and intestinal spirochetosis coinfection in Bangladesh, we studied both diseases in this study. Intestinal spirochetosis has negative effects on domestic swine and poultry industries (*3*). In humans, 1%–64% of colonic specimens demonstrate intestinal spirochetosis, with the highest prevalence in developing countries and immunocompromised populations (*4*). The etiologic agents of intestinal spirochetosis are members of the genus *Brachyspira* (formerly *Serpulina* and *Treponema*). *B. pilosicoli* isolated from humans cause disease in pigs and in chicken models of infection (*5*,*6*). *B. aalborgi* isolated from humans do not colonize animals (*7*). Surveillance of intestinal spirochetosis requires molecular tools because culture has limited sensitivity caused by the fastidious nature of *Brachyspira* spp. (*8*). Patients with symptomatic intestinal spirochetosis have chronic diarrhea or soft feces (*4*,*9*,*10*); these clinical signs may resolve with antimicrobial drug therapy (*9*). Histologic analysis of intestinal biopsy specimens showed densely packed spirochetes attached by 1 end to colonic epithelium, forming a false brush border (*11*). Invasion of colonic epithelial cells and bleeding may occur (*4*,*12*). Virulence mechanisms remain poorly understood. Research on a vaccine to protect pigs against intestinal spirochetosis has begun. However, there is no vaccine for protection against intestinal spirochetosis in humans.

Co-infection of cholera patients with additional pathogens has focused on enterotoxigenic *Escherichia coli* (ETEC); 13% of cholera patients in Bangladesh are co-infected with ETEC (*13*). The potential for pathogenic synergy between *V. cholerae* and other pathogens has been proposed but not investigated.

The Study

We defined the frequent presence of spirochetes in feces of patients with cholera. A 3-step method was used to establish the distribution of spirochetes in rice-water stool: 1) the presence of spirochetes in rice-water stool was determined by using dark-field microscopy; 2) the diversity of *Brachyspira* spp. within a subset of these patients was quantified by 16S rDNA analysis; and 3) the genetic diversity within the most abundant species was determined by analysis of NADH oxidase (*nox*).

Rice-water stool samples were collected from symptomatic cholera patients (≥15 years of age with no history of antimicrobial drug therapy) during the spring cholera outbreak of 2006 in Dhaka, Bangladesh, at the International Centre for Diarrheal Disease Research, Bangladesh, as part of a larger study (*14*). Samples were examined by dark-field microscopy for *V. cholerae* and other bacteria, and the presence of *V. cholerae*, lytic vibriophage, and ETEC was determined by using standard methods (*14*). Samples were preserved at 4°C in formalin, or cell pellets were resuspended in phosphate-buffered saline and 15% glycerol and stored at –80°C. Analysis showed that 36% (23/64) of samples that were positive for only *V. cholerae* also harbored spirochetes (online Technical Appendix, panels A–F, available from www.cdc.gov/EID/content/15/4/571-Techapp.pdf). Spirochetes were also found in 4/11 and 5/15 samples that harbored ETEC alone or both ETEC and *V. cholerae*, respectively. When we removed ETEC-positive samples as potential confounders, the presence of spirochetes was independent of lytic vibriophage in *V. cholerae*–positive stool samples (online Technical Appendix, panel G), which is in contrast to the documented trend concerning non–*V. cholerae* bacteria in rice-water stool (*14*). The ratio of spirochetes to

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V. cholerae was ≈1 and independent of lytic vibriophage (online Technical Appendix, panel H).

In the second step, 10 samples were chosen for molecular analysis on the basis of a large amount of spirochetes. Samples were heated (99°C for 10 min), and standard PCR for a diagnostic segment of the 16S rDNA gene (*15*) was performed by using genus *Brachyspira*–specific primers (5′-GTCTTAAGCATGCAAGTC-3′ and 5′-AACAGGCTAATAGGCCG-3′). Products were cloned and sequenced bidirectionally (GenBank accession nos. FJ599620–FJ599639). *B. pilosicoli* was the most common species, found in all 10 samples (online Technical Appendix, panel I). *B. aalborgi* was the second most common species (7 samples). A second set of panspecific 16S rDNA degenerate primers (5′-GTTTGATYCTGGCTYAGARCKAACG-3′ and 5′-CCSSTACGGMTACCTTGTTACG-3′) confirmed the presence of *Brachyspira* spp. and suggested that spirochetes of other genera were not present. The added resolution of *nox* analysis also identified *B. hyodysenteriae* at lower abundance.

Culture, purification, and microscopy of *B. pilosicoli* from glycerol stocks was used to cross-validate the molecular approach. Standard fastidious anaerobe agar (FAA) supplemented with bovine blood (10%), spectinomycin (400 μg/mL), and polymyxin B (5 μg/mL) was determined empirically to be the optimal medium for isolation of spirochetes. Dilutions of glycerol stocks from each patient were plated on FAA agar; single colonies were best obtained with an FAA overlay. Plates were incubated for 21 days at 37°C in an atmosphere of 94% H_2 and 6% CO_2. Most colonies gave rise to sigmoidal spiral cell morphotypes similar to the morphotype of the American Type Culture Collection (ATCC) (Manassas, VA, USA) control strain for *B. pilosicoli*. Additional controls were ATCC *B. hyodysenteriae*, *Helicobacter pylori*, and *Borrelia burgdorferi*. Subsequent molecular analysis of patient isolates confirmed them as *B. pilosicoli*. Consistent with previous studies, *B. aalborgi* was not cultured from patient samples.

In the third step, 5 samples were analyzed by using *nox* sequence comparisons that yield higher phylogenetic resolution. DNA was extracted and a *nox* segment was amplified by using degenerate primers (5′-GCYGGHACATGGGCDGCAAAAAC-3′ and 5′-CAAATACRCAAATAGCRTTAG-3′). Products were cloned and sequenced bidirectionally (GenBank accession nos. FJ599589–FJ599619). *B. pilosicoli* was the most common *nox* sequence found. A phylogenetic tree (Figure) demonstrated that individual patients harbored clonal lines of *B. pilosicoli* (patients B, D, and E) or more diverse strains (patient A). Overall, *B. pilosicoli* strains found in cholera patients were extremely diverse relative to the known outgroup species, which indicates the potential for detection of new species related to intestinal spirochetosis.

Conclusions

It has been casually observed for a century that stool from cholera patients harbors spirochete-like bacteria. We now define the major agents present as *B. pilosicoli* and *B. aalborgi*. More than one third of the cholera patients had spirochetes in their stools at densities equal to those of *V. cholerae*. The pathophysiology of intestinal spirochetosis in this setting and its relevance to human health remain unknown. Epidemiologic analysis of intestinal spirochetosis has so far relied on retrospective studies of colonic tissue collected for reasons secondary to the disease (*4*). We recommend a community-based prospective study of stool samples from healthy persons and patients with diarrhea by using the techniques described herein; animal reservoirs should be identified as a potential point of control. In the context of *V. cholerae* infection, we hypothesize that spirochetes may be present before *V. cholerae* infection and exacerbate the already devastating clinical course of cholera.

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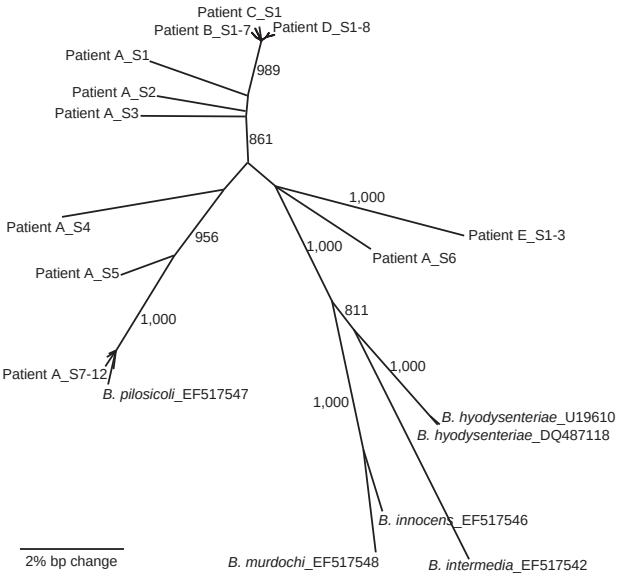

Figure. Neighbor-joining (NJ) phylogeny of NADH oxidase (*nox*) sequences of *Brachyspira pilosicoli* from 5 cholera patients (A–E). The *nox* sequences were PCR amplified, cloned, and sequenced from each patient (individual clones are appended _SX). Published sequences from known species are included for reference. NJ analysis was performed by using an NJ model and 1,000 bootstraps. Bootstrap values >800 are presented next to nodes. The scale bar indicates a 2% bp change (contiguous sequence ≈990 bp).

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Genetic Diversity of Toscana Virus

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Distribution of Toscana virus (TOSV) is evolving with climate change, and pathogenicity may be higher in nonexposed populations outside areas of current prevalence (Mediterranean Basin). To characterize genetic diversity of TOSV, we determined the coding sequences of isolates from Spain and France. TOSV is more diverse than other well-studied phleboviruses (e.g.,Rift Valley fever virus).

Toscana virus (TOSV) belongs to the family *Bunyaviridae* and genus *Phlebovirus*. According to the Eighth Report of the International Committee on Taxonomy of Viruses, phleboviruses are classified into 9 serocomplexes (*1*), 1 of which includes Rift Valley fever virus (RVFV), a deadly pathogen for cattle and humans. TOSV belongs to the sandfly virus serotype Naples serocomplex. Phleboviruses have segmented RNA genomes comprised of 3 units: L (large), M (medium), and S (small). Based on the analysis of the G_N glycoprotein (M segment), 4 lineages of TOSV have been proposed (*2*). Moreover, phylogenetic analysis has demonstrated that TOSV isolates from Spain differ from the original isolates from Italy, TOSV strain ISS Phl.3 (*3*).

TOSV is widespread in the Mediterranean Basin, and evidence of human infection has been found in Italy, France, Spain, Portugal, Cyprus, and Turkey. The main clinical manifestation is neurologic dysfunction (*4*). Although the virus is an important pathogen (*4*), little genomic information is available for TOSV. We believe that obtaining genome sequence information for viruses with poor representation in public databases is an urgent task for the virologic community, especially for programs of virus surveillance and study of emerging pathogens. The increasing popularity of nucleic acid–based methods of detection underscores the need for a deeper knowledge of sequence variability of wild-type strains to ensure the sensitivity and specificity of the diagnostic assays. We therefore characterized the genetic diversity of TOSV by determining the coding sequences of isolates from Spain and France.

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The Study

Viruses were recovered from Vero E6 (ATCC CRL-1586) cell cultures of cerebrospinal fluid samples (CSF) from 2 TOSV-infected patients and from a pool of sandflies. The first patient was a 30-year-old man living in a rural area of Granada, Spain. He had been admitted to hospital with headache, nausea, and fever; he was found to have meningeal and CSF pleocytosis. TOSV ESH 62100 was detected in an acute-phase CSF sample by reverse transcription–PCR (RT-PCR) (*5*). The second patient was a 57-year-old woman from Augbane (Marseille area), France, who had fever, malaise, photophobia, neck rigidity, and vomiting. TOSV-specific immunoglobulin M was detected in an acute-phase serum sample; a convalescent-phase sample indicated seroconversion. Her CSF sample was positive for TOSV by PCR (*6*) and was the source for the isolation of TOSV H/IMTSSA. TOSV EsPhGR40 was isolated from a pool of female sandflies captured in the metropolitan area of Granada (*3*).

Virus sequences were obtained by using conserved primers (primer sequences available on request). GenBank accession numbers of the new TOSV sequences were EF120631 and FJ153279–FJ153286. To assess the potential for establishing a simple cutoff for classification of TOSV genotypes (e.g., similar to programs created for mumps) (*7*), we used pairwise sequence comparison to compare these sequences with all published phleboviral sequences. Sequence analysis of the M segment was sufficient to enable determination of genotypes (Figure 1). Two different clusters were clearly distinguished. We propose to name those clusters genotypes A and B. The previously described TOSV lineages that include isolates from Italy all clustered in the proposed TOSV A genotype. Comparision with RVFV, for which 7 lineages (A to G) have been described (*8*), showed higher divergence in L and M segments and nonstructural (NS) and nucleoprotein (N) genes between the proposed TOSV genotypes than that found among RVFV lineages (Table).

Pairwise comparison demonstrated the following: 1) all TOSV S segments were highly conserved; 2) the L segment demonstrated less conservation than the N gene at the nucleotide or deduced amino acid levels; 3) TOSV M segments were the most divergent; and 4) variation in M segments was higher than that among RVFV strains but less

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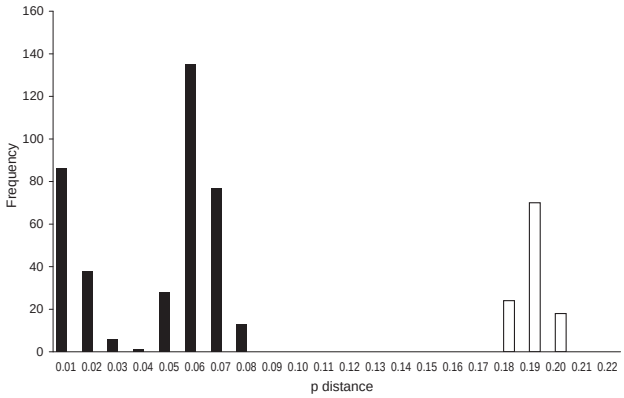

Figure 1. Histogram showing distribution of nucleotide pairwise (p) distances in the medium segment of Toscana virus. p distances are for nucleotides; frequencies are for intervals of 0.01. Validity of this method was confirmed by analysis of variance, comparing the scores of sequence comparisons within genotypes to those between genotypes. Black bars indicate intralineage distribution; white bars indicate interlineage distribution.

pronounced than that within the group of sandfly fever Sicilian viruses or sandfly fever Naples viruses (Table). With regard to the conserved S segments, the NS gene was more variable than the N gene, and despite a nucleotide pairwise difference of 12.1% in the N coding region, the amino acid sequences were completely conserved (100% identity);

Phylogenetic analysis of all phlebovirus sequences was performed by using the maximum-likelihood method available in PAUP* under tree bisection and reconnection branch swapping; the best-fit model of nucleotide substitution in each instance was determined by using MODELTEST (*9*) (parameter values available upon request) (Figure 2). Time to most recent common ancestor (TMRCA) was estimated for the M segment by using the data from 32 dated samples collected over 35 years and the Bayesian Markov Chain Monte Carlo approach (BEAST package; *10*). We applied a relaxed molecular clock with an uncorrelated exponential distribution of rates, a general time reversible + I + F4 model of nucleotide substitution and logistic population growth. Only the M segment had enough sequence representation to perform the analysis. Nonetheless, because glycoproteins are present on the surface of virions, they are the proteins most exposed to the selective pressures of the host.

The estimated mean rate of evolutionary change was 9.1×10^{-5} substitutions/site/year (95% highest probability density [HPD] = 2.5×10^{-4} to 2.7×10^{-6} substitutions/site/year). Under this rate the mean TMRCA was estimated to be 3,265 years, although with wide variance (178–11,000 years). Studies of RVFV, a closely related phlebovirus (*11*) have estimated the mean RVFV M segment rate to be 2.42×10^{-4} (95% HPD = 1.8×10^{-4} to 3.0×10^{-4} substitutions/site/year) (*8*). TOSV M segment TMRCA is higher than RVFV M segment TMRCA (117.3 years [variance 95–143 years]) (*8*). Although the wide variance of the TMRCA calculations might be affected by the scarce sequence information available, the differences observed might also be related to their biological differences. Both are phleboviruses transmitted by the bite of arthropods that could also be implied as amplifier hosts; however, because TOSV is believed not to be amplified on its

Table. Sequence differences among phleboviruses*

Comparison	Similarity of sequences (amino acid similarity), mean % ± SD			
	L segment	M segment	NS gene	N gene
Within TOSV	91.7 ± 0.7 (98.8 ± 0.5)	92.4 ± 0.6 (95.4 ± 0.9)	90.7 ± 0.8 (95.8 ± 1)	95.9 ± 0.8 (100)
Within TOSV A	NA	99.3 ± 0.1 (99.4 ± 0.5)	NA	100 (100)
Within TOSV B	98.2 ± 0.2 (99.7 ± 0.1)	95.5 ± 0.4 (98.2 ± 0.5)	97.3 ± 0.4 (98.5 ± 0.5)	98.5 ± 0.3 (100)
Between TOSV A and TOSV B	85.2 ±0.4 (97.0 ± 0.4)	81.8 ± 0.4 (90.0 ± 1.1)	83.6 ± 1.1 (89.6 ± 1.6)	87.9 ± 1.4 (100)
Within TOSV A G1 to G4	NA	99.3 ± 0.1 (99.3 ± 0.2)	NA	NA
Between TOSV A G1, G2, G3, and G4	NA	95.6 ± 0.4 (97.3 ± 0.5)	NA	NA
Within RVFV	97.7 ± 0.3 (99.8 ± 0.1)	95.4 ± 0.5 (98.6 ± 0.5)	97.2 ± 0.3 (98.5 ± 0.4)	97.6 ± 0.5 (99.8 ± 0.1)
Within RVFV lineages	98.8 ± 0.1 (99.5 ± 0.1)	98.8 ± 0.1 (99.5 ± 0.1)	98.8 ± 0.2 (99.1 ± 0.3)	99.1 ± 0.2 (99.9 ± 0.1)
Between RVFV lineages	96.6 ± 0.2 (99.2 ± 0.2)	96.6 ± 0.2 (99.1 ± 0.2)	97.0 ± 0.5 (98.0 ± 0.6)	97.5 ± 0.5 (99.6 ± 0.3)
Within SFSV	NA	76.5 ± 1.3 (80.2 ± 2.1)	99.7 ± 0.2 (98.9 ± 0.7)	100 (100)
Within SFNV	NA	72.2 ± 1.2 (72.9 ± 2.1)	NA	NA
Within PTV	96.5 ± 0.7 (100)	92.5 ± 0.8 (97.5 ± 0.9)	87.6 ± 1.4 (94.2 ± 1.6)	100 (100)
Between SFSV and PTV	NA	45.5 ± 1.8 (32.3 ± 3.2)	40.2 ± 1.9 (23.7 ± 3.1)	55.0 ± 3.6 (52.5 ± 6.4)
Between SFNV and TOSV	NA	57.2 ± 1.7 (53.6 ± 3.1)	NA	78.8 ± 2.8 (93.2 ± 3.3)

*Based on complete genome sequences. To estimate the evolutionary divergence over sequence pairs between groups, the average number of nucleotide differences per site over all sequence pairs between groups and among groups was calculated. Standard error estimates were obtained by a bootstrap procedure (500 replicates). For each pairwise sequence comparison, all positions containing alignment gaps and missing data were eliminated. L, large; M, medium; NS, nonstructural; N, nucleoprotein; TOSV, Toscana virus; NA, not applicable; RVFV, Rift Valley fever virus; SFSV, sandfly fever Sicilian virus; SFNV, sandfly fever Naples virus; PTV, Punta Toro virus.

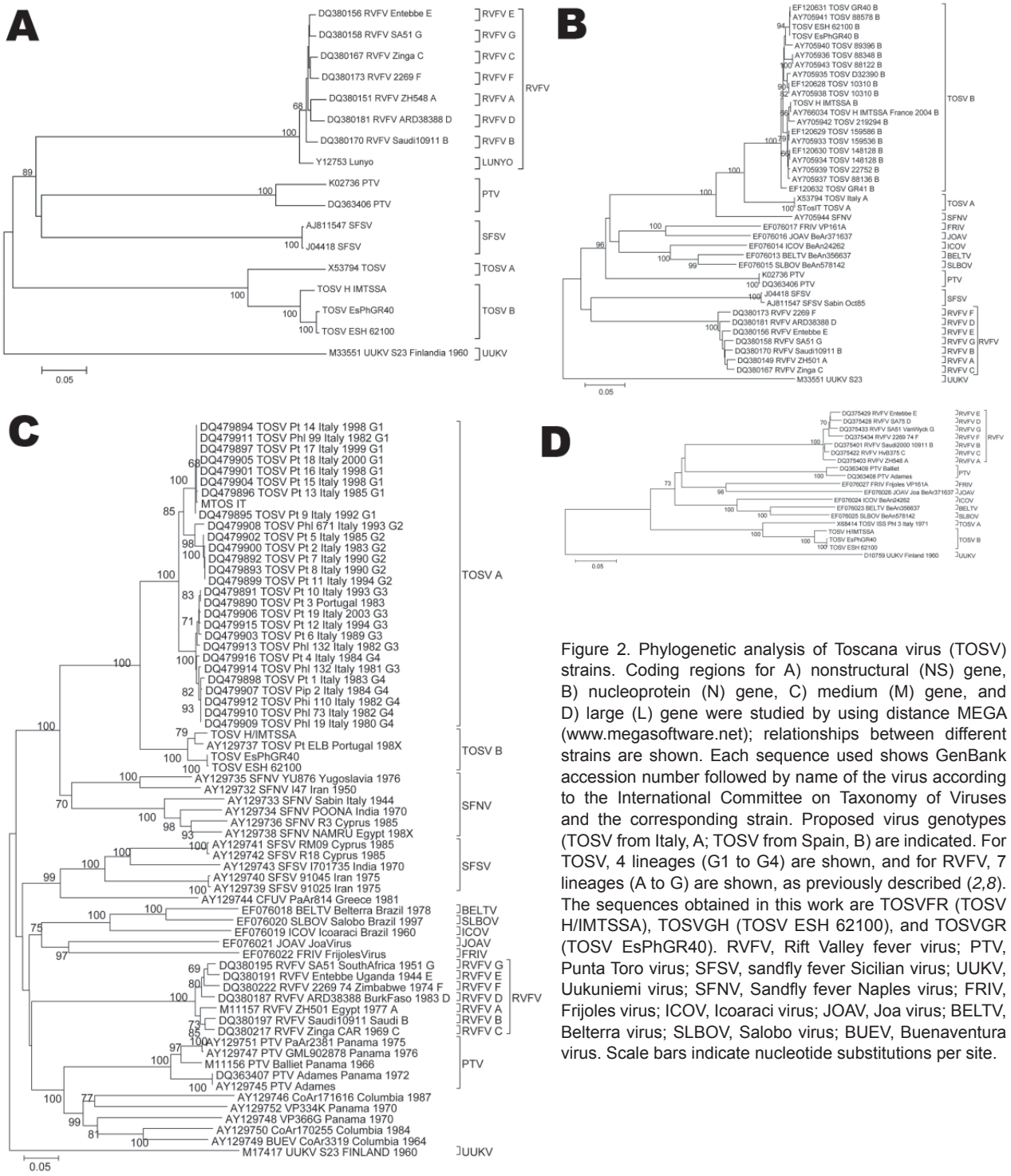

Figure 2. Phylogenetic analysis of Toscana virus (TOSV) strains. Coding regions for A) nonstructural (NS) gene, B) nucleoprotein (N) gene, C) medium (M) gene, and D) large (L) gene were studied by using distance MEGA (www.megasoftware.net); relationships between different strains are shown. Each sequence used shows GenBank accession number followed by name of the virus according to the International Committee on Taxonomy of Viruses and the corresponding strain. Proposed virus genotypes (TOSV from Italy, A; TOSV from Spain, B) are indicated. For TOSV, 4 lineages (G1 to G4) are shown, and for RVFV, 7 lineages (A to G) are shown, as previously described (*2,8*). The sequences obtained in this work are TOSVFR (TOSV H/IMTSSA), TOSVGH (TOSV ESH 62100), and TOSVGR (TOSV EsPhGR40). RVFV, Rift Valley fever virus; PTV, Punta Toro virus; SFSV, sandfly fever Sicilian virus; UUKV, Uukuniemi virus; SFNV, Sandfly fever Naples virus; FRIV, Frijoles virus; ICOV, Icoaraci virus; JOAV, Joa virus; BELTV, Belterra virus; SLBOV, Salobo virus; BUEV, Buenaventura virus. Scale bars indicate nucleotide substitutions per site.

mammalian hosts, it seems that the RVFV cycle includes mammals. The high conservation of RVFV genome sequences has been interpreted as indicating that the overall tolerance for mutation within the RVFV genome is low or that the viruses in the group have a relatively recent common ancestor (*12*). More studies are needed to understand the significance of the high degree of purifying selection observed in TOSV.

Conclusions

Our main goal, to improve the knowledge of sequence information for this neglected genus, was achieved by the

addition of 3 full genome sequences of TOSV. Geographic distribution may be different for each TOSV genotype. Whereas the TOSV A genotype circulates in Italy, France, and Portugal, the TOSV B genotype circulates in Spain, Portugal, and France. Both genotypes have been reported previously in France (*13*); however, the cocirculation of both genotypes in Portugal is confusing because sequences obtained independently in 2 laboratories from strains allegedly obtained from the same patient clustered in different clades (DQ479890 and AY129737) (*14*,*15*). Geographic differences in genotype distribution may relate to differences in vector distribution.

The distribution of phlebotomines is evolving with climate change and has implications for the epidemiology of vector-borne infectious diseases. Preexisting immunity almost certainly plays a role in limiting illness in the Mediterranean Basin; pathogenicity may be higher in naive populations outside areas of current TOSV prevalence. Thus, TOSV might be considered a threat for human health, and research and surveillance programs should aim to prevent its spread to new areas.

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Co-infection with Pansensitive and Multidrug-Resistant Strains of *Mycobacterium tuberculosis*

Michael P. Mendez, Mary E. Landon, Mary K. McCloud, Peter Davidson, and Paul J. Christensen

We report a case of a 23-year-old HIV-negative man with multidrug-resistant *Mycobacterium tuberculosis* that became evident while he was being treated for *M. tuberculosis* that was sensitive to all first-line drugs. This case should alert clinicians to consider co-infection as a possible cause of recrudescent disease.

A 23-year-old HIV-negative man from Somalia immigrated to the United States 1.5 years before seeking treatment for his symptoms. The patient had a 3-week history of dry cough, dyspnea with exertion, pleuritic chest pain, fatigue, weight loss, and night sweats. A chest radiograph showed extensive left hemithorax opacification, a left apical cavitary lesion, and right apical nodular lesions (Figure, panel A). Acid-fast bacillus (AFB) smears and culture were positive for *Mycobacterium tuberculosis*. Drug-susceptibility testing demonstrated sensitivity to all first-line drugs. He received directly observed therapy (DOT), which consisted of isoniazid (300 mg/d), pyrazinamide (1,000 mg/d), rifampin (600 mg/d), and ethambutol (1,200 mg/d).

One month after starting treatment, he had persistent fatigue and shortness of breath and had gained no weight; follow-up chest radiograph showed no change. As a result, he received a prednisone dosage of 40 mg/d with a tapered dose to no prednisone over 2 months (*1*,*2*). Thereafter, he improved rapidly both symptomatically and radiographically. His cough abated, and he was unable to produce sputum.

After 5 months of DOT, he again lost weight and felt fatigued. A repeat chest radiograph showed a new small cavitary lesion in the middle lobe of the right lung (Figure, panel B). At that time, repeat sputum sample was smear negative, but culture was positive for multidrug-resistant tuberculosis (MDR TB). Molecular characterization by spoligotyping and mycobacterial interspersed repeat units showed that the isolate from episode 1 differed genetically from that of episode 2 (Table) (*3*,*4*). Review of laboratory

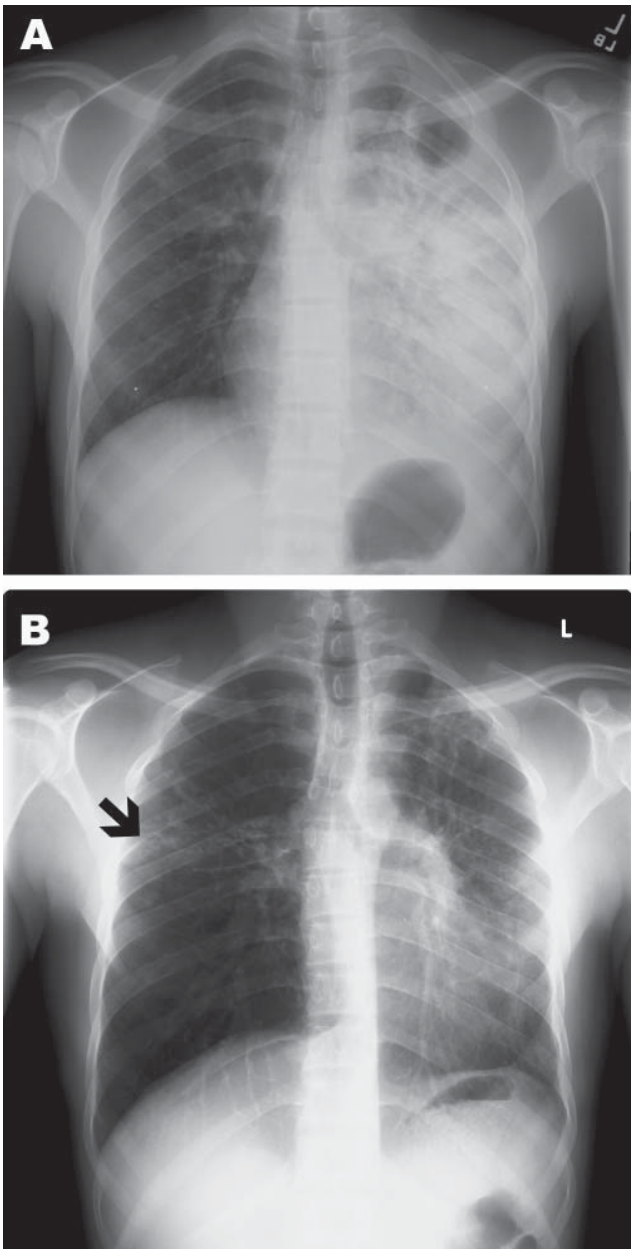

Figure. Poster-anterior chest radiographs of patient with multidrug-resistant tuberculosis. A) Radiograph taken at diagnosis, demonstrating dense consolidation of the left lower lobe and lingula. A left apical cavity is present. Minimal change is also noted in the right mid-lung zone. B) Radiograph taken after 5 months of directly observed therapy. Marked clearance is noted on the left; however, a new small cavitary lesion with surrounding infiltrate is noted in the right mid-lung zone (black arrow).

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Table. Selected analyses of sputum from patient with tuberculosis over the course of treatment during 2007*

Date	Isolate	Smear	Culture	Resistance†	Spoligotype	MIRU
Jan 4	1	Positive	Positive	None	000000004020771	224323-53313
Jul 12	2	Negative	Positive	I, R, P, E	777756777760771	224325153226
Sep 8	2	Positive	Positive	I, R, P, E	777756777760771	224325153226

*Molecular characterization was performed on Sep 8 to confirm the presence of the second isolate and reassess for presence of the original isolate.
MIRU, mycobacterial interspersed repeat units.
†I, isoniazid; R, rifampin; P, pyrazinamide; E, ethambutol.

records demonstrated that neither a switch in specimens nor cross-contamination were causes of this new finding. Repeat HIV testing was negative. Careful review of the patient's contacts, exposures, and travel history did not show any obvious source of reinfection. The patient did not travel outside of the county before or after initial treatment.

The patient received amikacin 15 mg/kg/d intravenously, moxifloxicin 400 mg/d, cycloserine 750 mg/d, and aminosalicylic acid 8 gm/d in accordance with drug-susceptibility testing. Because of the extent of previous disease, surgical removal of the diseased lung was not performed. He was kept in isolation until 3 sputum smears were AFB negative. He gained weight, his energy increased, and radiographic appearance of his lungs improved. At the time of his discharge, dosage of amikacin was decreased to 3×/wk, and cycloserine was decreased to 250 mg/d based on the serum levels of each drug. The patient was instructed to receive treatment for a minimum of 24 months after the first negative sputum culture.

Molecular characterization showed that the 2 strains of *M. tuberculosis* were not related (*3*,*4*). Thus, the cause of treatment failure most likely was not due to development of resistance by the infecting strain. Among the >35,000 *M. tuberculosis* isolates characterized by the Centers for Disease Control and Prevention's National Tuberculosis Genotyping Service (*5*), no exact genotype matches were found for the isolate from the first episode and 3 exact genotype matches were found for the isolate from the second episode (P. Moonan, pers. comm.). All 3 matched genotypes came from persons originating from the same African region (Somalia and Ethiopia), which suggests a phylogeographic lineage adapted to a particular human population (*6*). However, none of the matches clustered in the same US region as the current case. Further investigation determined that interstate transmission in the United States was unlikely. A review of risk factors for reinfection, such as travel to areas endemic for TB or exposure to high-risk populations, was negative, suggesting that the second strain was acquired before treatment. On the basis of this information, we speculate that the patient acquired both strains before traveling to the United States.

We report an immunocompetent person with 2 strains of *M. tuberculosis*, one sensitive to all first-line antituberculous drugs and one multidrug resistant. This case demonstrates the ability of genotyping information to identify simultaneous infection with multiple strains of *M. tuberculosis* in a patient in whom treatment failure is suspected. In addition, this case exemplifies the critical importance of clinical monitoring during DOT in the prompt recognition and treatment of patients who have unsuspected simultaneous infections with multiple strains of *M. tuberculosis*.

In patients who are not receiving DOT, a likely cause of treatment failure is nonadherence to the drug regimen (*7*). In patients who are receiving reliable DOT, deterioration may be explained by cryptic nonadherence, malabsorption, or laboratory error (*7*). In addition, exogenous reinfection should be considered as a possible reason for treatment failure or disease relapse (*8*–*11*). However, an underrecognized cause of treatment failure is mixed infection with ≥2 strains of *M. tuberculosis* (*12*–*15*). As reported previously (*12*), simultaneous infection with 2 competing strains should be considered when other common reasons are ruled out and a high index of suspicion is present. In the current case, the patient was receiving DOT and demonstrated clinical improvement while receiving an appropriate regimen based on drug-susceptibility testing. When his condition deteriorated clinically and sputum samples were culture positive, several possible causes of treatment failure were quickly discarded, including malabsorption, cryptic nonadherence, and laboratory error/contamination.

As has been previously hypothesized, we conclude that this patient originally harbored both strains (*12*). The effect of disease at the patient's initial presentation appears to have been predominantly due to the drug-sensitive strain, as evidenced by his initial clinical and radiographic improvement and sputum conversion. The successful treatment of the drug-susceptible strain and the inherent resistance of the second strain to the first-line medications enabled disease from the MDR TB strain to become apparent.

The initial therapy in this case was complicated by delayed clinical response. The use of corticosteroids as an adjuvant to antituberculosis medications in selected patients with severe disease has been documented to hasten resolution of symptoms and radiographic recovery without delaying clearance of organisms (*1*,*2*). Although the role of prednisone treatment in the emergence of the second strain is not clear, we believe the most important factor was the inherent antimicrobial resistance pattern of the second strain.

This case demonstrates the utility of DOT in assisting with prompt recognition of a new infecting strain and

highlights the use of genotyping in the assessment of possible treatment failure. Heightened awareness of possible infection with multiple strains, either from reinfection or coinfection, is critical when monitoring patients who are not improving or who recrudesce despite therapy guided by drug-resistance testing. The use of genotyping is a necessary tool in the evaluation of these patients. In particular, as in this case, a high index of suspicion for co-infection should be applied to persons originating from areas in which *M. tuberculosis* is hyperendemic.

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Enterovirus 71 Maternal Antibodies in Infants, Taiwan

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Enterovirus 71 (EV71) causes life-threatening disease outbreaks in young children in Asia. This cohort study was conducted to understand the dynamics of maternal EV71 antibodies in Taiwanese young infants. Approximately 50% of neonates had detectable EV71 neutralizing antibodies, which declined to almost undetectable levels by 6 months of age.

Transplacental maternal antibodies protect young infants from infectious diseases. On the other hand, maternal antibodies in young infants may impede vaccine effectiveness and confound interpretation of vaccine-induced immune responses. Thus, a need exists to understand the dynamics of pathogen-specific maternal antibodies in young infants (*1*–*3*).

Enterovirus 71 (EV71) was first isolated in California, USA, in 1969. Since then, EV71 has been isolated globally and causes life-threatening outbreaks in young children in Asia (*4*–*10*). National surveillance data and epidemiologic studies show that infants have an increased risk of severe EV71 infections in Taiwan (*6*–*11*). Consequently, vaccine development for EV71 in Taiwan should target infants. This cohort study was conducted to understand the dynamics of EV71-specific maternal antibodies in young infants in Taiwan.

The Study

Seropositive rates of EV71 neutralizing antibodies in preschool children have been found to be higher in rural areas than in urban areas in Taiwan (*11*). We chose Chang Gung Memorial Hospital (CGMH) as a study site because it has large obstetric and pediatric populations and serves residents from rural and urban areas in northern Taiwan (*7*). Pregnant women having prenatal examinations at CGMH were invited to participate in the study. Serum samples were obtained from participating pregnant women and their children to measure EV71 neutralizing antibody titers immediately before delivery for pregnant women; at birth for neonates (cord blood); and at 6, 12, 24, 36, and 48 months of age for infants. Institutional review board approvals were obtained from CGMH and from the National Health Research Institutes, according to the Helsinki Declaration. Informed consent was obtained from all mothers of participating infants. This report addresses the dynamics of EV71 maternal antibodies in young infants by 6 months of age.

Laboratory methods for measuring EV71 serum neutralizing antibody titers followed standard protocols (*7*) and used a local strain (TW/E59/2002 [B4 genotype]) and rhabdomyosarcoma cells. Serial serum samples obtained from each pregnant woman and her infant were tested in the same run to reduce assay variations. The starting dilution was 1:8, and the cutoff level for seropositivity was 8. Undetectable titer was assigned a level of 2 for calculation of geometric mean titer (GMT). For determining serostatus (positive or negative), serum samples were tested only at 1:8.

Under the assumption that levels of maternal antibodies decline exponentially and constantly, this study used paired serum samples collected at birth and at 6 months of age to estimate the biological half-life that represents an overall half-life and that is crucial for interpreting antibody responses in young infants. Longitudinal and cross-sectional methods of data analysis were used to estimate the biological half-life of pathogen-specific maternal antibodies (*1*).

Obtaining monthly serum samples from young infants to measure seroprevalence of maternal EV71-specific antibodies is unrealistic. Alternatively, the seroprevalence can be predicted mathematically. As has been shown in other viral pathogens, maternal antibody titers are assumed to follow a normal distribution after natural logarithm transformation and to experience a constant exponential decay over time after an infant's birth (*1*,*12*). If we assume normal distribution, 4 parameters (initial GMT at age *i*, SD of the distribution of antibody titers, decay rates of antibody titers, and cut-off for seropositivity) are crucial for estimating the seroprevalence in different ages (*12*).

Neutralization antibody titers were log-transformed to calculate the GMT and 95% confidence intervals. Statistical association between 2 nominal or ordinary variables was tested by using the χ^2 test, McNemar test, Fisher exact test, or Mantel-Haenszel χ^2 test, as appropriate. All statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) or SAS software (SAS Institute, Cary, NC, USA).

Serum samples from 459 pregnant women and their neonates were obtained from June 2006 to June 2008 and tested for EV71 neutralizing antibody serostatus. Seropositive rates of EV71 neutralizing antibodies in these pregnant

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women and their neonates were 63% and 51%, respectively. Seroprevalence in mothers was strongly associated with seroprevalence in their neonates, and neonates born to seronegative mothers were all seronegative ($p<0.01$, by McNemar test). In addition, the EV71 antibody titers in seropositive neonates were highly correlated with the EV7 antibody titers in their mothers ($R = 0.84$, $p<0.01$) (Figure 1).

From June 2006 through June 2008, a total of 309 neonates completed follow-up and blood collection at 6 months of age. EV71 neutralizing antibody titers in the serial serum samples from these 309 families were quantified. The seropositive rates of EV71 neutralizing antibodies in the family cohorts were 65% in the mothers, 50% in the neonates, and 1% in the 6-month-old infants, respectively (Table 1). Only 4 infants 6 months of age had detectable EV71 antibody titers; 1 showed seroconversion on the basis of antibody titers measured at birth (antibody titer <8) and at 6 months of age (antibody titer 512). This seroconverted infant did not develop any enterovirus-related symptoms (e.g., hand, foot, and mouth disease or herpangina). Of the 154 seropositive neonates, 3 remained seropositive and 151 became seronegative at 6 months of age (Table 2). In the 3 seropositive 6-month-old infants, the biological half-life of EV71 neutralizing antibodies was calculated as 39 and 42 days by using cross-sectional and longitudinal analyses, respectively. In the 151 seronegative 6-month-old infants, the biological half-life of EV71 neutralizing antibody was calculated as 53 and 60 days by using cross-sectional and longitudinal analysis, respectively (Table 2).

Seroprevalence rates were predicted by making the assumptions that the seropositive rate of EV71 neutralizing antibodies at birth is 50%, that the GMT of these seropositive neonates is 21.8 (SD 0.91), and that the half-life of maternal EV71 neutralizing antibodies is 42 days (1.4 months). Seroprevalence rates of EV71 neutralizing antibodies during each of the first 6 months of age were predicted to be 35%, 25%, 14%, 7%, 3%, and 1%, respectively (Figure 2). The predicted rate at 6 months of age was consistent with the observed seroprevalence at 6 months of age in our study population.

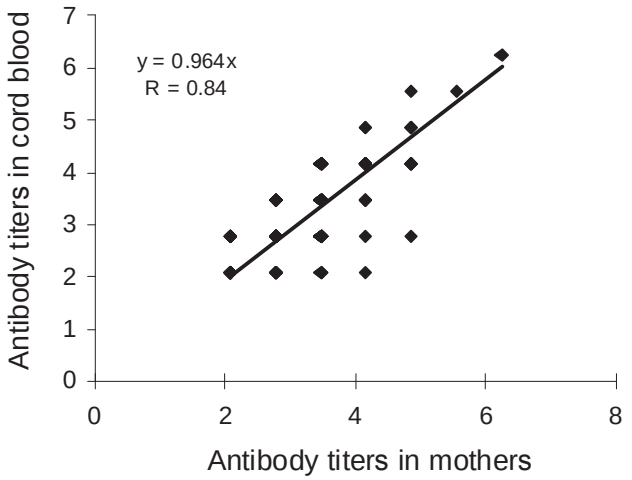

Figure 1. Scatter plot and correlation of enterovirus 71 neutralizing antibody titers (natural logarithm transformation) in 154 pairs of serum samples collected from seropositive neonates and their mothers, Taiwan. Values on the axes are logarithmic.

Conclusions

We found that ≈50–60% of pregnant women had serum EV71 neutralizing antibodies. These maternal EV71 neutralizing antibodies declined to undetectable levels in 99% of the 6-month-old infants. Two cross-sectional studies found similar seropositive rates in 30–49-year-old adults and in 6–11-month-old infants before the 1998 epidemic in Taiwan (*10*,*11*). In Singapore, a cross-sectional study found that 44% of 70 neonates had EV71 neutralizing antibodies in cord blood samples, but none of 52 infants 1–11 months old was seropositive (*13*).

Studies estimating the biologic half-life of EV71 maternal antibodies appear to be new. Theoretically, longitudinal analysis is more reliable and has narrower confidence intervals than cross-sectional analysis (*1*). Although only 3 infants had detectable antibody titers at 6 months of age, our study estimates the biological half-life of EV71 maternal antibodies to be 42 days, similar to the half-lives of antibodies to other pathogens calculated by using longitudinal analysis (*1*; M.-S. Lee, unpub. data).

Table 1. Distribution of enterovirus 71 neutralizing antibody titers in pregnant women, neonates, and 6-month-old infants in a cohort study, Taiwan

Antibody titer	No. (%) pregnant women, n = 307	No. (%) neonates, n = 309*	No. (%) 6-month-old infants, n = 309*
<8	107 (34.9)	155 (50.2)	305 (99.0)
8	79 (25.7)	44 (14.2)	3 (0.97)
16	48 (15.6)	48 (15.5)	0
32	34 (11.1)	24 (7.8)	0
64	24 (7.8)	29 (9.4)	0
128	13 (4.2)	6 (1.9)	0
256	1 (0.3)	3 (1.0)	0
512	1 (0.3)	0	1 (0.32)

*Two mothers delivered twins.

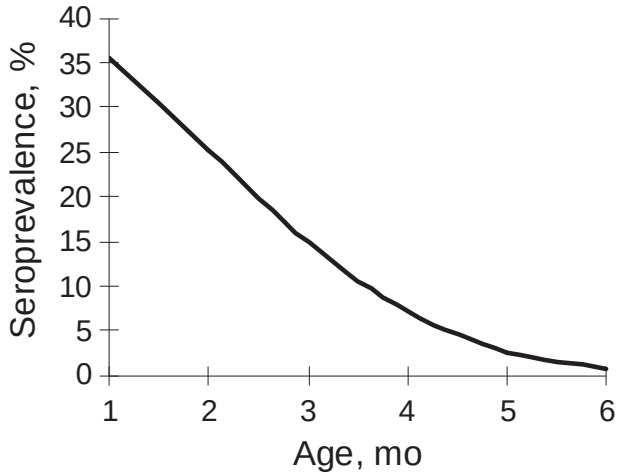

Figure 2. Predicted seroprevalences of maternal enterovirus 71 (EV71) neutralizing antibodies (antibody titer ≥8) in infants ≤6 months of age, Taiwan. Predictions are based on assumptions that 1) the seroprevalence in neonates is 50%, 2) the geometric mean titer (SD after natural logarithm transformation) in the seropositive neonates is 22 (0.91), and 3) the half-life of maternal EV71 neutralizing antibodies in young infants is 42 days.

Our prospective serologic study in northern Taiwan showed no seroconversion in young infants during 2007 and only 1 seroconversion during the first half of 2008. Based on the national enterovirus surveillance system, EV71 isolations were very low in 2006 and 2007 (*14*,*15*), findings consistent with our study.

A national program for developing EV71 vaccines was initiated in Taiwan in 2007. To improve vaccine development, the target population (those at high risk) needs to be identified. Several studies have shown that infants 6–11 months of age in Taiwan have the highest risk for severe EV71 infection and for death caused by this infection (*8–10*). Our serologic study found that 99% of 6-month-old infants have undetectable maternal EV71 neutralizing antibodies. Consequently, EV71 vaccines being developed in Taiwan should target infants <6 months of age.

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Table 2. Biological half-life of maternal enterovirus 71 neutralizing antibodies in 154 seropositive neonates, determined by cross-sectional and longitudinal analyses, Taiwan*

Antibody titers at 6 mo of age	No.	GMT at birth (SD†)	GMT at 6 mo	Cross-sectional mean half-life‡	Longitudinal mean half-life§
Detectable	3	203	8	39	42
Undetectable	151	21	2	53	60
Total	154	22 (0.91)	2	53	60

*GMT, geometric mean titer.
†After natural logarithm transformation.
‡Calculated on the basis of the GMT at birth and 6 months of age. For comparison, half-life is reported in days, assuming that1 month is equal to 30 days.
§Calculated on the basis of paired antibody titers in each individual.

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Correlation between Tick Density and Pathogen Endemicity, New Hampshire

Seth T. Walk, Guang Xu, Jason W. Stull, and Stephen M. Rich

To assess the endemicity of tick-borne pathogens in New Hampshire, we surveyed adult tick vectors. Pathogens were more prevalent in areas of high tick density, suggesting a correlation between tick establishment and pathogen endemicity. Infection rates in ticks correlated with disease frequency in humans.

Along the borders of the northeastern and the upper midwestern United States, black-legged ticks (*Ixodes scapularis*) are invading new areas (*1*–*3*). Because this tick is the principal vector of a number of human pathogens, defining and monitoring its possible expansion are imperative. Little information is available about *I. scapularis* invasions, including the relative rates of pathogen carriage as vectors expand their range and establish locally enzootic cycles. To assess the endemicity of 3 tick-borne pathogens (*Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*) throughout New Hampshire, we surveyed adult *I. scapularis* vectors.

The Study

During the fall of 2007, we established 16 sampling sites in the 10 counties of New Hampshire (Figure 1). Levels of reported human Lyme disease had varied among the counties in 2006. We categorized each site as high Lyme disease incidence (HLI), medium Lyme disease incidence (MLI), or low Lyme disease incidence (LLI) according to the reported number of Lyme disease cases per 100,000 persons in 2006 (*4*). As in the neighboring states of Massachusetts (Xu et al., unpub. data) and Maine (*5*), in New Hampshire, ticks are most abundant in coastal counties. The HLI sites (Strafford, Rockingham, and Hillsborough counties) were along the coast and had 37–104 reported Lyme disease cases per 100,000 persons; the MLI sites (Carroll, Belknap, Merrimack, and Cheshire counties) bordered the coastal counties and had 10–19 cases per 100,000 persons; and the LLI sites (Coos, Grafton, and Sullivan counties) were the most inland and had 5–12 Lyme disease cases per 100,000 persons.

We collected adult ticks rather than the conventionally sought nymphs (*6*–*8*) because we believe adults best indicate the pathogen pool in an area. Transovarial transmission of *B. burgdorferi* is negligible, and *I. scapularis* ticks acquire infection by feeding on infected hosts as larvae or as nymphs. Because larval ticks feed during late summer and early autumn, they are more likely to feed on migratory animals, such as birds; nymphal ticks feeding in early summer are likely to feed on resident hosts. Hence, adult ticks are more likely to have taken at least 1 resident blood meal from the site of origin, and their *Borrelia* infection rates (and genotype frequencies) are more representative of endemicity.

We visited each site twice within 1 month (October or November 2007) and sampled each site at each visit for

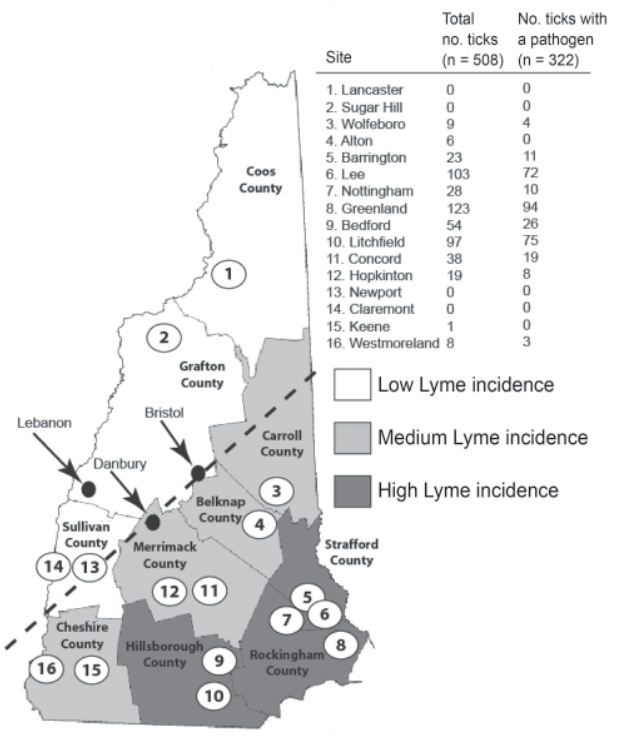

Figure 1. Results of *Ixodes scapularis* tick sampling and pathogen screening in New Hampshire. County names are shown in bold and sampling sites across counties of low (white), medium (light gray), and high (dark gray) Lyme disease incidence are numbered. Dashed line indicates the leading edge of the expanding *I. scapularis* range. Arrows and black dots indicate cities referred to in the discussion. Numbered open circles identify the locations of the 16 sample sites.

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≈25 minutes. A total of 509 adult ticks were collected by drag-sampling vegetation. As expected, ticks were most abundant at HLI sites and moderate at MLI sites; no ticks were found at the 3 LLI sites, including 2 sites (Newport and Claremont) that were close to where ticks have previously been reported (*9*). Likewise, we did not find ticks on 6 deer carcasses at hunter check stations in nearby towns (Newport, Danbury, and Bristol).

Ticks were transported to the laboratory, where they were frozen with liquid nitrogen and pulverized in Eppendorf tubes with plastic pestles (Kontes, Vineland, NJ, USA). DNA was extracted using Epicenter Master Complete DNA & RNA Purification Kits (Epicenter Technologies, Madison, WI, USA). Two duplex real-time–PCR reactions were developed (G. Xu et al., unpub. data) by using oligonucleotide primers and Taqman probes for real-time detection of total tick DNA and *B. burgdorferi* (duplex 1) and *A. phagocytophilum–B. microti* (duplex 2). The most common pathogen found was *B. burgdorferi*, followed by *B. microti* and *A. phagocytophilum* (Table). A total of 322 (63%) ticks carried at least 1 pathogen, and 40 (8%) ticks carried 2 pathogens. The prevalence of ticks positive for both *B. burgdorferi* and *B. microti* was greater than that of ticks positive for both *B. burgdorferi* and *A. phagocytophilum* and accounted for most (78%) coinfections. Neither of the observed coinfections (*B. burgdorferi–A. phagocytophium*, *B. burgdorferi–B. microti*) differed significantly from its expected random occurrence (contingency table analysis $p = 0.487$, χ^2 $p = 0.926$).

We found a significantly greater percentage of *B. burgdorferi*–infected ticks from HLI sites than from MLI sites (Figure 2). *B. burgdorferi* was twice as common in ticks from the HLI sites. Similarly, *B. microti* was more likely to be sampled from HLI sites (5.0%) than from MLI sites (2.7%), although this difference was not significant. Linear regression showed a strong correlation ($R^2 = 0.90$) between the entomologic risk index (total number of ticks × proportion of ticks infected [*6*,*8*]) in this study and the incidence of human cases of Lyme disease by county in New Hampshire in 2007.

Conclusions

I. scapularis was initially found in New Hampshire in 1985, near the town of Lebanon (*9*). Lebanon is located in a region where we were unable to sample ticks (Figure 1). It is tempting to hypothesize that *I. scapularis* was once more abundant in western counties. In 2001, A.T. Eaton noticed a distribution of ticks similar to what we found (*10*), suggesting that the current distribution has been stable for at least 7 years. Our result that high tick density areas had the highest overall prevalence of pathogens and the highest prevalence of coinfected ticks supports the finding of Hamer et al., who reported a significant difference in the rates of pathogen carriage between recently invaded and *I. scapularis*–endemic areas (*1*). The rates of infection we found are similar to those found by Swanson et al. from 5 other northeastern states (*11*). According to their meta-analysis, ≈40% (± 13%) of 2,109 adult and nymphal ticks were infected with *B. burgdorferi*, 21% (± 17%) with *A. phagocytophilum*, and 9% (± 8%) with either *B. microti* or *B. divergens*.

A noteworthy exception is the prevalence of *A. phagocytophilum*. We detected this pathogen at only 1 site (10 positive ticks from Greenland on 2 independent visits). Human anaplasmosis has been a reportable disease in New Hampshire for at least a decade (*12*), and cases have been rare (0 or 1 reported per year during 1998–2006). However, human anaplasmosis increased substantially in 2007 (3 cases) and 2008 (9 cases).

Human babesiosis appears to be following a similar trend (2 cases in 2005, 3 cases in 2006 and 2007, and 9 cases in 2008), although the disease has been reportable only since 2005. Lastly, we found a strong correlation between entomologic risk index and the incidence of human Lyme disease. This result contrasts starkly with a lack of correlation found by Falco et al. between the abundance of adult female ticks and reported cases of erythema migrans (a common clinical presentation of Lyme disease) in southern New York State (*7*). Improved reporting of Lyme disease by clinicians to state health officials may be responsible for this discrepancy. The data presented here suggest that

Table. Pathogen prevalence and coinfection in 2 regions of different tick density, New Hampshire*

Infection	No. ticks collected		
	HLI sites	MLI sites	Total
Single infection			
Borrelia burgdorferi	237	29	266
Anaplasma phagocytophilum	1	0	1
Babesia microti	13	2	15
Multiple infections			
B. burgdorferi + *A. phagocytophilum*	9	0	9
B. burgdorferi + *B. microti*	28	3	31
A. phagocytophilum + *B. microti*	0	0	0
B. burgdorferi + *A. phagocytophilum* + *B. microti*	0	0	0

*HLI, high Lyme disease incidence; MLI, medium Lyme disease incidence.

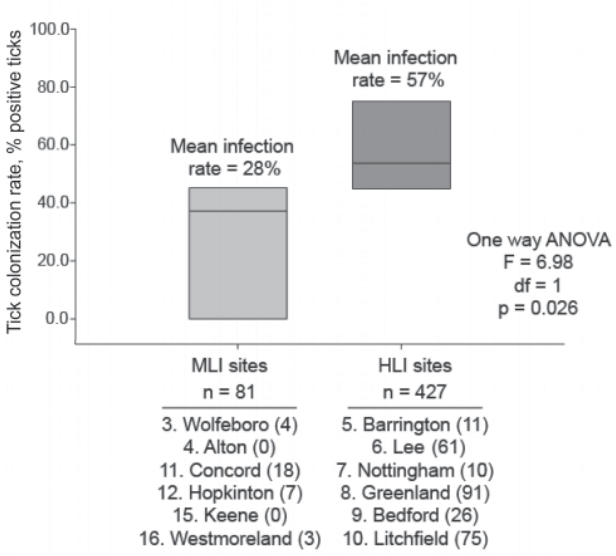

Figure 2. Analysis of variance (ANOVA) of *Borrelia burgdorferi* prevalence in *Ixodes scapularis* ticks isolated from New Hampshire counties of medium (MLI) and high (HLI) incidence of Lyme disease.

ERI estimates using adult ticks are accurate proxies for the yearly incidence of human Lyme disease in regions where Lyme disease is endemic.

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Lobomycosis in Offshore Bottlenose Dolphins (*Tursiops truncatus*), North Carolina

David S. Rotstein, Leslie G. Burdett, William McLellan, Lori Schwacke, Teri Rowles, Karen A. Terio, Stacy Schultz, and Ann Pabst

Lacazia loboi, a cutaneous fungus, is found in humans and dolphins from transitional tropical (Florida) and tropical (South America) regions. We report 2 cases of lobomycosis in stranded bottlenose dolphins (*Tursiops truncatus*), and 1 case of lobomycosis-like disease in 1 free-swimming, pelagic, offshore bottlenose dolphin from North Carolina, where no cases have previously been observed.

Lacazia loboi is a fungus (order Onygenales) that has not yet been cultured (*1*). Infection results in dermal and subcutaneous granulomas and 6–12-μm yeast-like bodies connected in chains by a small tubule (*2*); spread by the lymphatic system has been reported (*3*). Hematogenous spread and contiguous spread have not been excluded as means of propagation. Infections have been reported in humans (*4*) and dolphins, including Guiana dolphins (*Sotalia guianensis*) in tropical climates (Latin America) (*5*) and Atlantic bottlenose dolphins (*Tursiops truncatus*) in transitional tropical climates (Indian River Lagoon and Gulf of Mexico, Florida; Matagorda Bay, Texas; and Bay of Biscay, Europe) (*6*–*8*). We report 2 cases of lobomycosis in offshore (pelagic) bottlenose dolphins stranded off North Carolina in 2005 and 2008.

The Cases

KLC020

On August 20, 2008, a dead male Atlantic bottlenose dolphin was found stranded on the North Carolina coast. On the basis of overall length, rostrum length, and flipper size, the dolphin was identified as belonging to the offshore ecotype (*9*). Gross findings included numerous serpiginous and coalescing, raised, ulcerated-to-papillary nodules on the dorsum anterior to the dorsal fin and extending to the mid-body (Figure 1, panel A). Other gross lesions included verminous pterygoid sinusitis and pneumonia and mild dermal and retroperitoneal cestodiasis.

Dermal and subcutaneous granulomas composed of multinucleated giant cells, epithelioid macrophages, lymphocytes, and plasma cells were present in all skin sections and surrounded yeast-like structures. Fungi (6–10 μm) were connected in chains to adjacent fungal bodies by a thin neck (Figure 1, panel B). Other findings included parasitic migratory tracts in the brain, parasitized lungs, and pterygoid sinuses.

DNA was isolated from fresh frozen skin samples (DNeasy Tissue Kit; QIAGEN, Valencia, CA, USA) and amplified by using 28S rRNA generic primers and MicroSeq D2 LSU rDNA primers (Applied Biosystems,

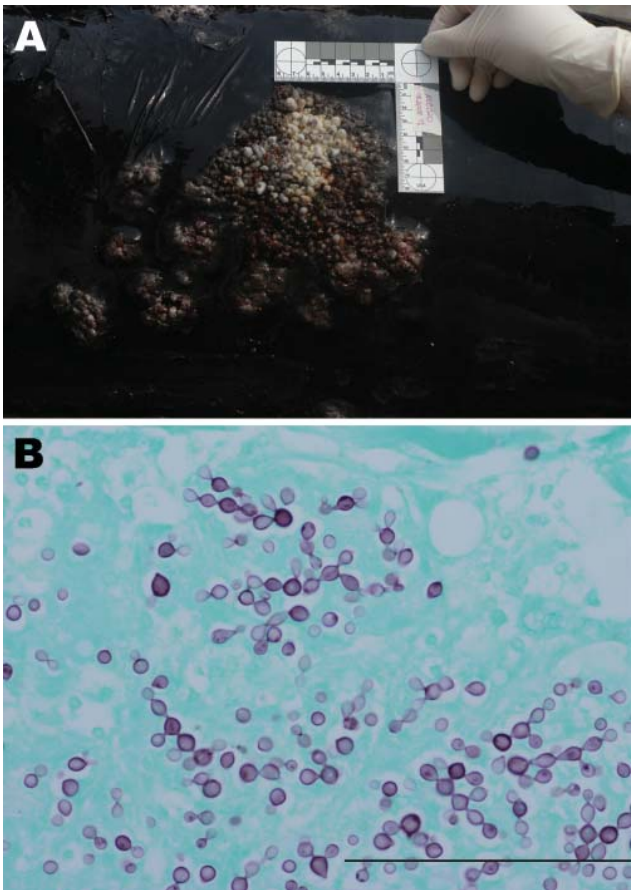

Figure 1. A) Serpiginous dermal nodules covering the dorsum of an offshore bottlenose dolphin (KLC020). B) Gomori methenamine silver–stained sections of dermis showing yeast-like structures connected by neck and arranged at various angles (magnification ×400). Scale bar = 100 μm.

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Foster City, CA, USA). Amplicons were sequenced at the University of Chicago Cancer Sequencing Facility and were most closely (97%) related to *Paracoccidiodes brasiliensis*. *Paracoccidioides* spp., *Lacazia* spp., and *Emmonsia* spp. are related fungi; validated sequences are not available for amplified regions for *Lacazia* spp.

AJW001

On March 5, 2005, a live male offshore Atlantic bottlenose dolphin was found stranded on Carolina Beach, North Carolina. The dolphin was in fair-to-good body condition. The most obvious gross finding was a few ulcerated dermal nodules scattered across the dorsum. Histologic findings from dermal nodules included granulomatous inflammation with numerous fungal yeast-like structures as in case KLC020. The dolphin also exhibited nonsuppurative meningoencephalitis, bronchointerstitial pneumonia, necrotizing hepatitis, and necrotizing adrenalitis associated with *Toxoplasma* spp.–like cysts and tachyzooites.

Live Sighting

On May 26, 2008, a free-swimming offshore bottlenose dolphin was sighted by a vessel survey team from Duke University Marine Laboratory at 35.66584°N, 74.79782°W, ≈60 km off Oregon Inlet on the Outer Banks of North Carolina (Figure 2). The animal had a large region of raised epidermal gray to white nodules over the entire dorsal surface cranial to the dorsal fin. These lesions are consistent with those seen in the other bottlenose dolphins in this report and lesions seen on bottlenose dolphins from the Indian and Banana rivers in Florida (*10*). The location of this sighting suggests that this dolphin is of the offshore ecotype (*11*,*12*).

Conclusions

Lobomycosis has been reported in the United States in coastal bottlenose dolphin populations in the Indian River Lagoon on the eastern coast of Florida, in the Gulf of Mexico off the western coast of Florida, and off the Texas Gulf coast. The Indian River Lagoon dolphin population has been assessed for temporal and spatial prevalence of lobomycosis (*10*). A prevalence of 6.8% was observed in that population from 1996 through 2006, and most cases were observed in the southern portion of this elongate body of water (*10*). The number of new cases per year, as determined from photograph identification studies, ranged from 1 to 9. Resolution of lesions has not been reported. In comparison, a prevalence of 3.9% was observed in a photograph identification study of *S. guianensis* from the Paranagua estuary in Brazil, a site of anthropogenic impact (*5*).

The cases in 2 stranded dolphins and in 1 photographed dolphin occurred in a subtropical climate of North America and involved an offshore rather than a coastal species. Information regarding unknown factors about these diseased animals, including host and pathogen range, pathogen molecular characterization, and environmental factors, could lead to a new range of organismal survival.

The offshore ecotype of bottlenose dolphins is generally found in waters >40 m deep but has been observed as close as 7.3 km from the coast at depths of 13 m (*12*). Bottlenose dolphins with offshore characteristics have been found as far south as the Florida Keys (*12*), but latitudinal movements of the offshore ecotype are not well understood. The 3 dolphins may have had a range that extended to the tropics where exposure could have occurred, rather than occurring in the region of stranding. A better understanding of movements of offshore ecotypes is needed so that potential exposure pathways can be inferred.

During preparation of this report, dorsal fin photographs from photograph identification projects along the mid-Atlantic coast identified 2 additional offshore *T. truncatus* that had skin lesions consistent with lobomycosis-like disease, a term used to describe gross observations that cannot be confirmed histologically (*5*). A more expansive study of photograph identification records could provide information on additional suspected cases. However, prevalence calculations from these studies will be negatively biased because the objective is generally to acquire photographs of the dorsal fin only. Dart biopsy of affected animals could confirm infection of dolphins with suspected cases but would require accuracy of sampling that may not be feasible in field conditions.

Because *L. loboi* has not been cultured, molecular techniques have been used to characterize the fungus. It is most related to the fungal order Onygenales, which includes *Emmonsia* spp. and *Paracoccioidies* spp. There are limited DNA sequences from *Lacazia* spp. for comparison, and PCR results for suspected *Lacazia* spp. are similar to

Figure 2. Free-swimming bottlenose dolphin (offshore ecotype) sighted off the Outer Banks of North Carolina with raised gray to white nodules over the dorsal surface, consistent with those of lobomycosis seen in other Atlantic bottlenose dolphins. *Xenobalanus* sp., a barnacle, is adhered to the tip of the dorsal fin. Image provided by Ari Friedlander, Duke University Marine Laboratory, Beaufort, NC, USA.

those for *P. brasiliensis* (*13*), which are consistent with our findings for samples from case KLC020. The host range of *P. brasiliensis*, method of exposure (inhalation), and propensity for multisystemic invasion makes this fungi an unlikely causative agent of the disease in the 2 stranded *T. truncatus* dolphins. Histologic features of *P. brasiliensis* were also consistent with lobomycosis.

Decreased lymphocyte populations, which indicates decreased immune function, have been observed in animals with lobomycosis from the Indian River Lagoon (*14*) compared with noninfected cohorts in capture-and-release studies. Systemic disease has been reported in dolphins with lobomycosis from this lagoon (*15*). Of the 2 dolphins from North Carolina with lobomycosis, 1 had disseminated *Toxoplasmosis* spp.–like protozoal infection, and the other had suspected parasitic migration to the brain. Despite no histologic evidence of lymphoid depletion, lymphocytic function could not be determined for either animal. In stranded cetaceans such as dolphins, concurrent systemic disease is not an unexpected finding. Whether the presence of the fungus predisposes the animal to infectious processes, lowers immunity, or is a sign of decreased immunity may be best addressed in long-term capture-and-release studies in areas where the fungus is endemic and where data may be available from animals before and after infection.

Confirmation of lobomycosis in 2 stranded cetaceans off the coast of North Carolina represents a change in the northern distribution of this organism. Additional information on distribution and movements of offshore population(s) is needed to understand the prevalence and potential sources of infection.

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Concurrent Chikungunya and Dengue Virus Infections during Simultaneous Outbreaks, Gabon, 2007

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An outbreak of febrile illness occurred in Gabon in 2007, with 20,000 suspected cases. Chikungunya or dengue-2 virus infections were identified in 321 patients; 8 patients had documented co-infections. *Aedes albopictus* was identified as the principal vector for the transmission of both viruses.

In the past 20 years, dengue virus (DENV) and chikungunya virus (CHIKV) have caused large and geographically wide ranging epidemics (*1*,*2*). Recent CHIKV outbreaks caused several million clinical cases in the Indian Ocean Islands and India (*3*). The virus has also dispersed to new regions, including Gabon in Africa and Italy (*4*,*5*). DENVs cause the most notable mosquito-borne viral disease in the world; ≈100 million infections occur annually worldwide, and the incidence has increased >30-fold in the past 50 years (*1*). Despite this tremendous expansion of both diseases, relatively few cases of Chikungunya fever have been reported in Africa (*3*,*6*,*7*), and few dengue-2 virus (DENV-2) epidemics have been reported (*8*,*9*). Simultaneous CHIKV and DENV-2 outbreaks have rarely been observed.

CHIKV and DENV-2 are frequently transmitted to humans by peridomestic *Aedes* mosquitoes. *Ae. aegypti* has been considered to be the principal vector in the urban transmission cycle, with *Ae. albopictus* and other anthropophilic *Aedes* spp. serving as secondary vectors (*10*,*11*). However, the actual situation is much more complex. First, *Ae. albopictus* was repeatedly shown to be a highly competent vector of CHIKV during the recent outbreaks in the Indian Ocean and Italy (*5*,*12*). Second, the overall distribution of *Aedes* mosquitoes is rapidly changing. Specifically, *Ae. albopictus* (the Asian tiger mosquito) has dispersed globally into new territories previously occupied by *Ae. aegypti*. As a consequence, the characteristics of DENV and CHIKV circulation and their outbreak dynamics are likely to be modified.

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The Study

We report an arboviral outbreak that occurred in Gabon, Central Africa, from March through July 2007, which showed the unexpected extent of the spread of *Ae. albopictus* populations in peridomestic urban areas. We also describe its association with atypical epidemiologic characteristics such as the co-circulation of CHIKV and DENV-2 and the frequency of human co-infections. The outbreak centered on the capital of Gabon; peaked from April through May 2007, in the heat of the long wet season; and subsequently moved north, where the virus sequentially reached several small towns along the route to northern Gabon and Cameroon (Figure 1). The outbreak generated ≈20,000 cases. Patients with suspected cases exhibited a dengue-like syndrome, including fever, arthralgia, and asthenia. Conjunctival hemorrhage, maculopapular rash, headache, and vomiting were also observed in the most severe cases.

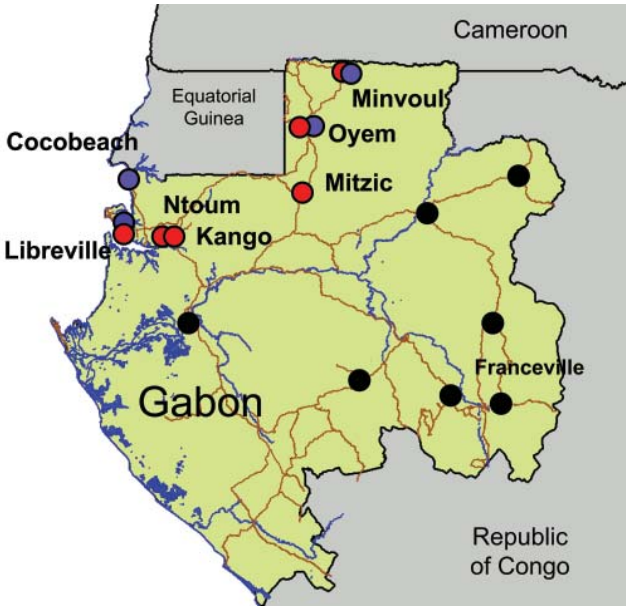

Figure 1. Chikungunya and dengue outbreaks in Gabon, 2007. Distribution of the outbreak and location of the 7 towns where suspected cases were laboratory confirmed by using quantitative reverse transcription–PCR assay are shown. Chikungunya cases are represented by red circles, dengue cases by blue circles, and cases negative for both viruses by black circles. Testing methods are described in the footnote to the Table.

During the course of the outbreak, 773 early blood samples (i.e., obtained during the first week after the onset of the disease) were collected from febrile patients who visited identified medical health centers in Libreville and other towns in Gabon (Table). Samples were tested for the presence of various arboviral RNA genomes by using the TaqMan quantitative reverse transcription–PCR (qRT-PCR) technology and specific primers and probes (protocols available upon request to the corresponding author). Among these 773 patients, 275 and 54 were positive for CHIKV and DENV, respectively, during May and July 2007 (Table), with 8 cases of co-infections. Using a dengue serotype-specific qRT-PCR assay, we showed that all DENV patients were positive for serotype 2 (DENV-2). In all 7 towns investigated on the route from Libreville to Cameroon (530 km), both CHIKV and DENV-2 human cases were reported, except in Cocobeach where only laboratory DENV-2 confirmed cases were observed (Figure 1).

To investigate this atypical scenario further, we analyzed 4,807 mosquitoes belonging to various species of *Aedes* (2,504 *Ae. albopictus*, 1,035 *Ae. aegypti*, 57 *Aedes simpsoni*), *Culex* (843 *Cx. quinquefasciatus*, 47 *Cx.* sp.), *Anopheles* (78 *An. gambiae*) and *Mansonia* (120 *M. africana*, 123 *M. uniformis*) in 15 different locations in Libreville where CHIKV or DENV-2 laboratory confirmed human cases were detected. Pools of 20 mosquitoes (constituted according to species and place of collection) were homogenized by using GenoGrinder 2000 (OPS Diagnostics, Bridgewater, NJ, USA) technology, and then tested for CHIKV and DENV-2 by qRT-PCR. We found that 7 and 3 groups of *Ae. albopictus* were positive for CHIKV and DENV-2, respectively, while no group containing other mosquito species was positive, indicating that *Ae. albopictus* was the only or at least the prominent vector of the 2 viruses.

These data provide evidence for the presence of CHIKV and DENV in Gabon and for their transmission to humans by *Ae. albopictus*. These epidemiologic results also confirm our previous observation that CHIKV strains isolated during the Gabon outbreak in 2007 belong to the Central African lineage and harbor the A226V mutation as a result of adaptation to *Ae. albopictus* through a mechanism of evolutionary convergence (*4*). More surprisingly, our results show that the spread of this mosquito in an area previously occupied predominantly by *Ae. aegypti* (*13*) was accompanied by the simultaneous emergence and transmission of DENV-2. One DENV-2 strain (designated as Libreville 2007), isolated from 1 febrile patient by using E6 Vero cells was further characterized by full-length genome sequencing (10,695 nt). Phylogenetic analysis showed that the DENV-2 Gabon 2007 strain belongs to the cosmopolitan, rather than the sylvatic, genotype (Figure 2). This cosmopolitan genotype includes mainly Asian but also related strains isolated in India, Australia, Mexico, the Indian Ocean, and Africa (Uganda, Somalia, and Burkina Faso), presumably the result of travel to these remote locations by viremic patients or the transportation of commercial goods by ship.

Conclusions

Taken together, these findings document CHIKV and DENV-2 co-circulation that resulted in large simultaneous outbreaks in regions where *Ae. albopictus* was shown to be the principal vector. Notably, we identified 8 patients with blood samples that tested positive for the presence of both CHIKV and DENV-2 genomes, indicating co-infection of these patients by both viruses. However, while unlikely, genetic exchanges between the 2 viruses, either by recombination or complementation, are not definitively excluded. Clinical examination of these patients (all adults, 5 women and 3 men) did not identify specific or severe symptoms, although given the limited number of cases and clinical and biologic investigations, this observation should be interpreted with caution.

Although the DENV cases were few, 8 of 48 (≈17%) DENV-2 positive patients from towns affected by the 2 outbreaks tested positive for CHIKV (Table). Extrapolation of this result suggests that the total number of DENV-2

Table. Positive test results for CHIKV and DENV-2 among febrile patients, by town, Gabon, 2007*†

Towns	No. patients tested	No. CHIKV+	No. DENV-2+	No. CHIKV+/DENV-2+
Libreville	686	249	45	6
Ntoum	3	1	0	0
Kango	7	3	0	0
Mitzic	6	4	0	0
Oyem	45	15	2	1
Minvoul	7	3	1	1
Cocobeach	19	0	6	0
Total	773	275	54	8

*CHIKV, chikungunya virus; DENV-2, dengue-2 virus; +, positive.
†RNA was extracted from 50 µL of plasma by using the ABI Prism 6100 Nucleic Acid PrepStation according to the manufacturer's recommended procedures (Applied Biosystems, Foster City, CA, USA). Fifty-microliter aliquots of extracted RNA were then used in 100-µL High Capacity cDNA synthesis reactions according to the manufacturer's instructions (Applied Biosystems). Finally, 10 µL of each cDNA reaction was then used as template for 50-µL quantitative PCRs that contained 200 nmol/L of probe and 900 nmol/L of each primer. The quantitative PCRs were then thermo-cycled in a 7500 Real-Time PCR system (Applied Biosystems) according to manufacturer's recommended procedures. The probe used for the CHIKV, DENV, and DENV-2 assays were FAM-labeled with TAMRA quencher (Applied Biosystems).

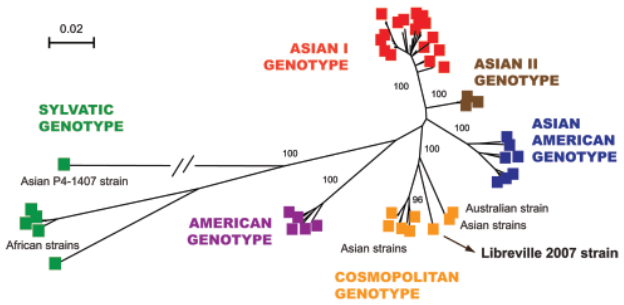

Figure 2. Phylogenetic relationships among dengue-2 virus (DENV-2) isolates based on full-length sequences (10,695 nt). A total of 85 DENV-2 genomes were compared with the human isolate obtained during the Gabon outbreak. Phylogeny was inferred by using neighbor-joining analysis. A neighbor-joining tree was constructed by using MEGA version 3.2 (www.megasoftware.net) with the Kimura 2-parameter corrections of multiple substitutions. Reliability of nodes was assessed by bootstrap resampling with 1,000 replicates. Branches are scaled according the number of substitutions per site, and the branch leading to the Thailand 94 strain was shortened for convenience. Bootstrap values are shown for major key nodes.

patients who are superinfected with CHIKV is likely to be high, which suggests that DENV-2 infection is not the antagonist for a secondary CHIKV infection. In contrast, only ≈3% of CHIKV+ patients were also DENV-2+; however, the starting period of time of infection or the sequence of infection by the 2 viruses cannot be assessed.

Although concurrent infections of dengue and chikungunya have been reported (*14*), such DENV-2 and CHIKV co-infections have never been previously associated with transmission by *Ae. albopictus*. Our study therefore provides a disconcerting example of the unexpected epidemiologic patterns that may be associated with the dispersal of both vectors (*Ae. albopictus* and *Ae. aegypti*) and pathogenic arboviruses (such as DENV and CHIKV). *Ae. albopictus* mosquitoes are now present in several temperate countries of the Northern Hemisphere where, given the opportunity, they could cause future arboviral epidemics. The recent sustained indigenous transmission of CHIKV by *Ae. albopictus* in northern Italy (*5*) provides a potential warning of what might occur much more frequently in the future in Europe and even in North America. Introduction of DENV or CHIKV in these regions are likely to generate indigenous transmission by *Ae. albopictus*.

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Gnathostomiasis Acquired by British Tourists in Botswana

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Infection with *Gnathostoma spinigerum* has been generally confined to Southeast Asia and Central and South America. However, gnathostomiasis was recently found in British tourists who had visited Botswana. Consequently, travel to Africa should now be considered a risk factor for gnathostomiasis.

In recent years, gnathostomiasis has increasingly been found in persons in countries where *Gnathostoma spinigerum* has not been endemic. However, *Gnathostoma* spp. should now be considered emerging imported pathogens. Apart from 2 previous reports of gnathostomiasis in Zambia and Tanzania (*1*), Africa has been considered free of this disease. Most persons seen in the West with gnathostomiasis have acquired the infection in Southeast Asia, particularly in Thailand and Japan (*2–4*), or in Central or South America, especially Mexico (*5*,*6*), where the main risk factor is consumption of raw or undercooked fish. Because few clinicians outside gnathostomiasis-endemic regions are familiar with the disease, diagnosis is often missed or delayed.

Our report describes a man with confirmed gnathostomiasis and another who probably had the disease; both had been on a fishing trip between Shakawe and Maun in northwest Botswana. A recent health alert from Johannesburg, South Africa, describes 2 clusters of infection with *Gnathostoma* sp. also acquired in the same region in Botswana as that visited by our 2 patients (*7*).

The Cases

The first person evaluated for possible gnathostomiasis was a British Caucasian man, 41 years of age, who came to the Hospital for Tropical Diseases (HTD) in London on September 30, 2008, 3 weeks after his return from the Okavango Delta in Botswana. He had been camping, walking barefoot, and swimming in and drinking river water. On several occasions, he had also eaten raw bream. About 2 weeks later, he reported intermittent abdominal discomfort, which was localized, but each morning, the pain moved to a different site and was accompanied by a palpable swelling over his spleen that resolved after 12 days. Pruritus then developed under his left arm and, within 24 hours, a painful subcutaneous lump developed on his anterior chest wall.

When he arrived at HTD, he was systemically well but had a raised nontender erythematous lesion below his left axilla. His eosinophil count was slightly high, 0.69×10^9 cells/L (reference count $<0.4 \times 10^9$ cells/L).

A presumptive diagnosis of migratory helminthic infection was made (gnathostomiasis or larval cestode infection), and he was treated with ivermectin, 200 μg/kg as a single dose, and albendazole, 400 mg 2×/d for 21 days. A week later, the lesion had migrated to his neck, but within 14 days, the lesion and eosinophilia had resolved. His initial serologic test result was negative for *Gnathostoma* spp. A subsequent sample was also negative, which may indicate that the antibody response had not developed sufficiently or that results were outside the sensitivity range.

A second British Caucasian man came to HTD on October 11, 2008, eleven days after the patient previously described, and 5 weeks after the new patient's return from the same trip in Botswana, where he had consumed the same foods and participated in the same activities. An erythematous, edematous, and pruritic lump (2 cm) developed above his left groin, lasted 4 days, and then subsided. As the lump decreased in size, he noticed a histamine-type track (left by a larva moving through tissue) toward his ribcage. One week later, the track mark had moved further up his chest. After another week, he reported a swollen, warm, and itchy right knee, which resolved within 24 hours, but 7 days later, similar symptoms developed in his right ankle.

He visited HTD again with a serpiginous, raised lesion on his back and surrounding erythema and eosinophilia (0.9×10^9 cells/L). He was treated empirically with albendazole, 400 mg 2×/d for 21 days, and praziquantel, 20 mg/kg as a single dose, for presumptive diagnosis of helminthic infection. Over the next 6 days, the serpiginous lesion migrated over his shoulder and neck, disappeared for 24 hours, then reappeared between his eyebrows, moved to his forehead and face, and then was felt inside his nose (Figure 1). On day 6, a spot developed below his left nostril, from which he expressed a larva. He brought it to HTD, where it was identified as *Gnathostoma spinigerum*.

Conclusions

Our patients received a diagnosis of typical cutaneous gnathostomiasis, but their lack of travel to a region in which the disease was known to be endemic was perplexing. The only previous reports of this infection from Africa were in

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3 persons who ate raw catfish from the Rufiji River in Tanzania (1994) and in 2 travelers who ate raw bream from the Zambezi River in Zambia (1998) (*1*). Before these reports, all reported cases of gnathostomiasis had been acquired in Southeast Asia and Central and South America.

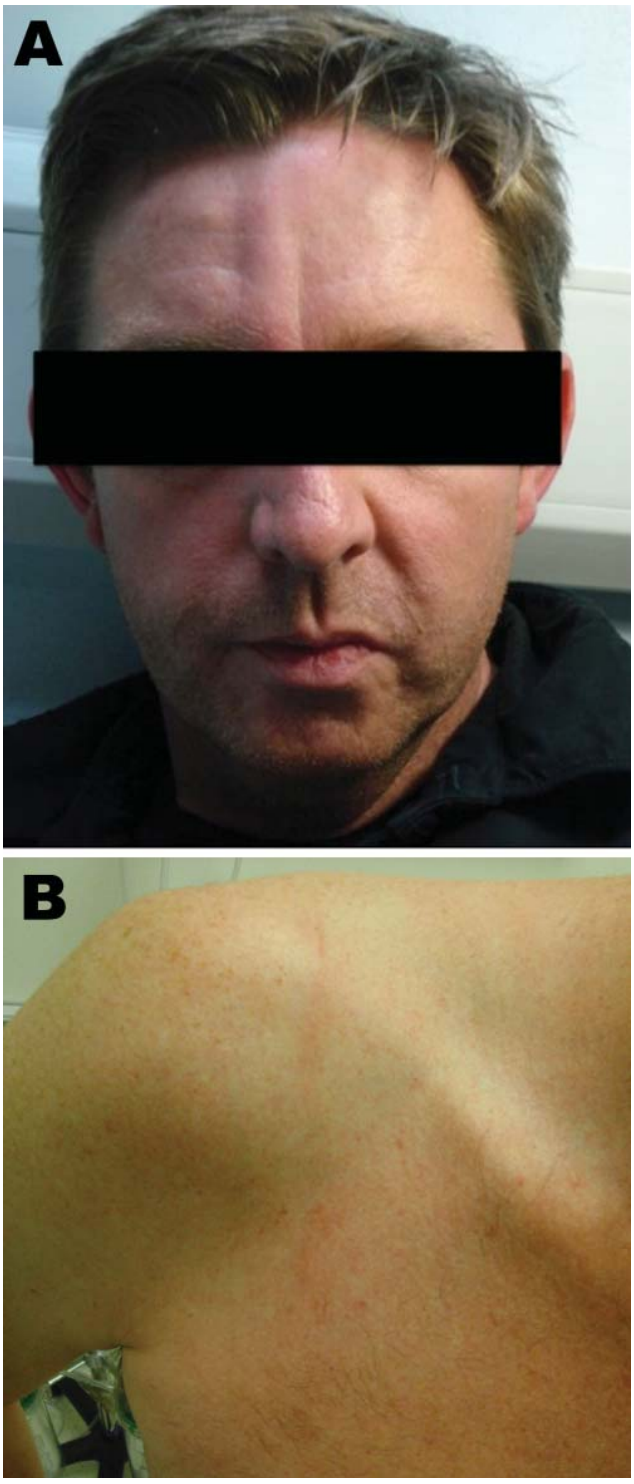

Figure 1. Cutaneous larva migrans on the forehead (A) and shoulder (B) of a male British tourist who had visited Botswana.

Gnathostomiasis is a nematode infection caused by the late third-stage larva (L3) of the helminth *Gnathostoma* spp. A foodborne zoonosis, it is endemic where people eat raw or undercooked fish that harbor the infectious L3. At least 4 species are known to cause human disease, the most common being *G. spinigerum*. Adult nematodes live in the stomach of definitive fish-eating hosts (commonly cats and dogs).When feces containing eggs are deposited in water, free-swimming first- and then second-stage larvae develop (*2*,*8*). Ingested by the first intermediate host (a copepod), they develop into early L3 forms. Second intermediate hosts (freshwater fish such as bream, catfish, snake-headed fish, sleeper perch, Nile tilapia, butterfish, or eel; frogs; snakes; chickens; snails; or pigs) ingest the copepods, liberating the larvae, which encyst in muscle and mature into L3 forms. When infected fish are eaten by a definitive host, the larvae mature into adults in ≈6 months (see life cycle of *G. spinigerum*, adapted from an illustration by Sylvia Paz Diaz Camacho, available from www.dpd.cdc.gov/dpdx/HTML/gnathostomiasis.htm). Humans are accidental hosts in which the parasite fails to reach sexual maturity.

Two alternative routes of human infection have been suggested: ingestion of water that contains infected copepods or direct skin penetration of food handlers through L3-infected meat (*2*,*8*). Symptoms in humans occur as the larva migrates through tissues, causing cutaneous and/or visceral larva migrans, which may begin within 24–48 hours after ingestion of infected meat. Initial nonspecific symptoms include fever, malaise, nausea, vomiting, diarrhea, and epigastric pain lasting 2–3 weeks and usually accompanied by a marked eosinophilia. Within 1 month, the cutaneous form may develop, with characteristic nonpitting edematous migratory swellings that may be painful, pruritic, or erythematous and may last 1–2 weeks. The swellings are typically due to 1 larva, occasionally due to 2 or more (*8*). Spontaneous larval extrusion, such as occurred in 1 of our patients, has been recorded.

Visceral gnathostomiasis occurs when the larvae migrate through the internal organs such as the lungs, gut, genitourinary tract, eye, ear, and central nervous system. This form causes more illness and deaths, with mortality rates reported at 8%–25%, than the cutaneous form (*9*,*10*). Pathogenicity is thought to result from direct mechanical injury by the larvae and by their release of secretions and excretions, which contain various compounds that cause tissue damage (*9*). The result is characteristic hemorrhagic tracts that may be seen at autopsy. Untreated, infected persons may have intermittent symptoms until the larvae die, after ≈12 years.

Diagnosis is suggested by eosinophilia, migratory lesions, and a history of geographic and food exposure. An immunoblot detecting 24-kDa–L3-specific antigen is considered diagnostic of *Gnathostoma* sp. and is the most

widely used serologic test because of its high sensitivity and specificity (*11*; P. Dekumyoy, pers. comm). Some laboratories use an ELISA for immunoglobulin G subclasses as a screening test (*12*). In our first patient, the larva was identified based on its morphologic features, including the number and size of cephalic hooks and the character and extent of spines on its body (Figure 2) (*2*,*13*).

For many years, treatment with various drugs was unsuccessful, and surgical excision of the larvae was the only effective management. Studies in the 1990s confirmed the efficacy of a 21-day course of albendazole, 400 mg 2×/d (*14*,*15*), and ivermectin, 0.2 mg/kg immediately or for 2 consecutive days (*15*).

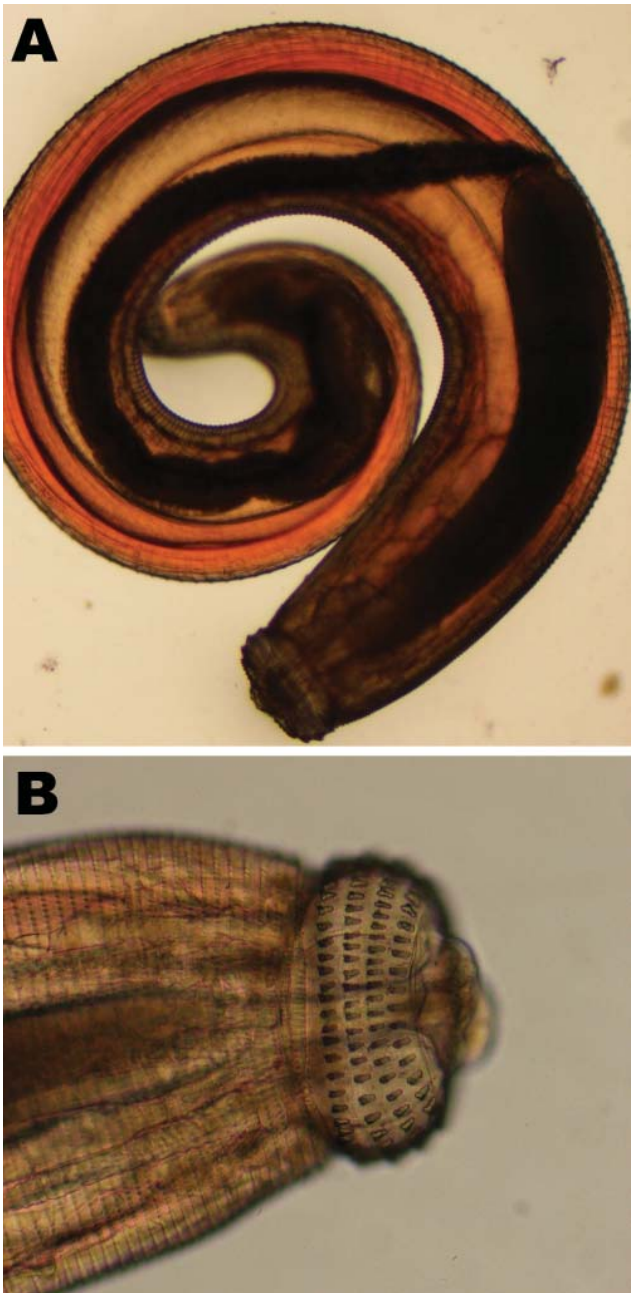

Figure 2. Third-stage larva of *Gnathostoma spinigerum*, which was expressed from the face of a male British tourist who had visited Botswana. Photograph shows entire larva (A) and larva head with hooks (B).

The area for risk of acquiring gnathostomiasis is expanding, and travelers and physicians need to be aware of this risk. Eradication of the organism is unlikely given its complicated life cycle, but local campaigns and appropriate pretravel advice can raise awareness and change people's eating habits. Travelers must realize that culinary adventures on exotic holidays can result in acquiring unwanted parasites that may have devastating consequences.

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Skin and Soft Tissue Infections (Patera Foot) in Immigrants, Spain

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An unusual skin and soft tissue infection of the lower limbs has been observed in immigrants from sub-Saharan Africa who cross the Atlantic Ocean crowded on small fishing boats (*pateras*). Response to conventional treatment is usually poor. Extreme extrinsic factors (including new pathogens) may contribute to the etiology of the infection and its pathogenesis.

Immigration is increasing from poor-resourced countries into Spain and other European countries (*1*). From the coasts of Mauritania and Morocco, each year ≈10,000 African people try to reach the coasts of the Canary Islands or the southern Iberian Peninsula by sea, aboard small boats (called *pateras*) (*2*). These boats, normally used for fishing, have capacity for only a few persons but these sea crossings are overcrowded with 40–50 persons and with minimal water and food provisions. The journey lasts several days, during which travelers are exposed to extreme conditions, including cold weather; deficient hygiene; prolonged sitting in the same position; and prolonged immersion of their feet in sea water possibly contaminated by traces of feces, urine, decaying food, or fuel–water emulsions. On arrival, many need medical care for hypothermia and dehydration. However, despite efforts of authorities to prevent humanitarian disaster, frequently these boats sink, and bodies are later found along the coast of the Canary Islands.

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Among the varied medical problems affecting these young, previously healthy immigrants are unexpectedly high numbers of severe skin and soft tissue infections (SSTIs), especially those involving the feet and legs. Clinical characteristics of these SSTIs were unfamiliar to our department, even though our unit collaborates directly with vascular surgeons, endocrinologists, and dermatologists to manage diabetes-related foot infections. The clinical picture comprises a painful cellulitis with minimal or imperceptible port of entry, deep abscesses, and tissue necrosis. Response to surgical debridement and broad-spectrum antimicrobial drugs is frequently poor, making amputation necessary in many cases. Here we describe the epidemiology, clinical features, microbiology, treatment, and outcome of 7 patients affected with severe SSTIs of the foot and leg and discuss the pathogenic role of *Shewanella algae* as an etiologic agent in this syndrome.

The Study

The 7 patients were treated at our Unit of Infectious Diseases and Tropical Medicine at the Hospital Universitario Insular of Las Palmas (Gran Canaria Island, Canary Islands, Spain). We defined the condition we call "patera foot" as all of the following: 1) acute SSTI involving the feet or legs, 2) direct relation of the infection to sea journey by overcrowded *patera* under extreme conditions, and 3) good health status before travel. For this report, we excluded patients with diabetes, chronic arterial or venous leg disease, edema, or any other predisposing conditions.

We performed the following basic interventions for all patients: registry of epidemiologic data; complete clinical history and physical examination; blood extraction for routine tests; blood cultures (if fever); cleaning and debridement of the affected area (if indicated by the vascular surgery team); and acquisition of cultures from the affected area by syringe (if abscess), skin punch (if cellulitis only), or deep infected tissue (if surgical debridement). Plastic surgery was performed when indicated. Amputation was carried out only after every effort was made to preserve the affected foot or leg.

All 7 patients whose conditions met the classification criteria were young, black, sub-Saharan men (Table). In all patients, we ruled out a defined immunodeficiency, specifically HIV infection; use of immunosuppressive agents; and indirect data suggesting primary immunodeficiency (lymphopenia or immunoglobulin deficiency). Organisms isolated from local specimens were gram-negative bacteria in all microbiologically positive cases. Four patients underwent amputation, including 1 transtibial and 1 transmetatarsal.

Patient 7, a 21-year-old man, was admitted to the intensive care unit because of shock secondary to severe SSTI and intense dehydration with acute renal failure and rhab-

Table. Clinical characteristics of 7 black male immigrants to Spain who developed severe skin and soft tissue infections (patera foot) following sea crossing from Africa on small boats (*pateras*)

Patient no.	Age, y	Country of origin	Isolated organism	Outcome
1	20	Ghana	Unknown	Transtibial amputation
2	38	Guinea	*Morganella morganii*	5th toe amputation
3	16	Mali	*Proteus vulgaris*	4th toe amputation
4	36	Guinea Bissau	*Enterobacter cloacae, Serratia* spp.	No amputation
5	27	Togo	Unknown	No amputation
6	20	Gambia	*Escherichia coli*	Transmetatarsal amputation
7	21	Côte d'Ivoire	*Shewanella algae*	No amputation; skin allograft

domyolysis. Bilateral painful enlargement of his lower extremities was evident, with disseminated round ulcers and serohemorrhagic, confluent blisters. The ulcers were covered with a fibrinoid, purulent exudate (Figure, panel A). After blood and skin samples were collected for culture, the patient began taking meropenem and linezolid . Skin samples grew gram-negative rods, initially identified as *S. putrefaciens* by using the API 20E system (bioMérieux, Marcy l'Etoile, France). By using experimental work (*3*–*5*) that allows differentiation between *Shewanella* species, we identified the organism as *S. algae* on the basis of its capacity to grow at 42ºC in 6.5% NaCl and to produce beta-hemolysis. The organism displayed in vitro susceptibility to ceftazidime, meropenem, piperacilin/tazobactam, ciprofloxacin, aminoglycosides, trimethoprim/sulfamethoxazole and aztreonam but resistance to amoxicilin/clavulamate, cefotaxime, and, notably, to imipenem (because *S. algae* is sensitive to other carbapenems i.e., meropenem). Four days after the patient was admitted to our unit, a large fluctuant area appeared on the dorsum of his left foot; drainage from the area consisted of a grossly purulent, foul-smelling material. Extensive debridement was necessary to control infection (Figure, panel B); 1 month later, a skin allograft was implanted (Figure, panel C). The patient was discharged, asymptomatic, after 70 days of hospitalization.

Conclusions

During sea crossing by *patera*, immigrants are exposed to extreme extrinsic conditions, such as cold weather and deficient hygienic conditions. Intrinsic factors, such as limited skin compliance related to young age and possibly race, may play additional roles in the pathogenesis of this syndrome. Black race may be an intrinsic factor because, to our knowledge, immigrants of other ethnic origin (Magreb countries) have not developed this syndrome.

The presence of gram-negative bacteria in all case-patients, especially *S. algae* in 1, instead of gram-positive cocci, indicates a source of infection related to water and illustrates the specific pathogenesis of this syndrome. *Shewanella* spp. are ubiquitous gram-negative bacteria; possible reservoirs include all types of water, oil emulsions, petroleum brines, protein-rich foods, and soil (*5*–*7*). Two *Shewanella* species, *S. algae* and *S. putrefaciens*, have been found in clinical specimens. Because automated systems are unable to distinguish between the 2 species, a number of infections attributed to *S. putrefaciens* probably correspond to *S. algae* (*4*). *S. algae* is considered a rare opportunistic pathogen for humans, frequently involving immunocompromised hosts (*6*,*8*,*9*), and are usually part of a polymicrobial infection (*6*,*10*,*11*), which may mask its clinical importance. The presence of chronic leg ulcers in the context of peripheral vascular disease occurs commonly in adults with *S. algae* SSTI (*6*,*10*,*12*,*13*), and the affinity of *S. algae* for necrotic or ischemic tissues has been well described (*12*,*14*,*15*).

The following sequence may explain why these young, previously healthy men developed such aggressive SSTIs. We speculate that specific etiologic agents (mainly GNB, including *S. algae*) present in densely contaminated water enter through macerated skin, then reach deep tissues that

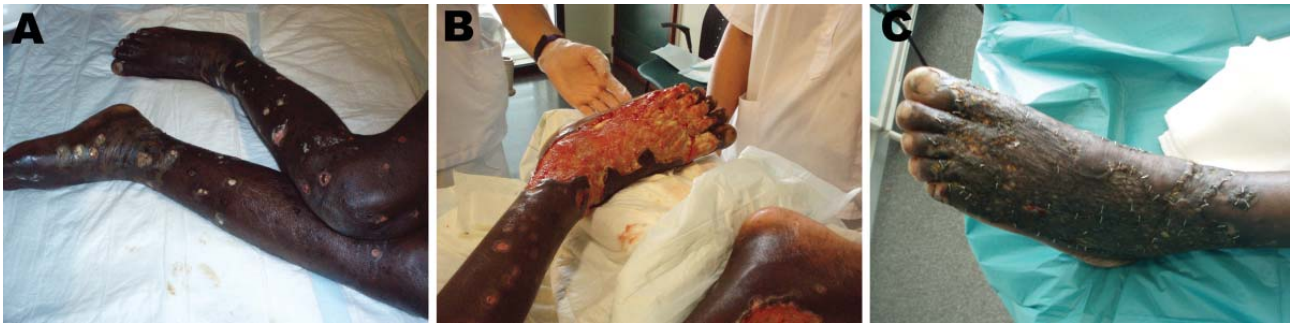

Figure. Progression of infection (patera foot) in case-patient 7, a previously healthy 21-year-old immigrant from sub-Saharan Africa who reached Spain by sea crossing on a small boat (*patera*). A) Initial severe skin and soft tissue infection of the lower limbs; B) extensive debridement of the left foot; C) left foot after skin allograft.

have been submitted to subacute ischemia from overpressure and deficient venous drainage, both related to forced, prolonged sitting. The ensuing inner inflammation, expanding against a young skin with limited compliance, further aggravates the ischemia and leads to necrosis, probably by a compartmental-like mechanism.

These cases appear to represent a new syndrome, with specific etiology, pathogenesis, clinical features, and response to treatment. GNB, including *S. algae*, are involved, and an ischemic mechanism may be crucial in the development of these destructive infections. The initial election of empirical therapy, always covering those pathogens, and early surgical evaluation are crucial in preventing major disability in these young people.

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Congenital Transmission of Chagas Disease in Latin American Immigrants in Switzerland

Yves Jackson, Catherine Myers, Alessandro Diana, Hans-Peter Marti, Hans Wolff, François Chappuis, Louis Loutan, and Alain Gervaix

International migration has changed the epidemiologic patterns of Chagas disease. Recently, 2 cases of Chagas disease transmitted from Latin American women to their newborns were diagnosed in Geneva, Switzerland. A retrospective study to detect Chagas disease showed a prevalence of 9.7% among 72 Latin American women tested during pregnancy in Switzerland.

Chagas disease, a zoonotic infection caused by *Trypanosoma cruzi*, is the most important endemic parasitic infection in Mexico and Central and South America because of the number of persons who become ill or die from this disease (*1*). An estimated 8–10 million persons are infected, and ≈14,000 persons die each year from Chagas disease (*1*,*2*). Historically, transmission by triatomine vectors has been the most common source of infection; however, the populations affected, transmission routes, and geographic distribution of Chagas disease cases have been greatly modified by urbanization and international migration. An estimated 14 million people from countries in which Chagas disease is endemic have moved to North America, Europe, Japan, and Australia. The number of persons currently infected by *T. cruzi* is probably >100,000 in the United States and >6,000 in Spain (*2*).

In Europe, vertical, transfusional, and transplantational routes have accounted for all cases of transmission. The risk for vertical transmission from an infected mother to her newborn is ≈5% (*3*). Vertical transmission is likely to go undetected in Europe because of lack of screening programs for at-risk pregnant women, who are usually in the long-lasting, chronic, asymptomatic phase of the disease and are unaware of their infection. An estimated 2,000 babies may have been born with *T. cruzi* infection in North America in recent years, and 2 cases of vertical transmission were recently reported from Spain (*4*–*6*). We report 2 additional cases of congenital infection with *T. cruzi*, detected in 2001 and 2006, at the Geneva University Hospitals in Switzerland. Subsequently, we conducted a retrospective serologic survey of pregnant Latin American immigrants to assess the potential for vertical transmission of Chagas disease in Switzerland.

The Cases

In 2001, a 31-year-old woman from Santa Cruz, Bolivia, delivered a 2,860-g, full-term, apparently healthy baby at the Geneva University Hospitals after an uncomplicated pregnancy. Like most undocumented immigrants recently arrived in Switzerland, she had received no medical supervision during her pregnancy. She stated that a blood test for *T. cruzi*, conducted in Bolivia, had been negative. Macroscopic examination of the fetal side of the placenta showed a 3.5-cm, subchorial, liquid-filled cyst (Figure 1). Histopathologic examination showed disseminated chorioamnionitis and associated funiculitis with large numbers of nonflagellated parasites. A recent infection with *Toxoplasma gondii* was ruled out by serologic testing. Congenital *T. cruzi* infection was confirmed by a positive blood microscopic examination for the infant, a positive serologic test result for the mother (immunofluorescence assay using killed *T. cruzi* parasites, Swiss Tropical Institute, Basel, Switzerland), and a positive blood PCR with TCZ1/TCZ2 primers for both the mother and the newborn. Electrocardiogram and echocardiogram of the newborn showed no abnormalities. The newborn received nifurtimox (10 mg/kg/d for 60 days) without notable adverse effects. Parasitemia became undetectable at the end of treatment, and serologic test result at 1 year of age was negative. The mother refused to be treated, claiming that she was feeling fine.

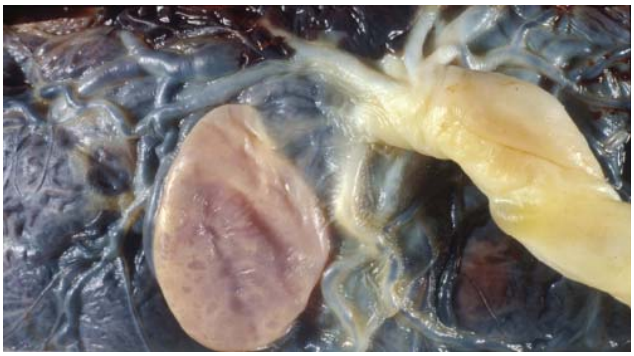

Figure 1. Fetal side of the placenta from Latin American pregnant woman who delivered her baby at Geneva University Hospitals, Geneva, Switzerland. A macroscopic subchorial liquid-filled cyst can be seen near the umbilical cord insertion.

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In 2006, a 25-year-old woman arrived in Switzerland from Santa Cruz, Bolivia, when she was 5 months pregnant. She delivered a 2,480-g, premature but healthy baby at 34 weeks' gestation at the Geneva University Hospitals. After discharge, histopathologic examination of the placenta showed funiculitis and chorioamnionitis with clusters of nonflagellated parasites. The mother had not been previously tested for *T. cruzi* but related that her father had died of Chagas disease–related heart complications. *T. cruzi* serologic testing by immunofluorescence was positive for the mother, and blood microscopic examination and PCR were positive for the newborn, confirming vertical transmission. Electrocardiogram and echocardiogram of the baby showed no abnormalities. The newborn began a 60-day course of nifurtimox (10 mg/kg/d) at 20 weeks of age and had no adverse effects. Blood PCR and serologic testing at 5 and 26 weeks after treatment was started, respectively, produced negative results. The mother was treated with nifurtimox after completion of breast-feeding and showed good tolerance to the drug.

The Study

In response to these 2 cases, in 2007, a retrospective serologic survey for *T. cruzi* infection was performed on stored serum samples from 72 undocumented pregnant Latin American women who had received prenatal care at the Geneva University Hospitals during the previous year. Median age was 30 years (range 20–43), and countries of origin were Bolivia (n = 30), Brazil (n = 22), Peru (n = 6), Ecuador (n = 5), Colombia (n = 4), Chile (n = 2), Honduras (n = 1), and unknown (n = 2). Serum samples were tested by IFA using *T. cruzi* parasites from in vitro culture (Swiss Tropical Institute). No confirmatory test was available. Of the 72 samples, 7 (9.7%) were positive, most from Bolivian women (n = 5). The seroprevalence among Bolivian women was 16.6% (5/30), consistent with prevalence found by similar surveys conducted recently in Bolivian maternity hospitals (*7*). Limitations of the study include the small number of samples tested and lack of a confirmatory test as recommended by the World Health Organization.

Conclusions

Only a small number of congenital cases of Chagas disease have been reported in countries in which this infection is nonendemic. The absence of routine screening programs for Chagas disease in pregnant women and newborns at risk most likely explains this low number, but other factors may be involved. Chagas disease affects immigrants, who frequently lack legal status and therefore experience difficulties (e.g., fear of deportation and financial and administrative constraints) in accessing quality healthcare during pregnancy. In Switzerland, undocumented immigrant women have poor access to medical supervision during pregnancy, so most consult a physician late in pregnancy or at time of delivery (*8*). Chagas disease is rare in Europe, and healthcare workers may simply not search for it, resulting in missed opportunities to diagnose the disease. In addition, up to two thirds of infected newborns are asymptomatic at birth, so congenital infection may go undetected if not actively sought.

Systematic screening of pregnant women at risk is likely to be beneficial in several ways. Treatment of infected mothers after completion of breast-feeding may reduce the risk for vertical transmission during subsequent pregnancies. Treatment of young women at the chronic, indeterminate stage of infection is likely to lower their risk for developing cardiac complications (*9*). Early screening and treatment of infected newborns are associated with high cure rates (*10*). Older children of mothers with newly diagnosed Chagas disease also benefit from screening and treatment (*11*). In addition, because immigrants with inadequate access to healthcare are at risk for being lost to follow-up after delivery, perinatal screening offers a good opportunity to screen other family members and offer treatment as needed.

Because most pregnant women receive their diagnosis during the chronic, asymptomatic stage of Chagas disease, screening with 2 sequential serologic tests is the most efficient strategy for detection of infection (*12*). PCR and parasitologic tests are ineffective for detection because they show lower sensitivity during this phase (*13*). In contrast, infected newborns usually have high levels of parasitemia. Therefore, microscopic techniques such as microhematocrit and concentration methods in umbilical cord blood

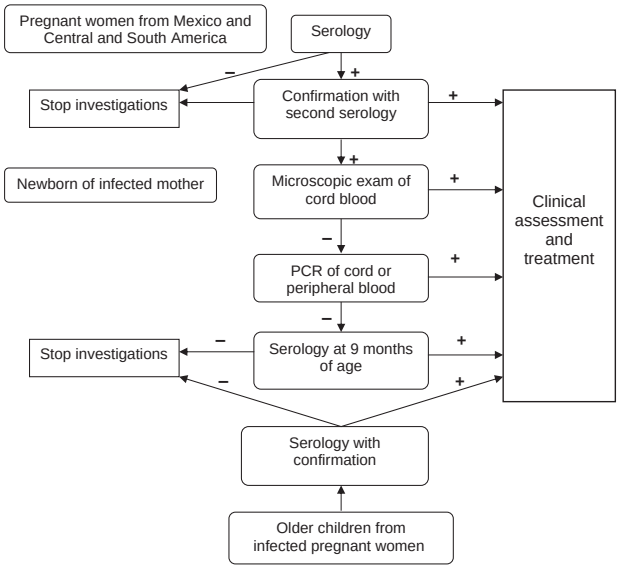

Figure 2. Algorithm for screening, diagnosis, and treatment of *Trypanosoma cruzi* congenital infection at Geneva University Hospitals, Geneva, Switzerland.

have fairly high (>80%) sensitivity (*14*). PCR is more sensitive for detecting Chagas infection in infants than in adults; however, few laboratories perform *T. cruzi* PCR in Europe.

Launched in January 2008, a program of systematic Chagas disease screening of pregnant women at risk and of newborns delivered by infected mothers is under way at the Geneva University Hospitals (Figure 2). All pregnant women from Mexico and Central and South America are screened by serologic testing. Newborns of infected mothers are screened by microscopic examination of cord blood after concentration (microhematocrit, Strout's method) and, if negative, by PCR. If PCR is negative, serologic testing is performed when the child is 9 months of age. Blood cultures are not performed because of time needed to obtain results. Examination of the placenta, which is an unreliable screening method, is also not conducted for diagnostic purposes (*15*). All infected mothers (after completion of breast-feeding), newborns, and their siblings are offered treatment for this potentially fatal disease. Prenatal and delivery care of Latin American immigrants is an opportunity to screen for Chagas disease and its potential vertical transmission. This strategy will help address this emergent health problem in Europe.

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We thank E. Couvreur for diagnosing Chagas disease from the placenta of the newborn described in the first case.

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Isolation of Genotype V St. Louis Encephalitis Virus in Florida

Christy L. Ottendorfer, Jason H. Ambrose, Gregory S. White, Thomas R. Unnasch, and Lillian M. Stark

We isolated and characterized St. Louis encephalitis virus (SLEV) from cloacal swabs of naturally exposed adult sentinel chickens in 2006. Phylogenetic analysis of SLEV strains isolated in Florida indicated that Brazilian SLEV circulated in 1972 and 2006; lineages were VA and VB.

In North America, before the introduction of West Nile virus (WNV; *Flavivirus*, *Flaviviridae*) in 1999, St. Louis encephalitis virus (SLEV; *Flavivirus*, *Flaviviridae*) was the most important agent of epidemic viral encephalitis (*1*). SLEV activity is restricted to the Western Hemisphere and outbreaks have occurred in North America since 1933 (*2*). The recent cocirculation of these closely related flaviviruses has raised the possibility that competitive pressures might alter the transmission cycle of WNV, SLEV, or both (*3*,*4*).

In Florida, periodic SLEV outbreaks since the 1950s led to the formation of an arbovirus surveillance program (*5*), anchored by the Florida Sentinel Chicken Arboviral Surveillance Network (*6*). SLEV is maintained in a mosquito-bird-mosquito cycle; amplification occurs in peridomestic birds and *Culex* spp. mosquitoes (*7*). Chickens are chosen as sentinels because they are susceptible to infection and develop antibodies after exposure (seroconversion) (*8*).

We isolated SLEV from naturally infected adult chickens and compared it with previously isolated strains. The envelope region of viral isolates was analyzed because of its biological importance and high immunogenicity in the host (*9*).

The Study

In Florida, SLEV transmission is sporadic with periods of low (enzootic) and high (epidemic) activity. SLEV was detected by sentinel chickens every year before introduction of WNV (1988–2007) (Figure). Since 2001, limited SLEV activity has been reported (*10*); SLEV may be in a natural decline, or transmission of WNV may influence SLEV cycles, as has been suggested in California (*4*).

In 2006, a total of 2,901 adult sentinel chickens were maintained at 275 sites of potential enzootic arbovirus transmission in 34 Florida counties. Blood was collected weekly from each chicken during peak transmission months (July–December) and tested with hemagglutination inhibition assay, immunoglobulin M antibody-capture ELISA, or plaque reduction neutralization test, as previously described (*11*). Sites with confirmed SLEV seroconversions were targeted for sample collection. For the first time since 2001, SLEV sentinel seroconversions (n = 40) exceeded WNV seroconversions (n = 30) (*10*).

In central and south Florida, 5 partner agencies targeted a subset (n = 15) of sentinel chicken sites with recent confirmed arbovirus transmission activity for cloacal swab collection from 95 chickens. During the weekly scheduled bleeding of the flocks, 1,338 cloacal swabs were collected in viral culturettes (Becton Dickinson, Franklin Lakes, NJ, USA); 529 swabs were retrospectively processed for molecular detection assays and virus isolation in Vero cells, as previously described (*12*). Viral RNA was extracted from cloacal swabs and first-passage cell cultures and amplified with real-time reverse transcription–PCR (RT-PCR) TaqMan assays for WNV and SLEV, as previously described (*13*). Two SLEV strains, FL06-S569 and FL06-S650, were detected by RT-PCR and cultured in Vero cells. Fourteen additional SLEV strains were obtained from the Florida Department of Health, Bureau of Laboratories–Tampa archive for phylogenetic analysis (Table).

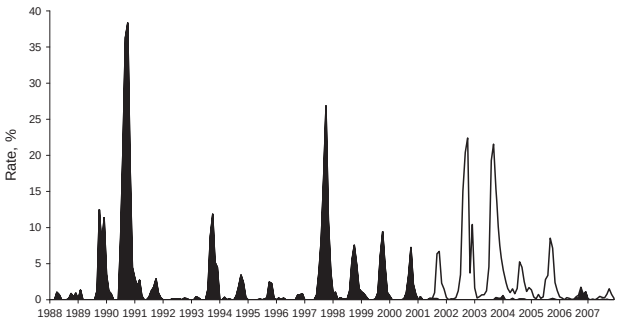

Figure. Rates of flavivirus seroconversion in sentinel chickens, Florida, 1988–2007. Black shading shows St. Louis encephalitis virus (SLEV); white shading shows West Nile virus (WNV). Because the number of susceptible sentinel chickens fluctuated during this time, the rates of seroconversion (no. positive chickens/total no. susceptible chickens × 100, per month) are presented rather than numbers of positive birds. SLEV seroconversion rates declined after the 2001 introduction of WNV despite continued surveillance, and an increased number, of susceptible birds located in regions historically at risk for SLEV enzootic transmission.

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To characterize SLEV strains, we amplified the envelope region using previously described primers (*9*) and the SuperScript III 1-step RT-PCR system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Sequences were aligned by using ClustalW 1.6 and phylogenetic trees drawn by using the maximum parsimony method, with 1,000 bootstrap replicates, in MEGA 4.0 software (*14*), including 60 other SLEV envelope sequences available in GenBank (*9*,*15*) and 4 related flavivirus outgroups (accession nos.: WNV NY99, AF196835; Japanese encephalitis virus, EF571853; Kunjin virus, AY274505; Murray Valley encephalitis virus, AF161266).

The phylogenetic tree places FL06-S569 and FL06-S650 into genotype VA (online Appendix Figure, available from www.cdc.gov/EID/content/15/4/604-appF.htm). This analysis further supports classification of SLEV into 7 lineages and 13 clades (IA-IB, IIA-IIE, III, IV, VA-VB, VI, VII), as previously suggested (*9*). FL06-S569 and FL06-S650 share 98% sequence identity with SLEV strains from South America, including Brazil (BeAn247377, BeAn242587) and Peru (75D90). Two nucleotide mismatches (silent transition mutations at positions 1083, 1404) were noted in the envelope region within the FL06-S569 and FL06-S650 isolates.

Envelope gene sequences were previously published for 6 Florida strains (*9*), and 9 additional archived Florida isolates were analyzed for this study. Reference strain FL72-M022 was isolated from an opossum from the Florida panhandle in 1972. FL72-M022 shares 97%–98% sequence identity with strains from Brazil (BeAn246262, BeAr23379, and BeH203235) and is placed in genotype VB. In contrast, SLEV reference strains isolated in Florida during 1952 and 1985 share 97%–99% homology with strains collected in Tampa Bay during 1962 (TBH-28, GHA-3) and in Mexico (65V310). The last large outbreak of SLEV in Florida occurred during 1990. Envelope sequence analysis demonstrated that strains isolated during 1989 and 1990 shared 98% homology with USA (V 2380-42), Guatemala (78A28), Tennessee (TNM 4-711), or Texas strains (83V4953, PVI-2419, 98V3181).

Conclusions

Despite detection of SLEV after the introduction of WNV, SLEV had not recently been cultured by existing statewide surveillance methods in Florida (*10*). Experimental evidence suggests that WNV cross-protective immunity in wild bird species may limit subsequent SLEV infections (*3*). In 2006, sentinel seroconversions supported this hypothesis; limited WNV activity may have enabled increased transmission of SLEV during the fall (Figure).

Partner agencies in the Florida Arbovirus Surveillance Network used a targeted strategy to preferentially sample sentinels located in "hot zones" of SLEV transmission activity for virus isolation and molecular analysis. Sequence analysis of reference strains and the 2006 SLEV isolates has shown the circulation of genotype V SLEV strains in Florida. The 2006 isolates do not represent a recent extension of the geographic range of strains of SLEV from Brazil because 1 genotype V strain was also collected during field studies in 1972. Instead, they support periodic circulation and maintenance of South American SLEV genotypes in Florida, where the diverse ecosystem may allow for evolution of the virus and periodic seeding of SLEV into the

Table. SLEV strains sequenced for phylogenetic analysis*

Strain	Designation	Location	Year	Host	Passage	GenBank accession no.
FL52-Miami	FL52	Miami, FL	1952	Human	SM1, Vero 1	EU906866
TBH-28	TBH-28	Tampa Bay, FL	1962	Human	SM11, Vero 2	EU906867
F72-M022	FL72	Walnut Hill, FL	1972	Opossum	SM3, Vero 1	EU906868
86-100309	FL85a	Indian River, FL†	1985	*Culex nigripalpus* mosquitoes	SM1, Vero 1	EU906869
86-100802	FL85b	Indian River, FL	1985	*C. nigripalpus* mosquitoes	SM2, Vero 1	EU906870
1A-059	FL89	Indian River, FL	1989	Northern cardinal	SM2, Vero 1	EU906871
3-594	FL90a	Indian River, FL	1990	Common grackle	SM1, Vero 1	EU906872
3A-038	FL90b	Indian River, FL	1990	Mourning dove	SM1, Vero 1	EU906873
3-582	FL90c	Indian River, FL	1990	Common grackle	SM1, Vero 1	EU906874
CXN GR8	FL90d	Indian River, FL	1990	*C. nigripalpus* mosquitoes	SM2, Vero 1	EU906875
FL06-S569	FLS569	Sarasota, FL†	2006	Chicken	Vero 1	EU906876
FL06-S650	FLS650	Sarasota, FL	2006	Chicken	Vero 1	EU906877
TRVL21647	TR58	Trinidad	1958	*C. coronator* mosquitoes	SM3, Vero 1	EU906878
TRVL43174	TR62	Trinidad	1962	*C. nigripalpus* mosquitoes	SM4, Vero 1	EU906879
BeAn70092	BR64	Belem, Brazil	1964	Kingfisher	?, SM1, Vero1	EU906880
BeAn156204	BR69	Belem, Brazil	1969	Chicken	SM2, Vero 1	EU906881

*SLEV, St. Louis encephalitis virus; SM, suckling mouse. Twelve strains collected over 5 decades in Florida were sequenced for phylogenetic analysis. Four South American strains of SLEV were acquired by the Bureau of Laboratories–Tampa before 1972 and sequenced as representative of genotype V SLEV. One control strain (TBH-28) was analyzed and used as a positive control in reverse transcription–PCR and sequencing assays. TBH-28 represents a Florida isolate of SLEV made during the 1960s, but the envelope sequence was previously published (GenBank accession no. AF205469).
†Indian River and Sarasota counties.

United States where the human population may have no immunity to the virus.

On the basis of placement into multiple lineages (IIA-IID, VA, VB) (online Appendix Figure), our data support the hypothesis that persistence of SLEV in Florida may differ from its activity in other regions of the United States. For example, the same or highly similar strains of SLEV can be locally maintained for more than a decade in California and Texas (*15*), whereas genetically similar strains of SLEV appear to be infrequently isolated, or maintained at levels below detection, over extended periods in Florida. Our findings suggest periodic introduction of different SLEV genotypes to Florida from the eastern United States and other countries (Mexico, Panama, and Brazil), with distinct North American (lineage II) genotypes isolated in epidemic years. The role of South American genotypes in enzootic or epidemic cycles of SLEV is unknown. In Florida, only the detection of North American genotypes has previously been reported (*9*,*15*), but the isolation of South American strains in 1972 and 2006 suggests a mechanism for the continued maintenance of SLEV. Further isolation and characterization of SLEV strains is needed to improve understanding of the mechanism(s) that favor the amplification of North vs. South American genotypes in Florida.

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Chagasic Cardiomyopathy in Immigrants from Latin America to Spain

To the Editor: An estimated 8 million persons in 21 countries in the Western Hemisphere are infected by *Trypanosoma cruzi*, the cause of Chagas disease. The global infection rate is 1.4% (*1*) and varies by geographic area from 0.1% to 45.4% (*2*). After infection, organ involvement, predominantly cardiac disease, will develop in 20%–30% after 10–30 years (*3*).

Worldwide, Spain is second to the United States in having the largest number of immigrants from Latin America (*4*). In 2008, immigrants accounted for 11.3% of the population in Spain. A total of 1,607,699 were from *T. cruzi*–endemic areas; of these, 239,942 are from Bolivia (*5*), the country with the highest prevalence of *T. cruzi* infection (*2*).

Imported Chagas disease may emerge in Europe. Chronic Chagas disease was diagnosed in 120 patients during 2003–2008 at the Tropical Medicine Unit, Ramón y Cajal Hospital, in Madrid, Spain. Of these patients, 22.5% had cardiac involvement and 95.8% were from Bolivia. Similar data have been observed in other cities in Spain and Europe (*6*,*7*).

Successful control programs for Chagas disease have been conducted in Latin America in recent years. However, because this disease may have a latency period of many years, infection in immigrants may vary depending on background prevalence in the country of origin.

To calculate Chagas disease prevalence in Latin American immigrants, we considered different values. The number of registered immigrants in Spain according to country of origin for 2007 (*5*) and infection prevalence rates in blood donors for different disease-endemic countries (1993–2002) (*2*) were recorded. The lowest and highest prevalence rates for each country were applied to the number of immigrants from that country living in Spain. Thus, estimates were obtained for the number of potentially infected immigrants for each country of origin. From these figures, a range for the total number of potentially infected immigrants was calculated. Taking into account that all blood donors, but only 80.2% of registered immigrants in Spain (*5*), are adults, we applied an age-correction factor of 80.2% to these figures (multiplying 80.2% by the total) (Table).

Two possible scenarios were then defined to estimate the number of chagasic cardiomyopathies that may arise in the immigrant population. For the best-case scenario, the lowest calculated number of potentially infected immigrants (29,485) was used with the lowest rate of progression to cardiac involvement (20%). For the worst-case scenario, the highest number of infections (98,030) was calculated and used with a 30% risk of progression to cardiac disease. On the basis of these estimates, 5,897–29,409 cases of chagasic cardiomyopathy may be diagnosed in the near future in Spain.

Information on the prevalence of *T. cruzi* infection has changed over time, and the immigrant populations may not be a representative group from disease-endemic areas. Thus, extrapolating these figures to the current population in Spain may pose some problems. However, prevalence data in Bolivia (9.9% in 2001) (*2*), the country with the highest rates of infection, are consistent with data from Spain (2005–2006), which reported a seroprevalence rate of 10.2% in blood donors from Bolivia (*8*).

In recent years, vector control programs in Chagas disease–endemic countries have influenced infection rates. However, most adult immigrants became infected during their childhood, particularly in Bolivia, before any vector control programs were started. Thus, estimated infection rates in adults should not be greatly biased. In a recent study in Spain, the average age of patients with Chagas disease who came to clinics was 35 years (*6*), a finding similar to that seen at our unit. At this age, one might expect infected patients to show cardiac involvement caused by Chagas disease, as well as other manifestations such as megaesophagus and megacolon (*3*).

Table. Immigrants in Spain from Chagas disease–endemic countries in South America potentially infected with *Trypanosoma cruzi*, 1993–2002*

Characteristic	No. immigrants, 2007†	Seroprevalence in blood donors, %‡	Potential no. infected immigrants§
Country			
Ecuador	420,110	0.1–0.2	420–840
Colombia	280,705	0.1–1.2	280–3,368
Bolivia	239,942	9.9–45.4	23,754–108,933
Argentina	145,315	4.4–5.5	6,393–7,992
Peru	120,272	0.1–0.2	120–240
Brazil	115,390	0.6–0.7	692–807
Venezuela	57,679	0.6–1.3	346–749
Paraguay	66,710	2.8–4.7	1,615–3,135
Chile	45,515	0.4–1.2	182–546
Uruguay	49,970	0.4–0.6	199–299
Total	1,541,608		36,567–122,232
No. adults¶	1,236,369		29,485–98,030
Estimated no. chagasic cardiomyopathies			5,897–29,409

*Infection determined on the basis of seroprevalence data from blood donors.
†Data obtained from the Instituto Nacional de Estadística (*5*).
‡Data obtained from Schmunis and Cruz (*2*).
§Calculated by applying seroprevalence data for blood donors in countries endemic for Chagas disease to no. immigrants from each of these countries living in Spain.
¶A correction factor for age was applied (80.2% of immigrants in Spain are adults).

Screening for Chagas disease should be recommended to all Latin American migrants, especially those from Bolivia. This screening would enable early treatment for persons in the chronic asymptomatic phase or those with mild cardiac involvement, persons for whom treatment has been recommended (*9*).

Current legislation in Spain makes screening all at-risk blood donors mandatory (*10*). However, screening of pregnant women from Chagas disease–endemic countries is not compulsory, although 46.8% of immigrants in Spain are female and birth rates in this group are higher than the national average for Spain (*5*). Detection of antibodies to *T. cruzi* during pregnancy would also be a useful public health strategy because it would enable early specific treatment of affected newborns. Screening of blood or organ donors would also be necessary in countries where there is no transmission by vectors.

T. cruzi infection may become a public health problem in countries in Europe that receive immigrants from disease-endemic areas. Thus, chagasic cardiomyopathy may soon have a serious effect on public health in Spain.

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Lethal Bluetongue Virus Serotype 1 Infection in Llamas

To the Editor: Since 1999, of the 24 known bluetongue virus (BTV) serotypes, five (1, 2, 4, 9, and 16) have spread extensively throughout portions of the Mediterranean basin. Since 2006, the range of serotype 8 (BTV-8) has extended northward into areas of Europe never before affected, causing the greatest epizootic of the disease on record. In 2008, a severe epizootic of serotype 1 (BTV-1) occurred in southwestern France; >3,340 outbreaks occurred in <4 months. We report 2 of these outbreaks, which indicate that BTV-1 can infect llamas and induce a lethal disease.

The first outbreak occurred in September 2008 on a sheep breeding farm in Crampagna, France. After bluetongue disease was suspected on the basis of clinical signs, all sheep and all 9 healthy llamas on the farm were tested for BTV by quantitative real-time reverse transcription–PCR (qRT-PCR) (Taqvet BTV "All genotypes," LSI, Lissieu, France). Of the 9 llamas, 7 had positive results; cycle threshold (Ct) values ranged from 28.1 to 36.2, indicating that these animals were sensitive to BTV infection. Serotype 1 was confirmed by a specific qRT-PCR. After 6 weeks, all infected llamas showed serologic response to BTV (ELISA ID Screen; Bluetongue, ID-Vet, Montpellier, France), and 1 llama remained positive by qRT-PCR, which suggests that viremia lasted at least 42 days in this animal. None of the 9 llamas showed clinical signs during the outbreak.

The second outbreak, also during September 2008, was identified on a llama breeding farm in Auzat, France. Two days before the onset of the disease, 1 female had aborted a 10-month-old fetus; she had no additional clinical signs. Of the 20 llamas on the farm, clinical signs were observed for

1 female and 1 male. The signs were anorexia and lethargy followed by acute respiratory distress with polypnea, dyspnea, hiccup-like breathing, and edema. The legs of the male became rigid with moderate paresis. Both animals died <24 hours after the onset of respiratory signs.

Postmortem examination of each of the 2 dead llamas 6–16 hours after death showed similar results: acute and severe hydrothorax, severe congestion and edema of the lungs, and fibrinous pericarditis. Histopathologic examination showed diffuse and severe congestion of the lungs, heart, and kidneys. Virus detection by qRT-PCR was positive for all postmortem tissue samples (spleen, lungs, kidneys, heart); Ct values ranged from 23.5 to 33.7. A 7-cm-long fetus that had been in utero in the female was also positive by qRT-PCR (Ct 38.6). From the aborted fetus, viral RNA was detected in the heart (Ct 39.4) and spleen (Ct 38.3); the blood of the female that had aborted was also positive. Infectious BTV was isolated (in a BioSafety Level 3 laboratory) from the lungs and the spleen of the 2 dead llamas and from both fetuses after 4–6 passages on baby hamster kidney–21 cells (ATCC-CCL10). The specificity of the cytopathic effect was confirmed by BTV-1 qRT-PCR and immunoperoxidase detection using a rabbit polyclonal serum directed against the VP7 viral protein.

The natural host range of BTV is limited to domestic and wild ruminants (*1*,*2*), although seroconversion with no disease in carnivores (*3*) and BTV-8 lethal disease in Eurasian lynx (*4*) have been reported. To date, serologic prevalence of BTV has been demonstrated in alpacas (*5*) but not in llamas or in guanacos (*6*,*7*). South American camelids have been considered to be resistant to the disease, although lethal bluetongue infection in 1 alpaca was suggested in a recent report (*8*).

Our isolation of the virus and detection of large amounts of viral genomes in blood and postmortem samples suggest lethal BTV-1 infection in llamas. In contrast to the clinical signs of paresis, acute respiratory distress syndrome was not frequently reported in domestic ruminants during the BTV-1 epizootic. Nevertheless, pulmonary edema is considered to be a feature of severe bluetongue disease because the lungs of ruminants are most susceptible to permeability disorders of the vasculature after BTV infection (*9*,*10*). Finally, bacterial isolation attempts from the lungs of the 2 llamas that died and PCR tests for bovine viral diarrhea virus and ovine herpesvirus type 2 were all negative. Thus, results of gross examination, histopathologic examination, and virus detection and isolation from postmortem samples indicate that BTV-1 infection was responsible for the death of the 2 llamas.

During the first outbreak, high prevalence of subclinical BTV-1 infection in llamas was detected. BTV-1 was also detected by qRT-PCR in 50% of the healthy llamas tested in the second outbreak and in 60% of those tested in a recent third outbreak (Escalquens, France, November 2008). Healthy llamas in northern France were also found to be positive for BTV-8 (B. Giudicelli, pers. comm.). BTV-1 isolation from 2 fetuses indicates that the strain currently circulating in southwestern France is competent to cross the placental barrier. These findings clearly indicate that llamas are currently infected with BTV-1 and that, although infrequently, the disease can be fatal.

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Spotted Fever Group *Rickettsia* sp. Closely Related to *R. japonica*, Thailand

To the Editor: In response to a recent report that suggested human infection with *Rickettsia japonica* in northeastern Thailand (*1*), we phylogenetically reexamined spotted fever group rickettsiae (SFGR) from Thailand. The organism had been isolated from a male *Haemaphysalis hystricis* tick found on Mt. Doi Suthep, Chiang Mai, northern Thailand, in December 2001. The strain was designated TCM1 and was not distinguishable from *R. japonica* by indirect immunoperoxidase stain using monoclonal antibody (*2*).

After propagating strain TCM1 in L-929 cell culture, we extracted DNA by using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). We subjected the DNA to sequencing that targeted a 491-bp fragment of rickettsial outer membrane protein A (*ompA*), a 394-bp fragment of the rickettsial genus–specific 17-kDa antigen gene, and a 1,250-bp fragment of citrate synthase gene (*gltA*). Direct sequencing of amplicons was performed as previously described. (*3*). Phylogenetic analyses based on o*mpA* indicated that strain TCM1 was closely related to and clustered within the same clade as *R. japonica* strain YH (98.4% identity) (Figure, panel A). Also, a 17-kDa antigen gene obtained from strain TCM1 showed 99.5% identity to the corresponding gene of *R. japonica* (Figure, panel B). Our phylogenetic analysis with *ompA* and 17-kDa antigen gene showed that strain TCM1 was closely related to *R. japonica* but distinguished from *Rickettsia* sp. PMK94 (which was closely related to *R. heilongjiangensis* from northeastern China) (*3*); another SFGR agent, *R. honei* from *Ixodes granulatus* ticks in Thailand (*4*), was apparently different from strain TCM1 (Figure). Phylogenetic analyses based on *gltA* (99.4%–99.6% identity) showed that strain TCM1 is also closely related to *R. japonica* and *Rickettsia* sp. strain PMK94 (data not shown). Thus, we describe the *R. japonica* group in Thailand. DNA sequences of strain TCM1 were determined and deposited in GenBank/EMBL/DDBJ under the following accession nos.: *ompA*, AB359459; 17-kDa antigen, AB359457; *gltA*, AB359458.

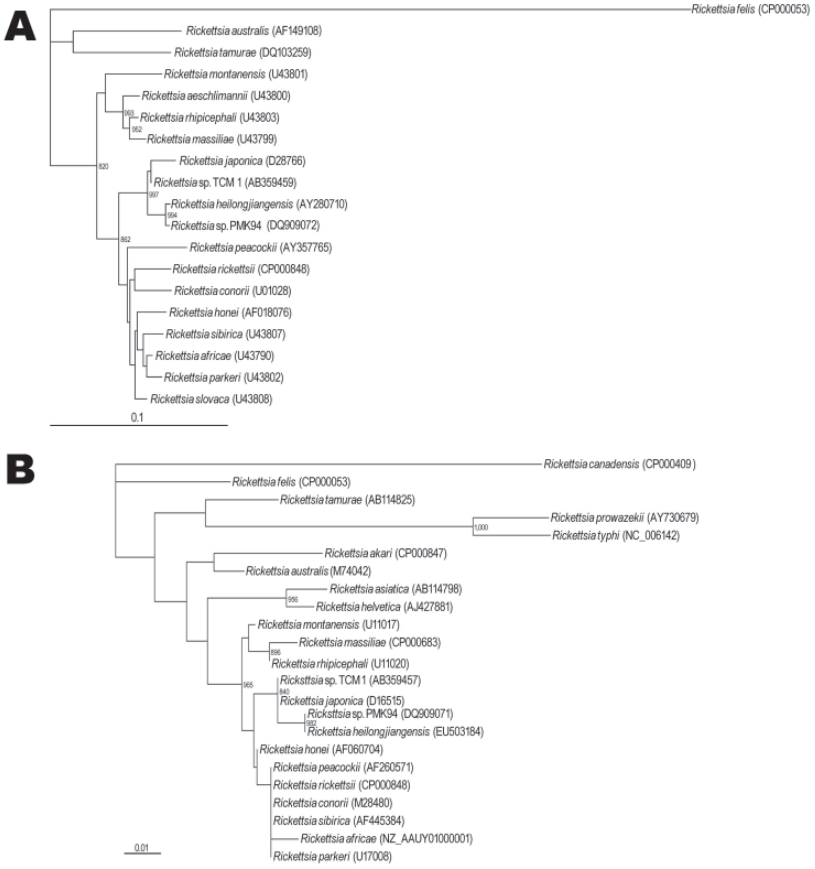

Figure. Phylogenetic analysis based on *ompA* gene (A) and rickettsial genus–specific 17-kDa antigen gene (B). Sequences were aligned by using the ClustalW software package (http://clustalw.ddbj.nig.ac.jp/top-j.html), and neighbor-joining phylogenetic tree construction and bootstrap analysis were conducted according to the Kimura 2-parameter method (www.ddbj.nig.ac.jp). Pairwise alignments were performed with an open-gap penalty of 10, a gap extension penalty of 0.5, and a gap distance of 8. Multiple alignments were also performed with the same values, and the phylogenetic branches were supported by bootstrap analysis with 1,000 replications (>800 were indicated). *Rickettsia felis* (CP000053) and *R. canadensis* (CP000409) were used as outgroups for *ompA* and 17-kDa antigen gene, respectively. The phylogenetic tree was constructed by using TreeView software version 1.5 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Scale bars indicate nucleotide substitutions (%) per site.

R. japonica is the specific pathogen of Japanese spotted fever, which has been found mainly in southwestern Japan (*5*). The present strain, closely related to *R. japonica*, is likely to have been isolated from *H. hystricis* in Thailand because *R. japonica* frequently has been isolated, or detected by PCR, from the same tick species in Japan (*6*). Such tick species–specificity of SFGR should be considered when speculating on any geopathologic relationships of rickettsioses among different SFGR-endemic areas. Previous reports on spotted fever–positive results of human serosurveys (*7*,*8*) and on a clinical case (*9*) in northern Thailand may provide epidemiologic background. In Asia, multiple species of rickettsiea (e.g., *R. japonica*, *R. heilongjiangensis*, *R. honei*) are the causative agents of spotted fever rickettsioses, so the agent closely related to *R. japonica* could cause spotted fever in Thailand. Additionally, *R. japonica* has been found in Korea (*10*), and our current study indicates that *R. japonica* and its genetic variants are widely distributed in Far Eastern countries, including Japan (Grant-in-Aid for International Cooperative Research, unpub. data). Therefore, the epidemiology and genetic variation of SFGR throughout Asia should be examined by molecular studies.

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Segniliparus rugosus Infection, Australia

To the Editor: Recently, a female teenager with cystic fibrosis who resided in tropical north Queensland, Australia, was found to be infected with *Segniliparus rugosus*. She was homozygous for the deltaF508 mutation, had well-preserved lung function, and regularly played competitive sports. Unlike many cystic fibrosis patients, she did not have a history of chronic *Pseudomonas aeruginosa* infections, but *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* had been previously isolated from her sputum. In May 2007, she described reduced exercise tolerance and increased cough with excess sputum production. Lung function testing showed modest spirometric decline. A computed tomographic scan of the chest showed significant mucus plugging and bronchiectasis, uncommon without previous *P. aeruginosa* infection. Sputum was 3+ smear positive for acid-fast bacilli (AFB), and *S. rugosus* was isolated from liquid culture. Empiric antimicrobial drug therapy was changed to rifabutin and co-trimoxazole because these drugs have been effective in previous cases (*1*). Clinically, the patient showed response to the treatment. After 12 months of treatment, her sputum was still 3+ positive for AFB, and *S. rugosus* was again found in culture. She was referred to a pediatric teaching hospital in Brisbane with worsening respiratory symptoms precipitated by influenza B infection. Antimicrobial drug therapy with intravenous imipenem, oral moxifloxacin, and co-trimoxazole for 2 weeks resulted in clinical improvement but little reduction in smear positivity.

The initial AFB smear-positive sputum specimen underwent routine decontamination with sodium hydroxide and neutralization and was inoculated into radiometric 12B vials (Bec-

ton Dickinson, Sparks, MD, USA). The culture was positive after 6 days' incubation and was referred to the state reference laboratory in Brisbane. The culture was smeared and stained with the Ziehl-Neelsen method, which showed short, pale AFB.

A crude boil DNA preparation was made from the positive culture, and an in-house multiplex PCR to identify *Mycobacterium* spp. was performed (*2*). No PCR products were detected, and the *Mycobacterium* genus-specific band was absent. Similarly, no *Mycobacterium* genus band was detected by using a PCR-hybridization method (Common Mycobacteria line probe assay, Hain Lifesciences GmbH, Nehren, Germany) (*3*). DNA sequencing was performed on the *Segniliparus rugosus* strain NQ1 (GenBank accession no. FJ593188), and a 1,250-bp fragment of the ribosomal 16S loci was obtained. A BLAST search (GenBank) was then performed on the sequence information obtained. The DNA sequence gave a 100% match to that of *Segniliparus rugosus* (GenBank accession no. AY608920) (*4*).

Drug susceptibility testing (DST) was attempted by using disk diffusion with Mueller-Hinton plates but was unsuccessful because of insufficient growth and indistinct margins. A commercially available microbroth dilution assay was used to determine the MICs for 12 antimicrobial drugs. The Sensititre Broth MIC Method for Rapidly Growing Mycobacteria, Nocardia, and Other Aerobic Actinomycetes (Trek Diagnostic Systems, Cleveland, OH, USA) was used according to manufacturer's instructions (*5*). However, problems still occurred because of inadequate growth of the organism using the testing media approved for mycobacteria (*6*,*7*). Superior results were obtained when the cation-adjusted Mueller-Hinton broth with TES buffer (Trek Diagnostic Systems), which is used routinely in this assay, was substituted with an internally prepared Middlebrook 7H9 broth (Becton Dickinson) (*1*). DST results are similar to those previously reported for other strains of *S. rugosus* (Table) (*1*).

The strain had rough, wrinkled colonial morphology on both blood agar and 7H11 plates. Growth was optimal at 35°C on 7H11 media. Arylsulfatase activity was weakly positive at 3 days and positive at 14 days. Growth on Lowenstein-Jensen (LJ) medium and on LJ with 5% sodium chloride medium occurred at 6–7 days. The strain was negative for both nitrate reductase and tween hydrolysis. Mycolic acid high-pressure liquid chromotography was performed (*8*), and the pattern obtained matched that of type strain CDC 945 (AY608920) previously reported (*4*). The pattern had a double cluster of adjoining eluting peaks with the last peak co-eluting with the internal high standard.

Only 3 other cases of *Segniliparus* spp. infection (none from Australia) have been reported. More remains to be learned about the efects of lung infections with *Segniliparus* spp. in cystic fibrosis patients. Although this patient improved clinically after treatment with antimicrobial drugs, she is still infected and will likely remain chronically infected. Because this genus is acid fast by the Ziehl-Neelsen method, laboratory workers and clinicians must be aware that AFB seen in pulmonary specimens from cystic fibrosis patients are not necessarily from the genus *Mycobacterium* and may be from the genus *Segniliparus*.

Table. Antimicrobial susceptibility patterns for *Segniliparus rugosus* strain in a cystic fibrosis patient, Australia

Antimicrobial agent	MIC, µg/mL
Amikacin	>128
Amoxicillin/clavulanic acid (ratio 2:1)	64/32
Cefoxitin	64
Ceftriaxone	>64
Ciprofloxacin	4
Clarithromycin	16
Gatifloxacin	0.5
Imipenem	1
Linezolid	64
Minocycline	>32
Tobramycin	>64
Trimethoprim/sulfamethoxazole	2/38

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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.

Multigenotype Q Fever Outbreak, the Netherlands

To the Editor: Q fever is a zoonosis caused by *Coxiella burnetii* (*1*). An ongoing Q fever outbreak has occurred in the Netherlands since 2007; incidence rates have increased >50-fold compared with the baseline rate (*2*). The source of this outbreak is unknown. Identifying the source of an infection is complicated because of difficulties in obtaining sufficient clinical and/or environmental samples for testing. Molecular diagnosis of Q fever has focused on the use of serum samples. Up-to-date genotyping of *C. burnetii* has depended on cultivation and enrichment of the isolate before analysis (*3*). We report multiple-locus variable-number tandem repeat analysis (MLVA) typing of *C. burnetii* for a variety of human and animal clinical samples obtained from different locations in the Netherlands (Table).

Severe pneumonia developed in patient 1 after close contact with sheep (ewes) and intimate cuddling with a newborn lamb. Patients 2 and 3 (a dairy goat farmer and his wife from another village) tested positive for Q fever after a large part of their goat herd aborted offspring. The farmer had no clinical symptoms; his wife had mild symptoms that disappeared spontaneously within 2 days. No samples from any of the goats were available. Two additional patients were tested, 1 of which lived in the same village as patients 2 and 3.

Swab specimens from all sheep and lambs tested in the first case yielded identical MLVA genotypes. The same genotype was also found in patient 1 but not in the other examined samples, implicating sheep as the origin of patient 1's infection. Although patients 2 and 3 lived together, the genotype found in patient 2 differed from the (partial) genotype found in patient 3. Yet another genotype was found in a patient from the same village (patient 4). However, an identical genotype found in patient 2 was found in a patient from a distant village (patient 5). The village had only 1 goat farm, and if this herd of goats was the source of infection for the farmer, his wife, and patient 4, it would have contained >1 genotype. At least 1 of the obtained genotypes has spread over a wider surface area in the Netherlands.

Our results show that the unprecedented, ongoing Q fever outbreak in the Netherlands involves multiple genotypes of *C. burnetii*. Because most of the genotypes differ only by a single repeat difference, they might represent microvariants of a hypervirulent strain that has been introduced in the Dutch animal population. MLVA schemes with up to 17 markers have been previously reported (*3*). In this "proof of concept" (applying direct genotyping of *C. burnetti* on clinical samples), we focused on the 3 shortest repeat units because we believed that these units might have the highest à priori chance of successful amplification in clinical samples (especially in serum/plasma). Similar genotypes as those reported here were found in the MLVA database (http://mlva.u-psud.fr), but these similarities need confirmation by using more markers. Although using only 3 markers may lead to poor discriminatory power, we were still able to distinguish 4 different genotypes in a relatively small collection of serum samples. We are currently exploring the use of additional MLVA markers.

Our results also show a poor correlation between DNA load and clinical symptoms. Multiple human and animal clinical samples, including serum and plasma, throat or genital swabs, or sputum and urine, may be useful for direct genotyping and outbreak source tracking.

Table. Genotyping results for human and animal clinical samples, Q fever outbreak, the Netherlands

Patient/animal no.	Sample	Ms27*	Ms28*	Ms34 *	Symptoms	Ct value†	Location
Patient 1	Plasma	3	3	8	Severe	34.4	1
Ewe 1	Vaginal swab	3	3	8	None	25.7	1
Ewe 2	Vaginal swab	3	3	8	None	16.3	1
Ewe 3	Vaginal swab	3	3	8	None	18.8	1
Lamb 1	Throat swab	3	3	8	None	27.9	1
Lamb 2	Throat swab	3	3	8	None	29.9	1
Lamb 3	Throat swab	3	3	8	None	28.9	1
Patient 2	Urine	3	3	7	None	31.7	2
	Throat swab	3	3	7		31.8	
Patient 3	Urine	NR‡	3	4	Mild	36.7	2
Patient 4	Sputum	4	3	7	Severe	34.2	2
Patient 5	Sputum	3	3	7	Severe	31.9	3
Nine Mile	Reference strain	4*	6*	5*			

*The allele-calling convention used was as published (*3*), resulting in a 4, 6, 5 code assigned respectively to the 6-bp repeat unit loci Ms-27, Ms-28, and Ms-34 for the genome sequence of the Nine Mile RSA-493 strain (GenBank accession no. NC002971.1). Primers for these markers were redesigned to amplify significantly shorter PCR products and were combined into 1 multicolor multiplex PCR. Primer sequences for Ms-27 were 5'-HEX-TCTTTATTTCAGGCCGGAGT-3' and 5'-GAACGACTCATTGAACACACG-3; for Ms-28, 5'-TMR-AGCAAAGAAATGTGAGGATCG-3'and 5'-GCCAAAGGGATATTTTTGTCCTTC-3'; for Ms-34, 5'-FAM-TTCTTCGGTGAGTTGCTGTG-3' and 5'-GCAATGACTATCAGCGACTCGAA-3'.
†Cycle threshold (Ct) value obtained by using real-time PCR targeting the IS1111a element.
‡NR, no result obtained. A full genotype was obtained only in samples with the highest DNA loads (Ct value ≤35).

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Correlation between Buruli Ulcer and Vector-borne Notifiable Diseases, Victoria, Australia

To the Editor: Buruli ulcer (BU) is a destructive skin disease caused by the toxin-producing environmental pathogen *Mycobacterium ulcerans*. Since the 1980s, BU has emerged as a major public health problem in rural West and Central Africa (*1*), where some researchers have suggested a role for aquatic insects as either reservoirs or vectors of *M. ulcerans* (*2*,*3*). However, this hypothesis remains unproven (*4*).

In contrast to the emerging BU–endemic areas in tropical rural West Africa, the climate of the Australian state of Victoria is temperate, yet locally acquired BU also has increased there in recent years (*5*). In addition, notifications have varied markedly from year to year for reasons not yet explained.

During the investigation of a new outbreak of BU in Victoria, we demonstrated that *M. ulcerans* is detectable by PCR in mosquitoes and that being bitten by mosquitoes increases the odds of being diagnosed with BU (*6*,*7*). However, *M. ulcerans*–positive mosquitoes might reflect only the presence of *M. ulcerans* in the local environment and play no role in transmission. To further investigate links between BU and mosquitoes, we compared patterns of notifications of BU with other notifiable diseases in Victoria. In particular, we were interested in any association between BU and the locally transmitted vector-borne alphaviruses Ross River virus (RRV) and Barmah Forest virus (BFV). Areas of BU and RRV/BFV endemicity overlap geographically, but areas with RRV and BFV are more extensive and include inland river systems where BU has not so far been reported.

Notification data for RRV, BFV, and other notifiable infections in Victoria are publicly available (*8*). Although BU was not made notifiable until January 2004 (before which notification was voluntary), since early 2000, most diagnoses were confirmed by culture or PCR at the Victorian Infectious Diseases Reference Laboratory, from which we obtained data for this report.

Our analysis showed that in the last 7 years (2002–2008), BU notifications correlated with combined RRV/BFV notifications ($r^2 = 0.52$, $p = 0.06$) (Figure). During the same period, no correlation was observed with tuberculosis, the other important mycobacterial disease in Victoria ($r^2 = 0.12$, $p = 0.43$); legionellosis, caused by a nonvectored water-associated pathogen ($r^2 = 0.04$, $p = 0.66$); or any other notifiable infectious disease (data not shown).

Although the environmental reservoir and mode of transmission of *M. ulcerans* remain unknown, mosquitoes are well known for transmitting RRV and BFV to humans, and year-to-year variation in incidence of these vector-borne viral infections is linked to changes in mosquito numbers (*9*,*10*). We are not implying that *M. ulcerans*, RRV, and BFV are transmitted simultaneously from the same reservoir species to the same humans or by the same mosquitoes. Also, environmental conditions that promote outbreaks of RRV/BFV infection might promote BU outbreaks without any other connection. However, we believe the correlation we have identified between BU and other mosquitoborne diseases is striking and further strengthens the link between mosquitoes and the transmission of *M. ulcerans* in Victoria.

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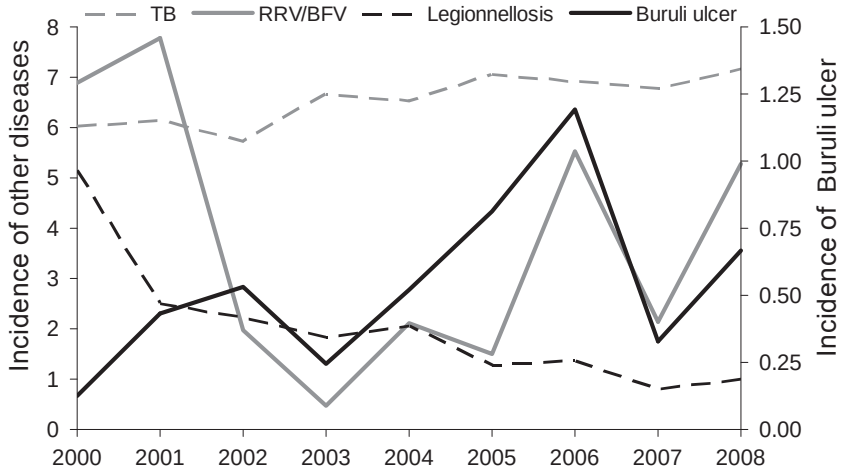

Figure. Numbers of cases per 100,000 inhabitants for selected notifiable diseases, Victoria, Australia, 2000–2008. Buruli ulcer is shown on the right y axis, other diseases on the left y axis: tuberculosis (TB), Ross River virus (RRV)/Barmah Forest virus (BFV), and legionnellosis.

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Avian Influenza Risk Perception among Poultry Workers, Nigeria

To the Editor: In Nigeria and other African countries, outbreaks caused by the Asian strain of highly pathogenic avian influenza virus (HPAI) subtype H5N1 have occurred in poultry. These countries do not have the capacity to effectively manage, eliminate, and control animal diseases, and humans generally live in close contact with poultry (*1*,*2*).

Before these outbreaks (2006) in other countries, effective risk communication had reduced chances of human infection (*3*,*4*), and the effect of news media reports in reducing infection also had been reported (*5*). However, risk evaluation, perception, or communication has not been reported from Africa, where poverty (*6*), inadequate primary healthcare facilities (*7*,*8*), and nonchalant attitudes to animal diseases predominate. In this study, we report the perception of poultry workers in Nigeria to avian influenza (AI).

To determine perception of AI, from November 2006 through January 2007, we surveyed a random sample of 200 poultry workers in 8 of the Nigerian HPAI virus (H5N1)–affected states: Kaduna and Kano (north); Plateau, Bauchi, Nasarawa, and Abuja (central); and Ogun and Lagos (south). We used pretested and previously evaluated structured interviews. Telephone interviews were used to confirm data collected from ≈15% of respondents, and data were evaluated by using descriptive statistics. All responses were evaluated according to published guidelines of the World Organisation for Animal Health (OIE), US Centers for Disease Control and Prevention, World Health Organization, OIE/Food and Agriculture Organization of the United Nations (FAO) Network on Avian Influenza, and Food and Drug Administration of the United Nations, taken from the organizations' websites.

One hundred thirty-five (68%) poultry farmer workers from 36 infected and 39 uninfected flocks responded to the interview. Farms evaluated had flocks of a few hundred (200–300) to >70,000 chickens. Eighty-nine percent of respondents were concerned about AI; 57% knew that AI has food safety implications. Eighteen percent were willing to eat chicken that had died or gotten sick from infection; 21% would eat chicken and eggs from infected farms. These surveyed workers stated that thorough cooking, frying, cleaning, and traditional cooking methods were sufficient to kill the HPAI virus. 23% were not aware of risk associated with processing of HPAI-infected meat.

Although 61% reported knowing some risk factors for AI (e.g., close association with infected birds, home slaughtering, unprotected personnel, eating and processing of infected carcasses), only 56% correctly described some risk factors. Sixty percent reported having heard about the AI virus before the outbreak in Nigeria; 55% reported knowing the symptoms in affected birds. Of the 67% who had some knowledge of the symptoms, 56% were familiar with differential diagnoses. Ninety percent erroneously believed AI was fatal only to birds, although 58% believed it could affect humans.

After the first wave of HPAI outbreaks in poultry in Nigeria (2006), 98% of respondents said they had gained some knowledge about AI, primarily through television but also through radio, newspapers, government, community public health messages, veterinarians, and the Internet or through journals and seminars. Although 21% of respondents had had their flocks tested for AI, they had difficulty distinguishing between clinical assessment and laboratory tests. Seven farmers had themselves been tested for AI exposure. Seventy-six percent of farmers were willing to be tested, but only 67.9% were willing to have their flocks tested.

Respondents were more concerned about the effect of AI on financial preservation of business interests than on public health risk. Knowledge about biosecurity and risk factors varied widely between urban/periurban (51 correct answers) and rural (25 cor-

Table. Comparison of positive responses to avian Influenza questionnaire, November 2006–January 2007*

Item	Nigeria	Italy	Thailand
Surveyed population	Farmers/farm workers	Farm workers	Consumers
Food safety knowledge	56.8%	58%	92%
Avian influenza concern	88.6%	69.7%	6
Knowledge of avian influenza	67.1%	63.8%	88%
Literacy level of respondents	47.7% higher education (degree, tertiary education)	One third high school and college	98%
Receipt of information after outbreaks	97.5%	91.8%	NA
Sources of information	TV, radio, newspaper	Mass media/health Worker/employers	NA
Economics of poultry production	Unwilling to quit poultry production in event of outbreaks	NA	Very important economic source
Percentage of urban respondents	40.2%	NA	41.9%

*NA, not available.

rect answers) workers. Most correct answers about knowledge of human infection by the HPAI virus also came from urban/periurban respondents. Forty percent of respondents who would not eat AI-infected chicken cited religious prohibition to eating dead animals. Seven respondents did not believe AI exists at all and viewed the outbreak situation as a diversionary tactics from the 2007 presidential election.

Our findings are similar to trends reported among poultry workers in previous studies (*3*,*4*) (Table). Our study showed that knowledge of food safety and risk factors and differentiation between HPAI and other poultry diseases is poor among the poultry farming communities of Nigeria. The belief by 90% of respondents that AI is lethal only in poultry further increases risk for human infection. The study also showed that farmers believe the news media (broadcast and print) are important in increasing public understanding of AI. Nearly all respondents agreed that poultry enterprise is profitable, albeit risky, and were not willing to abandon the business even in the event of an AI outbreak. Because the knowledge gap between the rural and urban communities further heightens the risk for human AI infection in Nigeria, public health messages about AI should target rural communities.

Previously, workers have indicated that socioeconomic factors prevent the rural and urban poor from accessing healthcare facilities (*8*). Lack of access to healthcare was evident in the response of workers who stated they would want to have themselves and their flocks tested if healthcare services were available and if government agencies would bear the cost of tests that may be unaffordable to most.

Since this survey, progress in disseminating knowledge of AI in Nigeria has been substantial. The country has established desk offices (state centers for coordination of surveillance activities in animals) to carry out regular surveillance for HPAI virus (H5N1), and farmers have tremendously improved their knowledge (*9*).

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Mycobacterium avium subsp. *hominissuis* Infection in a Pet Parrot

To the Editor: Tuberculosis is a chronic wasting disease in domestic birds (especially hens) and free-ranging birds worldwide (*1*). Most mycobacterial infections in birds are caused by *Mycobacterium avium* subsp. *avium* (mainly domestic birds) or by *M. genavense* (especially pet birds). Nontuberculous (potentially pathogenic) mycobacteria (i.e., *M. fortuitum*, *M. gordonae*, and *M. nonchromogenicum*) occasionally have been isolated from necropsied pet birds (*2*). Because potentially pathogenic mycobacteria also are increasingly problematic in immunocompromised human patients, they merit special attention. *M. avium* subsp. *hominissuis* can infect humans, especially immunocompromised per-

sons. *M. avium* subsp. *hominissuis* infections have been documented in pigs and cattle (*3*) and rarely in dogs (*4*), birds (*5*), and other animals.

We report a case of *Mycobacterium* infection in a female blue-fronted Amazon parrot (*Amazona aestiva*; pet bird) ≈6 months of age that was brought to a clinic because of inappetence over a 3-day period and polydipsia and yellow coloration of urine. Clinical examination showed slight emaciation, heavy biliverdinuria, ascites, and melena. By coprologic examination, 3 eggs of *Ascaridia* sp. worms were found in 1 field of view using 40× magnification. A Gram stain of fecal material showed sporadic gram-positive rods. On the basis of these signs, chlamydiosis was suspected. Differential diagnosis suggested liver cirrhosis, neoplasia, and Pacheco disease. Enrofloxacin (Baytril 2.5% injectable; Bayer AG, Frankfurt am Main, Germany) was administered subcutaneously (0.15 mL injected subcutaneously) and albendazole (Aldifal 2.5% suspension; Mevak a.s., Nitra, Slovakia) were administered orally (0.2 mL injected subcutaneously). The bird died 1 day later.

Necropsy showed ascites (clear yellowish fluid), hepatomegaly (stiff liver consistency, yellow-pink), mild splenomegaly, and hemorrhagic enteritis with thickening of the intestinal wall; the finding of hemorrhagic enteritis was unclear because the intestinal mucosa was hyperemic and covered with a thick layer of viscous mucus that contained blood. Twenty worms (*Ascaridia* sp.) were observed in the intestinal lumen.

Histopathologic examination showed diffused liver fibrosis with cystic dilatation of the bile ducts and focal extramedullary hematopoiesis. The hepatic parenchyma was nearly completely atrophic. Only some clusters of atrophic hepatocytes were observed. The other organs (kidneys, spleen, lungs, brain, and intestines) were free of histopathologic lesions. Hypertrophic cirrhosis (chronic active hepatitis) was diagnosed. Neither granulomatous nor other lesions were observed.

After Ziehl-Neelsen stain of tissue impressions, acid-fast rods were microscopically detected in the liver and intestine. Cultivation according to Matlova et al. (*6*) grew 6 acid-fast rod–positive isolates from 9 examined tissue specimens. A PCR assay confirmed *M. avium* spp., and a subsequent PCR assay for *M. avium* differentiation indicated *M. avium* subsp. *hominissuis* (IS*1245*+ and IS*901*–); both PCR assays were performed as described (*7*). The *M. avium* subsp. *hominissuis* isolate was classified as serotype 9. Typing of all isolates by IS*1245* restriction fragment length polymorphism (RFLP) analysis according to Van Soolingen et al. (*8*) showed 2 different multibanded IS*1245* RFLP types, which varied in only 1 band position (Table).

M. avium subsp. *hominissuis* is not considered an avian pathogen and rarely has been isolated from tuberculous lesions (*5*). However, our case study reports the isolation of *M. avium* subsp. *hominissuis* from multiple organs of 1 exotic bird that had developmental anomaly and liver fibrosis (Table). In addition to a few nonspecific gross lesions, nontuberculous lesions were observed in the liver, spleen, and intestinal organs.

The etiology of mycobacteriosis, especially in pet birds, is rarely identified. This may be because intravitam and postmortem findings are nonspecific. Infection with *M. avium* subsp. *hominissuis* may not lead to tuberculous lesions in birds, particularly when the infection occurs without complications. Susceptibility to mycobacterial infection, including *M. avium* subsp. *hominissuis*, depends on the host's immune and nutritional status, environmental conditions unfavorable for the host, and genetic factors (*1*,*9*). Consistent with these reports, in this case, the histologic findings such as fibrosis of the liver associated with cystic dilatation and intestinal ascaris infestation may have aggravated the intensity of the mycobacterial infection.

IS*1245* RFLP analysis showed isolates with 2 profiles that differ in the presence of only 1 band. The additional band in the rest of the isolates probably represents the transpositional event. The variability in 1 or 2 bands of 1 strain was also observed previously (*10*); therefore, we presume the bird was infected by only 1 strain of *M. avium* subsp. *hominissuis*. Unfortunately, the source of infection for this bird was not identified.

A multibanded IS*1245* RFLP profile was described in a *M. avium* isolate from a parrot (*4*), but no details about this case were given. Our findings suggest that owners of pet birds and their family members may be at risk from this pathogenic causal agent.

Table. Detection of *Mycobacterium avium* subsp. *hominissuis* in a female blue-fronted Amazon parrot (*Amazona aestiva*)*

Origin of examined tissue samples	Mycobacteria detection		PCR†		IS*1245* RFLP§
	ZN	Culture‡	IS*1245*	IS*901*	
Lung	–	+ (3)	+	–	a
Kidney	–	–			
Heart	–	–			
Liver	+	+ (15)	+	–	a
Intestine	+	+ (2)	+	–	b
Stomach	–	+ (8)	+	–	a
Bone marrow	–	+ (1)	+	–	a
Brain	–	–			
Musculus pectoralis	–	+ (34)	+	–	a

*ZN, Ziehl-Neelsen microscopy of homogenate for acid-fast rods; RFLP, restriction fragment length polymorphism; –, not detected/negative; +, detected/positive.
†PCR for IS*1245* and IS*901* was carried out according to the method described in (*7*).
‡Culture examination performed as described by Matlova et al. (*6*); colony-forming units per isolation are shown in parentheses.
§Standardized IS*1245* RFLP method was performed according to Van Soolingen et al. (*8*).

Hence, immunocompromised persons, children, and others involved in the breeding of exotic birds should avoid contact with birds with clinically suspected *M. avium* subsp. *hominissuis*.

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Mycobacterium colombiense and Pseudotuberculous Lymphadenopathy

To the Editor: *Mycobacterium colombiense* is a new species belonging to the *M. avium* complex (MAC). It is characterized by a unique internal transcribed spacer sequence and causing respiratory tract and disseminated infection in HIV-infected patients in Colombia (*1*). We report clinical and histologic features of lymphadenopathy resulting from *M. colombiense* infection.

A 25-month-old girl with an unremarkable medical history was hospitalized in the pediatric department of Timone Hospital, Marseille, France, due to development of swelling in a right subclavicular lymph node over a 1-month period. A 5-day course of oxacillin, which was administered orally, had been unsuccessful in alleviating the symptoms. The patient's general condition was excellent, and results of a physical examination were normal, with the exception of a 2-cm hard, immobile, yet painless, noninflammatory, enlarged lymph node. Due to the presence of the enlarged lymph node, a chest radiograph was performed, and results were normal. A hemogram indicated a hemoglobin concentration of 113 g/L, a leukocyte count 8.3×10^9/L consisting of 31% polynuclear neutrophils and 62% lymphocytes, and a normal blood smear. A platelet count indicated a concentration of 389×10^9/L, and the serum lactic dehydrogenase level was 440 UI/L. In addition, no biologic inflammatory syndrome was observed based on the concentration of C-reactive protein (<1 mg/L) and an erythrocyte sedimentation rate of 18 mm/h.

Fine-needle aspiration of the lymph node showed necrosis and mature, activated lymphocytes. These results suggested a possible diagnosis of lymphoma, and a surgical excision

biopsy was subsequently performed. Direct microscopic examinations were carried out after results obtained by Gram and Ziehl-Neelsen staining showed that the lymph node was negative for acid-fast bacilli. Histopathologic analysis indicated epithelioid cell granulomas containing giant cells and caseous necrosis without altered leukocytes, all of which are compatible with tuberculosis. Culturing of the biopsy specimen in BACTEC broth (Becton Dickinson, Courtaboeuf, France) at 5% CO_2 at 37°C yielded acid-fast bacilli after a 7-day incubation period.

After inactivating the cells and extracting the DNA by using a previously described method, we identified the isolate by PCR sequencing of the *rpo*B gene (*2*) and its demonstrated 100% sequence similarity to *M. colombiense* CIP108962^T (*1*,*2*). Accordingly, the isolate exhibited positive urease activity, a distinctive characteristic that differentiates *M. colombiense* from other MAC species (*1*,*2*).

Recently, *M. colombiense* was shown to be responsible for an enlarged lymph node in 1 child from Spain who did not show any evidence of HIV infection (*3*). In that patient, histopathologic examination showed granulomatous adenitis with necrosis. We report that *M. colombiense*–infected lymph nodes also yield clinical and histopathologic features evocative of tuberculosis. Indeed, MAC organisms remain the most prevalent agents demonstrated in diseased lymph nodes in children (*4*).

Because modern molecular tools used for the description of emerging MAC species have not been available in most previous reports, the real prevalence of *M. colombiense* may have been underestimated. In children, *M. hemophilum* (*5*), *M. avium* subsp. *avium* (*6*), *M. avium* subsp. *hominissuis* (*7*), *M. lentiflavum* (*8*), *M. bohemicum* (*9*), and *M. simiae* (*10*) have been demonstrated to be responsible for enlarged cervical lymph nodes (online Appendix Table, available from www.cdc.gov/EID/content/15/4/619-appT.htm). Because management and antimicrobial drug treatment of each of these different infections vary in terms of indication, choice of drugs, and duration, the accurate and rapid identification of the causative *Mycobacterium* species is absolutely necessary. This identification should use PCR sequencing of selected universal molecular targets, including the 16S rRNA and *rpo*B genes (*2*), as illustrated herein.

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Leptospira noguchii and Human and Animal Leptospirosis, Southern Brazil

To the Editor: Pathogenic leptospires, the causative agents of leptospirosis, exhibit wide phenotypic and genotypic variations. They are currently classified into 17 species and >200 serovars (*1*,*2*). Most reported cases of leptospirosis in Brazil are of urban origin and caused by *Leptospira interrogans* (*3*). Brazil underwent a dramatic demographic transformation due to uncontrolled growth of urban centers during the last 60 years. Urban slums are sites of poor sanitation that favors rat-borne transmission of leptospirosis among humans. Thus, this may explain the major involvement of serovar Copenhageni (*L. interrogans*). The predominance of *L. interrogans* is likely due to the underestimation of rural cases of leptospirosis.

Pelotas is a coastal city in Rio Grande do Sul State, in southern Brazil, with ≈400,000 inhabitants. This state has a typical temperate climate. However, the incidence of human leptospirosis is high (12.5/100,000 inhabitants in 2001) compared with the mean incidence in areas of Brazil where tropical and subtropical climates predominate (3.5/100,000 in the same year). Most cases in Rio Grande do Sul State (69%) occur in rural areas where the spatial distribution suggests an association with areas of rice field activities. Pelotas had an annual incidence of >50 cases per 100,000 inhabitants in 2001, which placed it among the cities with the highest incidence of leptospirosis in southern Brazil (*4*).

Before 2007, pathogenic serovars and strains in Brazilian collections included the following species: *L. santarosai*, *L. interrogans*, *L. kirshneri*, and *L. borgpetersenii*. However, our research group has recently reported the isolation of *L. noguchii* in Brazil from sheep (*5*). This species had been previously isolated from animals such as armadillo, toad, spiny rat, opossum, nutria, the least weasel (*Mustela nivalis)*, cattle, and the oriental fire-bellied toad (*Bombina orientalis)* in Argentina, Peru, Panama, Barbados, Nicaragua, and the United States (*1*,*6*). Human leptospirosis associated with *L. noguchii* has been reported only in the United States, Peru, and Panama, with the isolation of strains Autumnalis Fort Bragg, Tarassovi Bac 1376, and Undesignated 2050, respectively (*1*,*6*). The Fort Bragg strain was isolated during an outbreak among troops at Fort Bragg, North Carolina. It was identified as the causative agent of an illness characterized by fever, headache, myalgia, and a pretibial rash—Fort Bragg fever (*7*). We were not able to obtain data regarding the other 2 human isolates.

We report the isolation of 3 additional *L. noguchii* strains from Brazil, including 2 from cases of human leptospirosis. The first isolate (Bonito strain) was obtained from the blood culture of a 34-year-old man who exhibited fever, headache, myalgia, hemorrhages, jaundice, abdominal pain, diarrhea, and vomiting. The patient reported contact with rats, farm animals, and dogs before the onset of illness. Laboratory tests at admission to the Hospital Santa Casa de Misericórdia, Pelotas, showed an elevated level of serum bilirubin (total 21 mg/dL, direct 16 mg/dL) and a slight increase in liver enzyme levels (alanine aminotransferase 2×, aspartate aminotransferase 1.5× above reference levels). An acute-phase serum sample showed a titer of 25 against serovars Autumnalis and Bratislava by microscopic agglutination test (MAT).

The second isolate (Cascata strain) was obtained from the blood culture of a 16-year-old boy who exhibited headache, fever, flulike symptoms, and myalgia. He reported previous contact with rats and dogs. The patient was not hospitalized, and an acute-phase serum sample showed a titer of 25 against saprophytic serovar Andamana by MAT. Both patients were from the rural area of Pelotas. Unfortunately, convalescent-phase serum samples were not obtained from these patients.

A third isolate (Hook strain) was obtained from a male stray dog with anorexia, lethargy, weight loss, disorientation, diarrhea, and vomiting. The animal died as a consequence of the disease. The isolate was obtained from a kidney tissue culture. No temporal or spatial relationship was found between the 3 cases.

Serogrouping was performed by using a panel of rabbit antisera. Bonito, Cascata, and Hook strains were classified as Autumnalis, Bataviae, and Australis, respectively. Serogroups were confirmed by the strong and specific reaction of hyperimmune sera against these isolates, with the reference strains of the respective serogroups. Species identification was accomplished by sequencing nearly the full length of the 16S rRNA gene, as previously described (*5*). The sequences of the Hook, Cascata, and Bonito strains were deposited in GenBank under accession nos. EU349494–EU349496.

In addition, the *rpoB* gene sequence was determined and used for further confirmation of the species. The *rpoB* sequence for the strains Hook, Cascata, Bonito, and the *L. noguchii* reference strains were deposited in GenBank under accession nos. EU349497–EU349505. BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) alignment confirmed the new isolates as *L. noguchii*. The 16S rRNA gene sequence was also used for taxonomic analysis of *L. noguchii* (Figure). The topology-based dendrogram demonstrates sequence relatedness among strains isolated in Pelotas and the *L. noguchii* Autumnalis, Australis, and Bataviae strains deposited in GenBank (Figure). No molecular or serologic characterization at the serovar level was performed.

We report herein the occurrence of *L. noguchii* species in southern Brazil. The 3 isolates obtained belong to distinct serogroups. Information presented here places *L. noguchii* among the prevalent *Leptospira* species that are able to cause human and animal leptospirosis in southern Brazil.

Isolation procedures and DNA sequencing were conducted at the Federal University of Pelotas. Serogrouping of the isolates was performed at the Gonçalo Moniz Research Centre.

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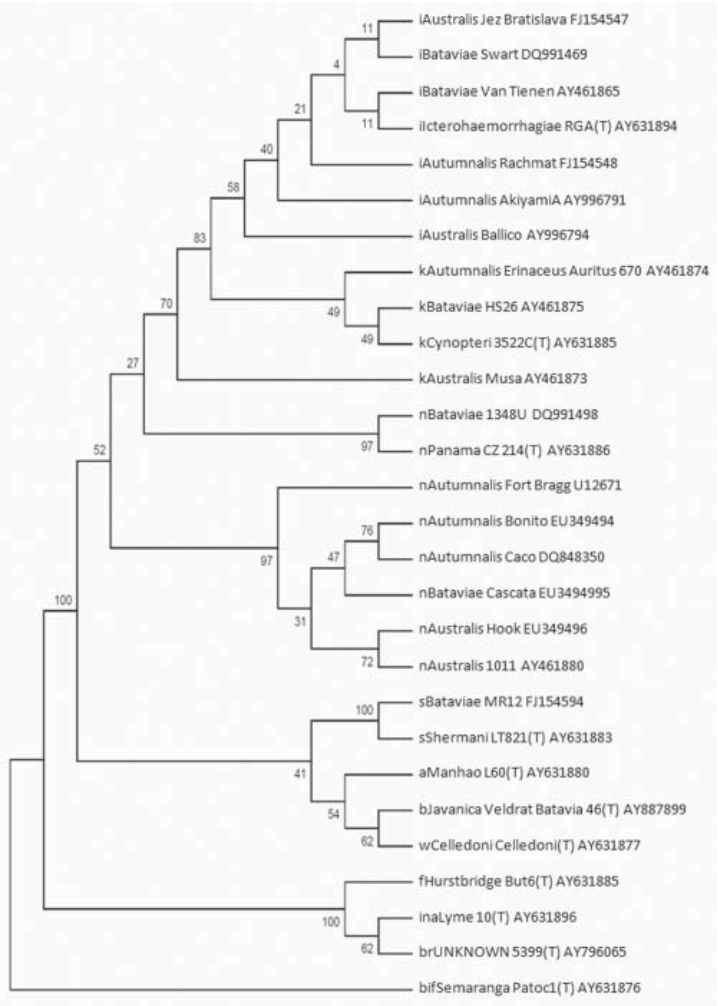

Figure. Dendogram constructed by using the neighbor-joining algorithm, based on a 1,180-bp sequence of the 16S rRNA gene demonstrating the position of the Brazilian strains (Bonito, Cascata, and Hook) within the *Leptospira noguchii* species. This dendogram summarizes, by bootstrap-based topology, the evolutionary relationship among *L. noguchii* strains. The bootstrap consensus values are indicated over each root. The initial lowercase letters indicate the respective species to which each strain belongs: i, *L. interrogans*; k, *L. kirschneri*; n, *L. noguchii*; b, *L. borgpetersenii*; w, *L. weilii*; s, *L. santarosai*; a, *L. alexanderi*; f, *L. fainei*; in, *L. inadai*; br, *L. broomi*; bif, *L. biflexa*. (T) indicates the type-strain for each species. The GenBank accession number follows the strain identification.

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Aquaculture and Florfenicol Resistance in *Salmonella enterica* Serovar Typhimurium DT104

To the Editor: In a letter recently published in Emerging Infectious Diseases, Smith (*1*) discussed evidence that he mistakenly believes to undermine the hypothesis that the florfenicol resistance gene present in some isolates of the epidemic *Salmonella enterica* serovar Typhimurium DT104 strain originated from a florfenicol resistance plasmid present in *Vibrio damsela* (*Pasteurella piscicida*) that infected fish farms in Japan in the 1990s (*2*). Smith correctly states that the florfenicol resistance gene was present in *S. enterica* serovar Typhimurium DT104 strains isolated in the United States in 1985, before the gene was documented in *V. damsela* in Japan (*1*,*3*). He is also correct in noting that this particular florfenicol resistance gene was detected in a plasmid in *Klebsiella pneumoniae* in France in 1969 (*1*,*4*).

However, an earlier report by Briggs and Fratamico (*5*) clearly established that the florfenicol resistance genes and the tetracycline resistance genes *tetG* and *tetR* in the *Salmonella* genomic island 1 (SGI1) were surrounded by non–antimicrobial-drug resistance DNA. This DNA is homologous to DNA sequences in plasmids PASPPFLO and pJA8122 (see Figure 1 and Table 2 in reference 5) (*5*–*7*). In addition to antimicrobial drug resistance genes, PASPPFLO and pJA8122 contain cloned DNA segments of indigenous R plasmids found in *V. damsela* and *V. anguillarum*, respectively; these cloned DNA segments span sequences that extend beyond their florfenicol resistance and *tetR*/*tetG* genes (*5*–*7*). For example, the region of the florfenicol resistance gene in SGI1 contains 763 nt of the non–antimicrobial-drug resistance portion of the original *V. damsela* plasmid; the region of *tetR*/*tetG* contains 468 nt of the non–antimicrobial-drug resistance DNA segment of the *P. piscicida* plasmid (*5*–*7*).

The presence of these non–antimicrobial-drug resistance R plasmid DNA sequences in SGI1 constitutes a molecular signature that firmly establishes the aquaculture origin of the florfenicol resistance and the *tetR*/*tetG* genes in the *S. enterica* serovar Typhimurium DT104 strain studied by Briggs and Fratamico and in the SGI1 of other bacteria (*5*). These R plasmid DNA sequences in SGI1 also confirm direct or indirect horizontal gene transfer between bacteria in the aquaculture environment and *S. enterica* serovar Typhimurium DT104 (*5*–*7*).

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In Response: In his letter (*1*), Cabello makes 2 observations regarding the debate concerning the origin of the *flo*R gene in *Salmonella enterica* serovar Typhimurium DT104. The first observation is that the plasmid PASPPFLO contained cloned segments of an indigenous *Vibrio damsela* plasmid. However, PASPPFLO is not the name of a plasmid but is the GenBank locus identifier associated with the sequence (GenBank accession no. D37826) of a 3,745-bp region of the *V. damsela* plasmid pSP92088 that contained pp-*flo* (*2*,*3*).

The second observation is that sequences flanking the *floR* gene in *S. enterica* serovar Typhimurium DT104 (GenBank accession no. AF071555) are homologous to those flanking the pp-*flo* gene sequenced from the *V. damsela* plasmid pSP92088 (*4*). On the basis of this homology, he seems

to assume that these flanking sequences must have originated in *V. damsela* and, therefore, that they constitute a molecular signature that firmly establishes the aquaculture origin of this florfenicol resistance. What Cabello does not mention is that sequences flanking a wide range of *floR* genes, including those in plasmid R55 (GenBank accession no. AF332662), are also homologous to those found in *S. enterica* serovar Typhimurium DT104 (*5*,*6*).

These data suggest that during horizontal transfer between species and genera, the association of *floR* with its flanking regions has been conserved (*5*,*6*). However, the data provide no evidence for postulating a unique association of these flanking sequences with *V. damsela*, and, therefore, do not provide evidence for an aquaculture origin of *floR*. If Cabello believes that sequences flanking *floR* in *S. enterica* serovar Typhimurium DT04 constitute a molecular signature that firmly establishes the aquaculture origin of *floR* in *S. enterica* serovar Typhimurium DT104, he should provide some explanation as to how this signature was also present in the R55 plasmid detected in a *Klebsiella pneumoniae* strain isolated in 1969 (*5*,*7*).

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The Status of Infectious Disease in the Amazon Region

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The Amazon River basin region is a vast territory with an area >7 million km^2, encompassing parts of 9 South American countries: Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, and Venezuela. The Amazon River, the longest river in the world, traverses the region from west to east, fed by multiple tributaries. The region also contains the largest tropical rainforest in the world, situated on a massive plain whose altitude is near sea level. With a climate characterized by high temperatures and humidity and copious rainfall, the region has the densest and most varied ecosystem in the world.

Conditions in the region are favorable for the transmission of numerous tropical diseases, which pose particular risks for populations exposed to precarious housing and working conditions. Many of these are well-known diseases whose epidemiologic characteristics are changing as the result of accelerating population, environmental, and climate changes. Others are novel diseases, which are being discovered in the region regularly.

Malaria is the most important endemic disease in the region because of its high incidence. It is naturally transmitted by mosquitoes of the genus *Anopheles.* Slow-flowing, nonpolluted, shaded waters in the region provide this vector with a favorable environment for reproduction, and dense forest enables the adult vector to live longer than in other climates. Climatic conditions favor the development of *Plasmodium* spp. in these mosquitoes. Intense human migration from rural to urban areas contributes to malaria transmission in peripheral areas of Amazonian cities.

Tegumentary leishmaniasis is another high-incidence disease in the region. Multiple animal species serve as reservoirs for *Leishmania* spp. in the rainforest, as do the disease's primary vectors, insects of the genus *Lutzomya*. Arboviruses are highly endemic to the region, particularly Oropouche virus and Venezuelan equine encephalitis virus. Yellow fever is also endemic; nonhuman primates are the principal reservoirs of the disease during its sylvatic cycle. Vaccination is an essential means of protection against yellow fever for both the local population and visitors. *Aedes aegypti* mosquitoes in Amazonian urban centers pose an ever-present risk for yellow fever transmission and are also responsible for the high incidence of dengue.

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For reasons yet to be determined, persons living in the Amazon region have a high prevalence of leprosy and viral hepatitis. Mycoses such as Jorge Lobo disease, caused by *Blastomyces loboi*, are also characteristic of the region (*1*).

The transmission of known pathogens through novel modes, the emergence of pathogens not previously detected in the region, and the emergence of newly recognized pathogens have been reported with increasing frequency in the Amazon region. As illustrated by articles in this issue about adiaspiromycosis (*2*), malaria (*3*,*4*), dengue (*4*), Chagas disease (*5*), Kaposi sarcoma–associated herpesvirus (*6*), suspected Brazilian purpuric fever (*7*), and other infections, new diseases continue to emerge and old ones continue to undergo epidemiologic change throughout the vast Amazon River basin.

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High Incidence of Diseases Endemic to the Amazon Region of Brazil, 2001–2006

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In Brazil, reportable diseases are the responsibility of the Secretariat of Health Surveillance of the Brazilian Federal Ministry of Health. During 2001–2006, to determine incidence and hospitalization rates, we analyzed 5 diseases (malaria, leishmaniasis [cutaneous and visceral], dengue fever, leprosy, and tuberculosis) that are endemic to the Amazon region of Brazil. Data were obtained from 773 municipalities in 3 regions. Although incidence rates of malaria, leishmaniasis, tuberculosis, and leprosy are decreasing, persons in lower socioeconomic classes with insufficient formal education are affected more by these diseases and other health inequalities than are other population groups in the region.

A structured intervention to address the most prevalent diseases endemic to Brazil started when the Oswaldo Cruz Institute in Rio de Janeiro was created in 1900 and research began (*1*). Brazil has a federative political system composed of 3 levels of government: federal (union), states, and municipalities. All are considered autonomous bodies by the Federal Constitution of 1988 and none have authority over the others. Brazil has 26 states, 27 federal districts (also known as federative units), and 5,564 municipalities. Considerable demographic disparities exist among the states on the basis of their resident populations in 2007 (*2*). The 27 federative units are divided into 5 geographic regions: North, Northeast, Southeast, South, and Central-West.

The 5 geographic regions in Brazil are analytical units that are included in any epidemiologic analyses of this country. Historically, the North and Northeast regions, which include most of the Amazon River Basin, have the greatest social inequalities and the highest prevalence of disease. Furthermore, the quality of epidemiologic data is lower in the North and Northeast regions than for other regions of Brazil.

Despite advances in the Brazilian public health system (the Single Health System [SUS]) and the stated principles of universal and equitable healthcare contained in the Brazilian Constitution, many inequalities still exist with regard to access to healthcare services and to training and distribution of healthcare professionals (*3*). For example, according to Ministry of Health data, despite having the second highest number of medical schools in the world (175, second only to India) (*4*) and accepting ≈17,000 medical students each year, Brazil has a glut of physicians in the South and Southeast regions but nearly none in >1,300 municipalities (*5*).

In Brazil, health inequities among different groups are even more striking and directly associated with social and economic conditions (*6,7*). Reinforcement of support networks for promotion and protection of individual and collective health is Brazil's greatest challenge, especially for states in the Amazon region of Brazil.

Demographic density in the Amazon region of Brazil is low (4.7 persons/km^2 in 2007); many areas are nearly bereft of healthcare facilities. Paradoxically, an intense urbanization process is taking place in the region, and estimates in 2000 showed that ≈70% of the population lived in urban areas. The urbanization trend contrasts with the rural lifestyle of traditional populations (indigenous groups, river dwellers, rubber tappers, quilombolas) in the region. Quilombolas are descendants of former slaves who escaped from slave plantations that existed in Brazil until the abolition of slavery on May 13, 1888. Because of their isolated status, their primary occupations are mineral extraction or subsistence farming.

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Our study involved 5 reportable (compulsory notification) diseases (malaria, leishmaniasis [cutaneous and visceral]), dengue fever, and leprosy) of the 44 reportable diseases with national coverage and 10 diseases with sentinel surveillance in Brazil. We determined the incidence and hospitalization rates of patients with these 5 diseases in states in the Amazon region of Brazil during 2001–2006.

Amazon Region of Brazil

The Amazon region of Brazil (Amazon River Basin), as defined by Brazilian legislation, comprises 773 municipalities in 3 geographic regions and 9 states (the entire North region, a large portion of the state of Maranhão [183 of 217 municipalities] in the Northeast region, and the entire state of Mato Grosso in the Central-West region) (Figure 1) (*8*). This region has a population of ≈23.6 million inhabitants, an area of 4.97 million km^2 (≈60% of Brazil), and a demographic density 10× less than the national average.

The National Notifiable Disease Information System (Sistema de Informação de Agravos de Notificação Compulsória), created in 1993, is a national electronic surveillance system that contains a variety of diseases in 1 integrated database. This system accepts reports on cases and outbreaks. Relevant data are obtained from notifying health centers on standardized forms. Data are entered into the system, in most instances, by personnel from the Municipal Health Secretariats (Secretarias Municipais de Saúde). These data are transferred electronically according to a preestablished data flow: Municipal Health Secretariats → regional health coordination units (within states) → State Health Secretariat (Secretaria Estadual de Saúde) → Federal Ministry of Health.

The National Notifiable Disease Information System database, the malaria database (National Malaria Database [no longer in existence]), and Informational System of Epidemiological Surveillance of Malaria are managed and monitored by the federal government in the Secretariat of Health Surveillance/Ministry of Health (*9*). Case definitions are established by the Secretariat of Health Surveillance/Ministry of Health and are based on recommendations of the World Health Organization (*9*).

Data from the SUS Hospitalization Information System Sistema de Informações Hospitalares do Sistema Único de Saúde (SIH-SUS) were also included. These data include ≈80% of hospitalizations in the study region. This system records data according to the International Classification of Diseases, 10th revision (ICD-10) (*10*).

Missing Data

For the resident population >10 years of age, a detailed analysis of the proportion of cases with missing data for education level (Figure 2) showed large reductions in leishmaniasis, leprosy, and tuberculosis over the period of evaluation. However, incidence rates of 13% for dengue fever and 16% for malaria were maintained in persons for whom information on education level was not provided.

Figure 1. Map of Brazil showing the Amazon region (green).

Calculation of Indicators

Indicators (Table 1) for data analysis were calculated according to definitions of indicators and basic health data (Indicadores e Dados Básicos – Brasil – 2007; http://tabnet.datasus.gov.br/cgi/idb2007/matriz.htm) of the Inter-Agency

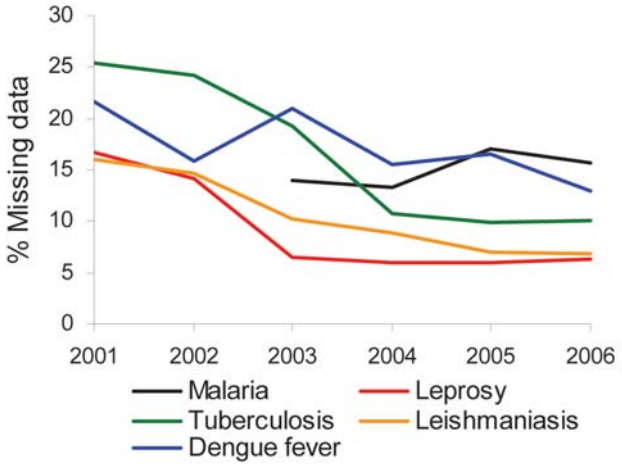

Figure 2. Distribution of cases of 5 diseases with missing information on patient education level among persons >10 years of age, Amazon region of Brazil, 2001–2006. Data for malaria were obtained from National Malaria Database (2003–2006); data for other diseases were obtained from the National Notifiable Disease Information System/Secretariat of Health Surveillance/Ministry of Health; population data were obtained from the Brazilian Institute of Geography and Statistics.

Table 1. Incidence indicators for 5 reportable diseases, Brazil, 2008*

Disease	Indicator	Criteria	Method of calculation
Malaria	Annual parasitic index for malaria	No. positive examination results for malaria/100,000 residents in a given geographic area in the year under consideration (codes B50-B53; ICD-10).	(No. positive test results for malaria/total resident population) × 100,000
Leishmaniasis	Incidence rate for forms of leishmaniasis	No. new and confirmed cases of leishmaniasis (all forms)/100,000 residents in the local population of a given geographic area in the year under consideration (code B55; ICD-10)	(No. new/confirmed cases [all forms] among residents/total resident population) × 100,000
Dengue fever	Incidence rate for dengue fever	No. new dengue fever cases (classic and dengue hemorrhagic forms)/100,000 residents in the local population of a given geographic area in the year under consideration (codes A90-A91; ICD-10)	(No. new/confirmed cases [all forms] among residents/total resident population) × 100,000
Leprosy†	Coefficient of leprosy detection	No. newly diagnosed cases/100,000 residents in the local population of a given geographic area in the year under consideration	(No. new cases among residents/total resident population) × 100,000).
Tuberculosis	Incidence rate for tuberculosis	No. new and confirmed cases of tuberculosis (all forms)/100,000 residents in the local population of a given geographic area in the year under consideration (codes A15–A19; ICD-10)	(No. new/confirmed cases [all forms] among residents/total resident population) × 100,000

*Source: Pan American Health Organization (*11*). ICD-10, International Classification of Diseases, 10th revision.
†The technical control program of the Secretariat of Health Surveillance/Ministry of Health began using a multiplication factor of 100,000 population in August 2008 to make its detection rate data for leprosy comparable with the incidence data for other diseases.

Health Information Network, which is composed of government agencies and institutions of higher education and research (*11*). Data were analyzed by using SAS statistical software (*12*) and stratified by age group, education level, and year during 2001–2006; an aggregation of the 773 municipalities in the Amazon region of Brazil formed the basic unit of analysis. For different education levels, missing data were redistributed proportionally among all age groups >10 years of age, as per the statistical technique used.

For denominators of the incidence rates, populations projected by the Brazilian Institute of Geography and Statistics during 2001–2006 were used for classification of age groups. Demographic projections were specifically developed for these population groups by education level. These projections were made by using average geometric rates of annual population growth (*13*) obtained from the 1996 population count and expanded sample data from the 2000 demographic census (*13*,*14*). We also used a correction factor for a section of persons with ≥12 years of formal education because the Federal Ministry of Education had indicated that during 2001–2005, the average annual increase in university enrollments in the North region, which was used as a proxy indicator for the Amazon region of Brazil, was ≈12% (*15*).

Hospitalization rates for each disease were calculated by using total registered hospital stays and the corresponding ICD-10 code as registered in the national SIH-SUS database as the numerator and the resident population as the denominator as per methods of Siqueira et al. (*16*). To facilitate comparison with other data, all indicators were adjusted to a rate/100,000 population. The total number of persons that purchased private health plans, according to data from the National Agency of Supplementary Health, was subtracted from the denominator (*17*).

Incidence and Hospitalization Rate

Malaria was the vector-borne disease with the highest incidence in the region; the number of new cases gradually increased from 1,530/100,000 in 2001 to 2,365/100,000 in 2006. However, a reduction in the hospitalization rate for this disease was also observed and, as expected, the most affected age group was young adults 15–49 years of age (Table 2).

Dengue fever, which reemerged in Brazil in the 1980s, is endemic to the Amazon region of Brazil and has maintained its epidemiologic pattern of epidemics in isolated areas. In 2001, it reached its peak incidence (283.8/100,000) and became the most common vector-borne disease in the region until 2003 (Table 2).

Leishmaniasis transmission has become an urban problem, particularly in outlying areas of major and mid-sized cities in the Amazon region of Brazil. This problem is apparent despite a reduction in incidence from 79.9/100,000 in 2001 to 60.7/100,000 in 2006 and constant hospitalization rates (Table 2)

Tuberculosis incidence was lower in the study area than in the rest of Brazil (incidence rate 62/100,000 in 2006). The incidence of tuberculosis in the Amazon region of Brazil has remained constant (≈45.5 cases/100,000); concentration of cases was higher among the elderly, and the hospitalization rate was ≈10.0 (Table 2).

Leprosy showed little variation in incidence and hospitalization rates in most of the disease-endemic areas in the study region. A higher frequency was noted among adults and the elderly (Table 2).

We calculated disease distribution per age group. The highest incidence rates for leishmaniasis and dengue fever were among persons 30–49 years of age, the highest incidence rate for malaria was among persons 15–29 years

of age, the highest incidence rate for leprosy was among persons 50–69 years of age, and the highest incidence rate for tuberculosis was among persons >70 years of age. Malaria is the most prevalent disease in the Amazon region of Brazil, although its incidence has decreased over the study period (Figure 3).

Table 3 shows incidence rates for the 5 diseases by patient education level. Resulting inequalities between various social and cultural groups are apparent. For example, the incidence of malaria is highest among groups with a lower education level.

Leishmaniasis, even with an overall reduction in incidence in 2006, still showed differences in disease rates. The incidence rate for this disease was 3-fold greater for persons with 1–3 years of schooling than for persons with ≥12 years of formal education.

Dengue fever, with its predominantly urban presence, affected persons in the highest socioeconomic classes. For

Table 2. Incidence and hospitalization rate, by age group, for 5 reportable diseases, Amazon region of Brazil*

Age group, y	2001		2002		2003		2004		2005		2006	
	Incid.	Hosp.	Incid.	Hosp.	Incid.	Hosp.	Incid.	Hosp.	Incid.	Hosp.	Incid.	Hosp.
Malaria												
0–4	1,745.9	68.1	1,481.5	55.0	1,545.5	54.0	1,682.3	55.6	2,185.0	58.4	2,057.8	51.4
5–14	1,591.2	45.1	1,339.3	34.2	1,714.9	28.6	1,920.3	33.3	2,610.5	33.9	2,428.9	26.8
15–29†	1,464.4	87.1	1,280.5	66.9	2,227.1	63.2	2,465.4	68.6	2,967.1	70.0	2,573.0	52.9
30–49		86.5		64.9	2,118.1	59.0	2,376.7	62.3	2,869.0	60.9	2,469.6	47.0
50–69		76.7		57.1	1,558.5	48.2	1,813.2	51.8	2,326.1	57.8	2,025.7	42.7
≥70		58.4		43.3	689.9	41.0	788.4	47.9	1,102.7	63.5	964.5	41.0
Total	1,529.6	72.7	1,319.4	55.5	1,898.5	50.8	2,119.2	55.0	2,661.4	56.5	2,364.9	43.8
Leishmaniasis												
0–4	28.6	11.4	32.6	10.0	44.2	11.5	43.8	10.8	39.6	16.5	33.5	16.6
5–14	37.0	2.6	37.0	2.2	45.9	1.7	42.2	2.1	34.1	2.7	29.2	3.0
15–29	106.1	2.1	108.3	2.2	131.1	1.6	117.9	1.8	97.6	2.0	75.4	2.0
30–49	113.7	2.5	116.4	2.4	135.1	2.6	124.0	2.1	106.0	2.3	84.1	2.6
50–69	97.2	4.0	99.9	4.0	107.3	4.3	98.3	3.4	92.4	3.7	75.3	3.2
≥70	68.3	58.0	66.9	43.3	76.6	40.7	68.9	47.7	62.6	63.3	61.2	41.0
Total	79.9	5.0	81.8	4.3	97.3	4.2	88.9	4.3	75.7	5.6	60.7	5.2
Dengue fever												
0–4	86.7	8.4	48.1	13.0	57.8	15.1	29.4	10.1	38.4	13.3	44.4	12.0
5–14	152.3	21.7	96.7	30.0	112.1	32.7	52.9	21.3	87.7	28.6	85.7	25.7
15–29	347.0	55.8	199.4	72.2	235.3	74.3	123.8	50.0	197.8	65.7	165.4	51.8
30–49	423.5	64.8	259.2	85.9	298.0	81.0	165.3	55.3	256.9	72.4	221.5	56.0
50–69	358.2	82.2	251.4	109.0	275.4	109.6	154.7	71.8	227.7	91.1	216.3	68.4
≥70	258.7	88.6	160.2	115.7	168.6	114.9	105.0	91.1	157.1	113.7	151.6	78.9
Total	283.8	46.6	173.0	61.6	199.6	61.8	106.6	41.8	166.4	54.6	147.9	43.3
Leprosy												
0–4	3.2	0.0	5.6	0.0	4.6	0.1	4.3	0.0	3.3	0.0	2.7	0.1
5–14	33.1	0.6	35.5	0.7	35.1	0.6	33.5	0.5	31.4	0.2	23.5	0.4
15–29	91.4	3.6	94.1	3.9	98.0	3.5	91.1	3.6	80.8	3.4	63.0	4.7
30–49	129.8	8.6	135.3	8.7	133.3	7.5	124.7	9.0	119.0	7.2	94.8	8.0
50–69	186.7	22.4	185.6	23.4	183.6	19.8	184.8	18.8	175.6	13.0	144.9	16.1
≥70	145.4	31.5	157.1	40.8	148.3	26.9	152.3	20.4	153.4	19.7	127.4	19.7
Total	84.8	5.8	87.9	6.2	88.0	5.1	83.8	5.2	78.0	4.2	61.9	5.1
Tuberculosis												
0–4	10.8	2.3	10.8	3.1	10.2	3.8	9.4	5.5	7.9	6.4	6.9	1.1
5–14	8.7	1.7	8.4	1.3	7.6	1.6	7.4	1.5	7.4	1.4	6.5	0.6
15–29	58.0	9.0	56.2	7.6	55.5	11.6	55.8	12.8	51.5	9.4	48.2	5.8
30–49	77.7	15.8	77.1	14.8	78.2	16.3	75.5	16.8	72.7	14.5	70.0	12.5
50–69	109.3	27.6	107.9	21.9	105.3	25.4	108.6	26.8	109.7	24.0	106.4	21.2
≥70	144.4	33.4	145.2	32.2	127.5	32.8	141.6	37.6	136.2	35.1	134.3	26.6
Total	51.3	10.1	50.5	8.9	49.6	10.8	49.5	11.7	47.4	10.0	45.1	7.2

*Values were standardized by the Inter-Agency Health Information Network (Pan American Health Organization, 2008) (*11*) for each disease. Malaria: annual parasitic index/100,000 population; leishmaniasis, dengue fever, and tuberculosis: incidence rate/100,000 population; leprosy: detection coefficient/100,000 population. Incid., incidence; Hosp., hospitalization rate/100,000 population. Data for malaria were obtained from the National Malaria Database (2001 and 2002) and Information System of Epidemiological Surveillance of Malaria (2003–2005), and data for the other diseases were obtained from the National Notifiable Disease Information System/Secretariat of Health Surveillance/Ministry of Health. Incidence data were obtained from the Brazilian Institute of Geography and Statistics, and hospitalization data were obtained from the Hospitalization Information System/Ministry of Health.
†For malaria in 2001 and 2002, this age group corresponds to ≥15 years of age because the Ministry of Health used this age group to tabulate the data.

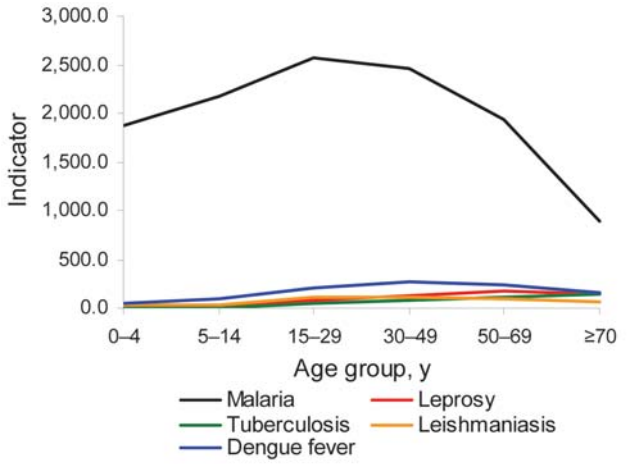

Figure 3. Incidence of 5 diseases by age group, Amazon region of Brazil, 2001–2006. Data for malaria were obtained from National Malaria Database (2003–2006); data for other diseases were obtained from the National Notifiable Disease Information System/ Secretariat of Health Surveillance/Ministry of Health; population data were obtained from the Brazilian Institute of Geography and Statistics. Values were standardized by the Inter-Agency Health Information Network (Pan American Health Organization, 2008). Malaria: annual parasitic index/100,000 population; leishmaniasis, dengue fever, and tuberculosis: incidence rate/100,000 population; leprosy: detection coefficient/100,000 population.

each year of the study, we noted an illness pattern that affected the middle and upper classes more than other socioeconomic groups.

Incidence rates for leprosy and tuberculosis were highest for groups with lowest levels of education. Incidence rates were 2× greater for groups with <1 year and 1–3 years of formal education.

Conclusions

As is occurring in the rest of Brazil, the Amazon region of Brazil is undergoing an epidemiologic transition; infectious and parasitic diseases are decreasing and noncommunicable chronic diseases are increasing. However, vector-borne and mycobacterial diseases still constitute a public health problem in this tropical region.

Despite decreases in incidence rates of malaria, leishmaniasis, tuberculosis, and leprosy, our study shows that these diseases are more common in persons with insufficient education of lower social classes than in other population groups. This reality is more stark and systematic in the Amazon region of Brazil than in other areas of this country. According to a report of the National Commission on Social Determinants in Health (*6*), Brazil is among countries with the most skewed income distribution in the world, demonstrating the effects of social determinants as a main risk factor for illnesses in the region.

Cases of autochthonous malaria in Brazil have decreased in 2007 and 2008. Data for December 2007 showed 455,899 cases (incidence rate 1,931.40/100,000 residents). This finding represents a reduction of 2,899 hospitalizations for this disease, a decrease of 32.9% from 2006 to 2007. This decrease was caused primarily by improved control of urban malaria (*18–20*). Integration of disease surveillance activities into the primary care network has been the main force behind improved control of malaria.

Different forms of leishmaniasis are considered by the World Health Organization (*21*) to be a worldwide public health problem. In the Amazon region of Brazil, the incidence of leishmaniasis has decreased sharply from 108.6 cases/100,000 inhabitants in 2003 to 95.9 in 2004, 80.2 in 2005, and 62.0 in 2006; the degree of exposure was largely associated with disorganized occupation of new areas. Therefore, cases tended to occur among populations in recent land settlements and former rainforest areas. *Leishmania* (*Viannia*) *guyanensis* are protozoa enzootic to these areas. However, Campbell-Lendrum et al. (*22*) reported increasing domestication of cutaneous leishmaniasis and its possible dissemination in households in large cities in the Amazon region of Brazil.

Three serotypes of dengue virus (DEN-1, DEN-2, and DEN-3) are currently circulating in different regions of and have different regional manifestations. Brazil has the most cases of dengue fever in the Western Hemisphere; ≈70% of all cases are reported, and of this total, 17% are concentrated in the Amazon region of Brazil (*23*). When the risk factors for a dengue epidemic in Goiânia in the Central-West region of Brazil were analyzed, Siqueira et al. (*24*) noted a higher dengue prevalence among those with a lower educational level. This finding differs from our results and again highlights the specificities of disease in this region. Hospitalization rate for this disease increased for most of Brazil (*16*,*25*), but decreased in the Amazon region of Brazil during the study period from 61.6/100,000 in 2002 to 43.3 in 2006. In September 2008, data on the isolation of DEN-4 in the Amazon region were revised and now show that there is no evidence that this serotype circulates in the Amazon region of Brazil.

Two thirds of the world's cases of tuberculosis are in Africa, the People's Republic of China, and India (*26*). However, Brazil still has an elevated incidence rate for this disease; 42.9 cases/100,000 occurred in the Amazon region in 2007. Success in control of tuberculosis results from early detection of new cases and an effective treatment regimen. Tuberculosis incidence has remained constant in the Amazon region but the hospitalization rate for this disease has decreased. This decrease is the result of effective investment of resources in expanded access to treatment in the primary care network. This investment has been

made through greater focus on family health and strategies to strengthen the local autonomy and capacity to provide quality care, together with better health surveillance and monitoring activities.

Incidence of leprosy in Brazil has shown an upward trend since 1980. However, in 2006, the number of new cases decreased to 61.9/100.000. Using negative binomial and Poisson distributions to analyze trends in the incidence of leprosy, Penna and Penna (*27*) and Martelli et al. (*28*) reported that there should have been a constant incidence, not a sharp decrease, for this disease in 2006. Leprosy is a chronic disease that is not expected to show extensive epidemiologic changes in a short period. Our results suggest that fewer reports of leprosy cases were caused by reduced new-case detection during the study period. However, hospitalization rates for this disease in 2006 are similar to those of previous years.

The SUS celebrated its 20th anniversary in 2008 and represents an ambitious example for confronting historic social inequalities in the national context through guaranteeing the right to universal healthcare. Integration of primary care and health surveillance programs has shown excellent results in the Amazon region of Brazil; particular success has occurred with malaria control, for which the number of blood slides prepared has increased from 150,000 to 450,000. Consequently, the numbers of persons who start treatment during the first 48 hours of onset of malaria symptoms has increased considerably. Conversely, factors that contribute to the presence of malaria, dengue fever, tuberculosis, leprosy, leishmaniasis, and other dis-

Table 3. Incidence of 5 reportable diseases, by level of education, Amazon region of Brazil*

Education level, y	2001†	2002†	2003	2004	2005	2006
Malaria						
<1	–	–	3,501.2	2,141.0	2,619.9	2,578.4
1–3	–	–	3,934.6	4,199.2	5,393.4	4,420.6
4–7	–	–	1674.0	1,883.4	2,313.3	2,041.8
8–11	–	–	582.1	682.9	815.3	763.0
≥12	–	–	1,405.5	1,197.3	1,502.4	1,125.9
Total	–	–	2,360.1	2,025.1	2,473.7	2,094.3
Leishmaniasis						
<1	146.0	121.3	115.9	100.0	90.7	66.6
1–3	19.9	101.0	144.8	142.6	122.3	96.0
4–7	181.0	120.0	125.1	109.1	95.2	74.2
8–11	31.2	45.1	55.3	47.8	42.0	33.9
≥12	24.8	65.0	64.4	43.6	33.3	26.2
Total	95.4	95.0	108.6	95.9	80.8	62.0
Dengue fever						
<1	150.2	121.8	101.4	53.4	79.7	78.4
1–3	37.0	114.5	180.8	118.5	171.8	129.9
4–7	405.5	250.5	209.1	112.2	185.9	161.6
8–11	269.5	178.0	228.9	120.1	185.1	156.7
≥12	456.8	326.1	405.0	204.2	271.4	226.9
Total	251.0	187.3	205.4	114.3	177.6	151.4
Leprosy						
<1	182.8	166.9	147.9	138.7	128.5	100.7
1–3	33.1	110.5	137.2	135.7	126.7	101.0
4–7	175.0	121.0	102.6	94.5	88.4	70.2
8–11	43.6	57.1	63.9	58.5	56.9	42.4
≥12	44.8	83.2	88.5	69.7	58.9	45.4
Total	107.2	108.2	105.9	97.7	90.0	69.3
Tuberculosis						
<1	107.3	99.4	87.5	82.3	78.4	69.5
1–3	19.2	59.1	76.1	70.4	71.3	65.5
4–7	95.5	64.0	49.8	49.4	47.2	46.0
8–11	38.9	38.4	39.2	44.5	40.9	39.5
≥12	43.5	71.4	55.8	54.6	49.5	42.2
Total	63.8	61.1	58.6	57.3	54.3	50.2

*Values were standardized in the Inter-Agency Health Information Network database (Pan American Health Organization, 2008) (*11*) for each disease. Malaria: annual parasitic index/100,000 population; leishmaniasis, dengue fever, and tuberculosis: incidence rate/100,000 population; leprosy: detection coefficient/100,000 population. Data for malaria were obtained from the National Malaria Database (2001 and 2002) and Information System of Epidemiological Surveillance of Malaria (2003–2005), and data for the other diseases were obtained from the National Notifiable Disease Information System/Secretariat of Health Surveillance/Ministry of Health.
†This information was not systematized for malaria in the National Malaria Database in 2001 and 2002.

eases in the Amazon region of Brazil are social inequality associated with poor funding of the public health system, imperfections in the integrated approach among the 3 spheres of government, and accelerated and disorderly urbanization of the metropolitan areas.

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Acute Conjunctivitis with Episcleritis and Anterior Uveitis Linked to Adiaspiromycosis and Freshwater Sponges, Amazon Region, Brazil, 2005

Marcia O. Mendes, Mario A.P. Moraes, Ernesto I.M. Renoiner, Marta H.P. Dantas, Tatiania M. Lanzieri, Carlos F. Fonseca, Expedito J.A. Luna, and Douglas L. Hatch

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Learning Objectives

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

- Describe the mechanism of infection for adiaspiromycosis.
- Identify the age group most susceptible to ocular adiaspiromycosis.
- Describe presenting symptoms associated with ocular adiaspiromycosis.
- Describe the frequency of ocular lesions associated with adiaspiromycosis.
- Identify risk factors for ocular adiaspiromycosis.

Editor

Beverly Merritt, Technical Writer-Editor, *Emerging Infectious Diseases.* Disclosure: *Beverly Merritt has disclosed no relevant financial relationships.*

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We conducted an epidemiologic investigation of an outbreak of ocular disease among children to determine whether the disease was linked to *Emmonsia* sp., a rarely-reported fungus and an agent of adiaspiromycosis. Using an unmatched case–control study design, we compared case-patients with asymptomatic controls randomly selected from the population. Scleral biopsies were analyzed microscopically. Of 5,084 children examined, 99 case-patients were identified; mean age (+1 SD) was 11.0 ± 4.4 years. Symptoms included photophobia (57%), ocular pain (42%), and blurred vision (40%). In the multivariate analysis, risk factors included diving in the Araguaia River (odds ratio 5.2; 95% confidence interval 2.4–12.0). Microscopy identified foreign bodies consistent with adiaconidia. This outbreak probably resulted from foreign-body–type reactions to adiaspiromycosis conidia after initial irritation caused by conjunctival contact with spicules of sponges in the river. Symptomatic children responded to corticosteroid treatment. Adiaspiromycosis is a preventable cause of ocular disease in the Amazon region.

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Adiaspiromycosis, caused by the fungus *Emmonsia* sp., was first identified in Brazil during pathologic examination of lung tissue in a patient with pneumonia who died unexpectedly during treatment (*1*). Conidia of *Emmonsia* sp. are commonly present in the environment, mainly in soil and dust, and some studies have shown that pulmonary infection most often results from inhalation (*2–4*). Conidia, which also affect other mammals, including marsupials and rodents, do not cause infection; rather, disease is caused by the robust, multicellular immunologic response in tissue against the growing conidia, which results in noncaseating granulomas (*2*). Human pulmonary adiaspiromycosis has been reported in the literature from multiple countries, including Russia, the Czech Republic, Guatemala, Brazil, and the United States (*1–4*); disseminated infection may occur in immunocompromised persons. Diagnosis is most frequently made by experienced pathologists who microscopically exam tissue using various stains, including periodic acid-Shiff, which shows large, round, multiwalled structures with surrounding foreign-body–type mixed cellular reactions (*3–5*).

On October 26, 2006, local ophthalmologists notified the State Health Secretariat in Tocantins, located in the northern Amazon region of Brazil, of an unusual outbreak of conjunctivitis with ocular nodules of unknown etiology among children. Illness was identified in 17 children, 16 of whom were <15 years of age; all were residents of Araguatins (population = 29,336), a city located along the slow-moving Araguaia River. The disease had remained underdiagnosed and underreported in Araguatins until an ophthalmologic service was initiated in the neighboring city of Augustinópolis, where the initial case-patients were referred, examined, and subsequently reported to local health authorities. Shortly after the condition was reported to Brazil's Ministry of Health, a team of epidemiologists, laboratorians, and ophthalmologists began an investigation with the following objectives: 1) determine the magnitude of the problem, 2) identify the cause, and 3) implement prevention and control activities.

Methods

Screening

Because 16 (94%) of the 17 initial case-patients reported were 5–15 years of age, active searches were conducted in 40 of the 41 primary schools in Araguatins. Health workers were trained by ophthalmologists to identify children with clinical signs that were similar to the initial 17 case reports. Children with blurred vision, or any of the following, were referred for an ophthalmologic examination: conjunctival injection or inflammation, nodules on conjunctiva or sclera, or cornea with any discoloration or opacification.

History

We defined a case of confirmed ocular disease (COD) in a child with any of the following physical signs: conjunctival injection or inflammation, nodules on sclera, or conjunctival or corneal opacities with anterior uveitis identified during ophthalmologic examination (including by slit-lamp and microscopy). Patients with COD were interviewed by using a standardized semistructured questionnaire; parents served as proxies for young children. Information was collected about basic demographic characteristics, duration and type(s) of symptoms, source of drinking water, frequency and specific locations where children had exposure to the local freshwater river, and similar illness in family members.

Case–Control Study

We hypothesized that exposure to the Araguaia River played a role in the chain of events resulting in ocular disease. This hypothesis was tested by using an unmatched case–control study design, based on an estimated 90% of case-patients having prior ocular exposure to river water; and by using 80% power, an α level of 0.05, and a case:control ratio of 1:3, which yielded a study sample size of 62 case-patients and 186 controls. The 62 case-patients included in this study were randomly selected from a total of 91 children with COD identified and interviewed. Two separate control groups were selected for interviews. The first group (community controls) included 186 asymptomatic persons ranging from 5 to 20 years of age living in households systematically selected from randomly chosen residential blocks in the urban area of Araguatins municipality. A second control group (household controls) comprised all asymptomatic residents of case-patient households.

Statistical Analysis

In the univariate analysis of the case–control study data, categorical variables were tested by using a χ^2 test, and continuous variables were compared by using a Kruskall-Wallis or *t* test, as appropriate. The odds ratio (OR) was used as the measure of association, 95% confidence intervals (CIs) were calculated, and $p<0.05$ was considered significant. Using a stepwise backward elimination strategy to calculate the adjusted OR, an unconditional logistic regression model was used for the multivariate analysis.

Laboratory Methods

Serologic tests from children with COD included ELISA tests for onchocercosis (immunoglobulin [Ig] G), toxoplasmosis (IgM), and toxocariasis (IgG). Blood smears and aqueous humor from selected patients were examined microscopically for evidence of microfilaria. Biopsy samples from COD case-patients with scleral nodules or corneal

abnormalities were fixed in formalin, stained with hemotoxylin and eosin, and periodic acid-Schiff, and examined microscopically. Soil samples were examined for helminth eggs and larvae. Water samples were collected from areas of the Araguaia River where case-patients reportedly swam. These samples were examined for 1) freshwater sponges, which were identified to species, and 2) silicious spicules (gemmoscleres) of these sponges; details of the methods and results of this sampling have been published (*6*,*7*).

Results

In addition to the initial 17 COD case-patients who were examined by ophthalmologists and reported to the Ministry of Health, a total of 5,084 children 5–15 years of age (corresponding to 83% of this age group in the population) were examined at 40 schools by health workers. During these active searches, of 235 students triaged and referred for evaluation of possible ocular abnormalities, 64 (27%) were categorized by ophthalmologists as having COD and 103 (44%) had sequelae. In addition to the total 81 COD case-patients identified above by November 26, 2005, COD was diagnosed for an additional 18 by January 26, 2006, identified initially by local clinicians or through patient self-referral.

Of the 99 COD case-patients identified, 91 (92%) were interviewed, of whom 70 (77%) were male and the mean age (±1 SD) was 11.0 ± 4.4 years. Ocular-related signs and symptoms were conjunctival hyperemia (89%), conjunctival nodule (70%), excessive tearing (63%), conjunctival pruritus (60%), photophobia (57%), ocular pain (42%), and blurred vision (40%); other reported symptoms included headache (37%) and generalized pruritus (16%). The number of COD case-patients peaked during the dry season (July–September) (Figure 1) when schools were closed and contact with the local river was most common. Of those interviewed, 88 (97%) resided in urban areas of Araguatins, and 3 (3%) resided in rural areas.

Ophthalmologists identified unilateral ocular lesions in 73 (80%) of interviewed COD case-patients; in 18 (20%) patients, the lesions were bilateral. Unilateral subconjunctival nodule(s) of sclera (Figure 2), some of which extended to the corneal limbus, were identified in 43 (47%) case-patients and were present bilaterally in 12 (13%). Unilateral corneal opacities (Figure 3) were observed in 32 (35%) case-patients, bilaterally in 18 (20%).

Neighborhoods most commonly affected in the city of Araguatins included Centro, where 61 (67%) of all case-patients resided (prevalence of 8 cases per 100,000 inhabitants), Vila Cidinha (6 per 100,000), and Nova Araguatins (3 per 100,000). Seventy-five (82%) case-patients attended an urban school in Araguatins, and 3 (3%) attended a rural school (Table 1); 13 (14%) of case-patients were not enrolled in a school.

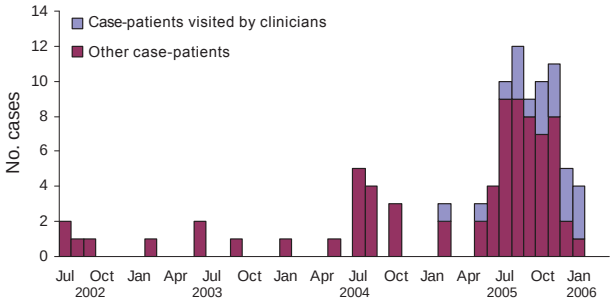

Figure 1. Date of symptom onset for 84 patients in whom confirmed ocular infection was diagnosed, Araguatins, Brazil, 2002–2005.

Of the 62 COD case-patients randomly selected to participate in the case–control study, 48 (77%) were male. Case-patients were significantly younger (mean 11.4 ± 3.5 years) than household controls (mean 25 ± 17.8 years; p<0.001, Student *t* test), but age distribution was similar to that of community controls (13 ± 6.0 years; p = 0.4, Student *t* test). In univariate analysis, male sex was significantly associated with disease when case-patients were compared with 178 household controls (OR 4.7, 95% CI 2.3–9.8, p<0.001); and with 186 community controls (OR 4.5, 95% CI 2.2–9.4, p<0.001).

Environmental exposures most strongly associated with increased risk for disease, which was significant when compared with both household and community controls (Table 2), were swimming or diving in the Araguaia River and frequenting Cais beach on the bank of the Araguaia River. Fishing in the river was associated with disease but only when case-patients were compared with community controls. Factors not significantly associated with disease

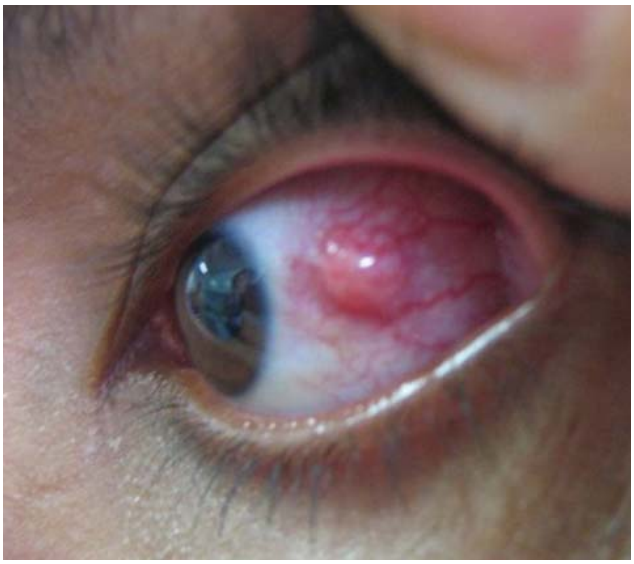

Figure 2. Conjunctival infection and opaque scleral nodule with vascularization in case-patient with confirmed ocular disease, Araguatins, Brazil. Source: Dr Leandro Alencar/Dr Carlos Franklin.

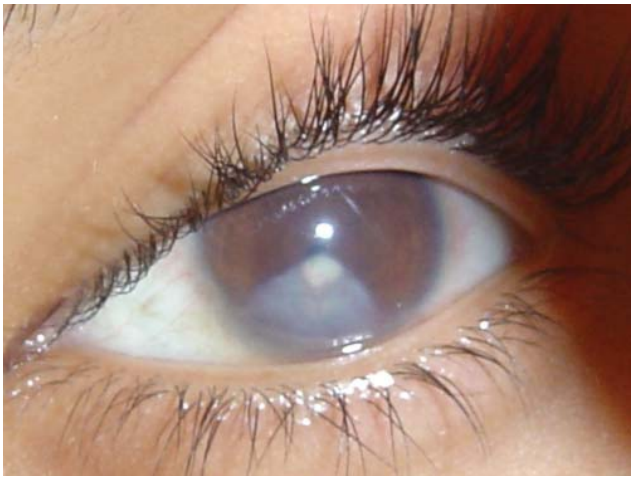

Figure 3. Diffuse opacification of lower quadrant of anterior chamber and cornea with anterior sinéquia in case-patient with confirmed ocular disease, Araguatins, Brazil. Source: Dr Leandro Alencar/Dr Carlos Franklin.

Table 1. Prevalence of confirmed ocular disease in schools surveyed, stratified by urban area, Araguatins, Brazil, 2005

School zone*	No. case-patients identified	No. students	Prevalence, %
Urban	75	3,299	2.3
A	11	179	6.2
B	28	664	4.2
C	14	615	2.3
D	9	379	2.4
E	1	88	1.4
F	4	331	1.2
G	4	331	1.2
H	4	581	0.7
Rural	3	1,785	0.2
I	1	26	3.8
J	1	52	1.9
L	1	108	0.9

*Area where schools are located.

(using either control group) were drinking untreated river water, washing clothes in the river, contact with various types of domesticated animals, a history of exposure to ticks, or a history of allergies. Frequency of river contact was also significantly associated with disease (Table 3). According to multivariate analysis, factors most strongly associated with disease were being of male sex, frequenting the Cais beach area, and diving underwater in the Araguaia River (Table 4).

Among 32 case-patients treated with corticosteroid (oral and/or topical prednisone) by ophthalmologists, disease was resolved or cured in 25 (78%); 7 (22%) case-patients had more severe symptoms and were referred to the Sao Geraldo Hospital in Belo Horizonte, Minas Gerais State.

Among those with nodules, 14 had biopsy samples taken under sterile surgical conditions for diagnostic purposes. Microscopic examination of nodules identified microulcerations of corneal epithelium (Figure 4), and a mixed acute inflammatory response mainly consisting of leukocytes, with some eosinophils, and lymphohistocytic and diffuse neutrophilic infiltrates with edema. Twelve case-patients (13%) had a granuloma of the anterior chamber of the eye unilaterally; 1 case-patient had bilateral anterior chamber granulomas. In addition, in 2 biopsy samples, subconjunctival inflammation was present surrounding large, 200–600-micron, thick-walled, spherical foreign bodies (Figure 5) consistent with adiaconidia of *Emmonsia* sp. fungus, a cause of adiaspiromycosis.

Onchocerciasis, toxoplasmosis, toxocariasis, and microfilaria were discarded as possible etiologies for COD. All 17 samples tested for onchocerciasis were nonreactive for IgG, and no evidence of microfilaria was found in blood smears (n = 17), aqueous humor (n = 1), or biopsy samples of cutaneous nodules (n = 6) examined microscopically. All samples tested for toxoplasmosis (n = 46) were nonreactive for IgM antibodies. Serologic tests for detection of IgG for toxocariasis were reactive for 59 (88%) of 67 COD case-patients, 14 (74%) of 19 household controls, and 53 (64%) of 82 community controls; helminth eggs and larvae, including *Toxocara* spp., were found in 7 of 10 soil samples.

Table 2. Results of univariate analysis of case–control study on relationship between various exposures to freshwater rivers and confirmed ocular disease, Araguatins, Brazil, 2005*

Exposure	No. (%) case-patients exposed, n = 62	Household control group, n = 178			Community control group, n = 186		
		No. (%) exposed	OR	95% CI	No. (%) exposed	OR	95% CI
Contacting river water	61 (98)	132 (74)	21.3	3.0–424.2	145 (78)	17.2	2.5–344.8
Diving underwater	49 (83)	67 (38)	8.1	3.7–18.4	90 (49)	5.1	2.3–11.5
Visiting Cais Beach	50 (81)	97 (55)	3.4	1.6–7.2	52 (30)	10.1	4.7–21.9
Fishing	30 (48)	74 (42)	1.3	0.7–2.4	54 (29)	2.3	1.2–4.3
Drinking untreated river water	23 (38)	62 (35)	1.1	0.6–2.2	50 (27)	1.7	0.9–3.3
Washing clothes in river water	6 (10)	43 (24)	0.3	0.1–0.9	29 (16)	0.6	0.2–1.6

*OR, odds ratio; CI, confidence interval.

Table 3. Results of univariate analysis of case–control study on relationship between frequency of exposure to Araguaia River and odds of confirmed ocular disease, Araguatins, Brazil, 2005*

Frequency	No. (%) case-patients exposed, n = 62	Household control group, n = 178		Community control group, n = 186	
		No. (%) exposed	OR†	No. (%) exposed	OR‡
Weekly	25 (40)	20 (11)	6.9	13 (7)	10.8
Once every 2 weeks	4 (7)	5 (3)	4.4	7 (4)	3.2
Vacations	25 (40)	109 (61)	1.3	121 (65)	1.2
Rarely§	8 (13)	44 (25)	1.0	45 (24)	1.0

*OR, odds ratio.
†χ^2 test for trend 25.7; p<0.0001.
‡χ^2 test for trend 35.3; p<0.0001.
§Fewer than 3×/year.

Discussion

These results confirm the existence of an outbreak of conjunctivitis and severe ocular disease probably caused by adiaspiromycosis, mainly among school-aged children. Risk factors for COD identified in this investigation included diving underwater and frequenting a specific beach (Cais) on the Araguia River in the Amazon region of Brazil. The precise reasons eye contact with river water increased risk remain unclear. Perhaps exposure to freshwater sponge spicules caused an initial conjunctival irritation, as suggested in previous publications (*6–8*). However, the microscopic identification of probable adiaconidia of *Emmonsia* sp. fungus in the scleral biopsy samples from children with severe disease in this outbreak suggests that conjunctival irritation was most likely followed by conjunctival exposure to conidia of this fungus, perhaps in dust caused in part by dry environmental conditions, similar to the exposure of respiratory mucosa described in case reports of pulmonary adiaspiromycosis (*1–5,9*). Adiaspiromycosis causes an inflammatory and often granulomatous response in tissue because of the presence of nonbudding, thick-walled adiaconidia of *Emmonsia* sp. fungus (*10*). Disease is thought to result from exposure to conidia (through inhalation or mucosal contact with dust); these conidia subsequently cause a marked inflammatory response and enlarge to become adiaconidia ranging in diameter from 300 to 600-micorons (*1–5,9,10*).

Boys were at higher risk than girls most likely because boys had more facial and eye contact with the river water while swimming and diving. To minimize bias, we randomly selected asymptomatic controls among persons 5–25 years of age in the community, but some selection bias may have resulted because boys and adolescents were absent at the time of interview (only 42% of community-based controls were boys). The clinical characteristics of conjunctivitis in this outbreak were unusual for several reasons. First, unlike conjunctivitis caused by common bacterial or viral pathogens, neither purulent conjunctival discharge nor hemorrhage was reported, and family members of case-patient households were not commonly affected. In addition, disease was characterized by unusual, single or multiple, white, opaque scleral nodules, often with hyperemia or local edema, and in some cases with opacification (changes in the normally transparent characteristics of the cornea or superficially on scleral tissue) extending to the limbus, or angular corneal opacities and anterior uveitis with granulomas in the anterior chamber. We believe that the clinical improvement of nearly all patients treated with corticosteroids also argues strongly against a bacterial cause or fungal species other than *Emmonsia* because conidia of *Emmonsia* sp. enlarge and cause a localized inflammatory response but do not commonly have the potential to disseminate.

Characteristics of this outbreak are similar to those of an outbreak of anterior uveitis and granuloma previously reported in India, where the etiology was traced to trematodes (*11*). Although the thick-walled foreign body we observed microscopically on slides from 2 case-patients was initially suspected to be trematodes, the round, apparently spherical shape, thick walls, and vacuous central area with lack of organized, internal structures is most consistent with the adiaconidia of the *Emmonsia* sp. Morphologic appearance differs from that of the fungus *Coccidiodes immitis*, in which spores contain internal microsporules (*10*).

Table 4. Results of multivariate analysis of case–control data showing independent effect of type of exposures to Araguaia River and risk for confirmed ocular disease, Araguatins, Brazil, 2005*

Exposure to Araguaia River	Household control group		Community control group	
	aOR (95% CI)	p value	aOR (95% CI)	p value
Swimming	3.1 (0.4–26.8)	0.3	2.1 (0.2–19.4)	0.5
Diving underwater	4.6 (1.9–10.6)	0.0004	2.7 (1.1–7.1)	0.04
Visited Cais Beach	3.2 (1.4–7.1)	0.005	9.9 (4.3–22.9)	0.00001
Male gender	3.4 (1.6–7.2)	0.001	4.7 (1.9–11.0)	0.0004
Fishing in river	–	–	1.2 (0.5–2.7)	0.6

*Unconditional logistic regression model used. aOR, adjusted odds ratio; CI, confidence interval.

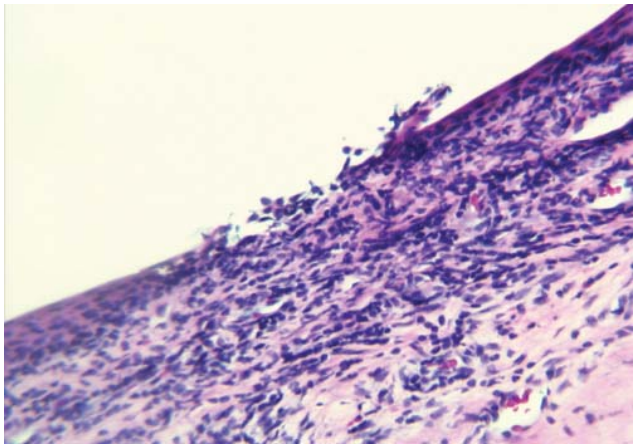

Figure 4. Scleral nodule biopsy sample, showing microulceration of corneal epithelium (magnification ×20, hematoxylin and eosin stain), Araguatins, Brazil. Source: Department of Pathology, University of Brasília.

The natural history of this disease is unknown. However, we identified COD case-patients in several stages of disease, including patients with sequalae. Moreover, after obtaining school surveys, we identified ≈5% of children with ocular abnormalities; COD was diagnosed in one third of children after an ophthalmologic exam. We educated the population about risks for eye contact with river water; active searches were conducted to identify all ill persons in the population and in neighboring cities, and health officials limited recreational access to the Araguaia River. These findings suggest that the extent of this problem may be more widespread in the Amazon region than is currently recognized.

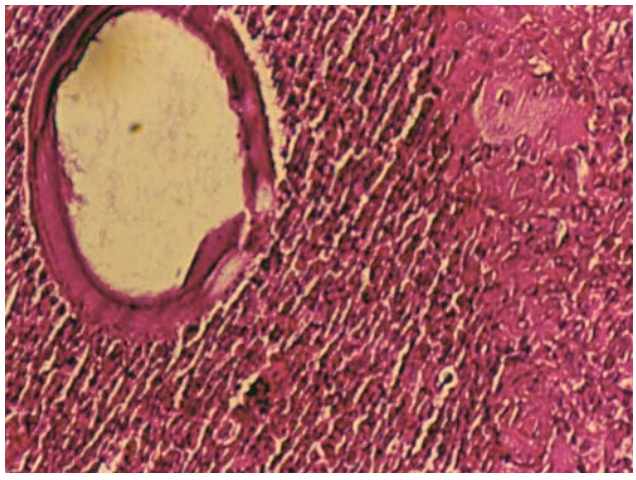

Figure 5. Scleral nodule biopsy specimen, showing diffuse, subconjunctival mixed-cellular infiltrate surrounding large, thick-walled adiaconidia of *Emmonsia* sp. (magnification ×200, hematoxylin and eosin stain), Araguatins, Brazil. Source: Department of Pathology, University of Brasília.

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Human Febrile Illness Caused by Encephalomyocarditis Virus Infection, Peru

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Etiologic studies of acute febrile disease were conducted in sites across South America, including Cusco and Iquitos, Peru. Patients' clinical signs and symptoms were recorded, and acute- and convalescent-phase serum samples were obtained for serologic examination and virus isolation in Vero E6 and C6/36 cells. Virus isolated in Vero E6 cells was identified as encephalomyocarditis virus (EMCV) by electron microscopy and by subsequent molecular diagnostic testing of samples from 2 febrile patients with nausea, headache, and dyspnea. The virus was recovered from acute-phase serum samples from both case-patients and identified with cardiovirus-specific reverse transcription–PCR and sequencing. Serum samples from case-patient 1 showed cardiovirus antibody by immunoglobulin M ELISA (acute phase <8, convalescent phase >1,024) and by neutralization assay (acute phase <10, convalescent phase >1,280). Serum samples from case-patient 2 did not contain antibodies detectable by either assay. Detection of virus in serum strongly supports a role for EMCV in human infection and febrile illness.

Encephalomyocarditis virus (EMCV; family *Picornaviridae*, genus *Cardiovirus*) is a group of closely related virus strains belonging to 1 serotype with a wide host range (*1*). Infection with EMCV is associated with sporadic cases and outbreaks of myocarditis and encephalitis in domestic pigs, in numerous species of nonhuman primates, and in other mammalian species (*2*–*8*). The disease is often fatal—frequently, sudden death is the first indication of infection—and most outbreaks have been associated with captive animals, such as those found in piggeries, primate research centers, and zoos. Virus isolation and serologic studies indicate EMCV is distributed worldwide, but clinical disease in humans or domestic animals is relatively infrequent. Although disease transmission is poorly understood, rodents appear to be the natural reservoirs (*1*). Rodent infestation has been implicated in the genesis of several epizootics; disease transmission apparently results from close contact between rodents or their excreta and individuals of susceptible mammalian species (*1*,*2*,*4*,*5*,*8*,*9*). In several instances, rodent control measures have interrupted disease transmission and halted institutional epizootics (*4*).

Human EMCV infection and disease have been documented by virus isolation from several specimen types, including serum, stool samples, cerebral spinal fluid, and throat washings (*10*–*12*). However, because this disease is so infrequent in humans, positive association with EMCV is difficult to establish. In addition, results of several early studies were questionable because EMCV was isolated by using laboratory mice; researchers could not unequivocally establish that the virus did not originate from the mice used for isolation and passage rather than from human clinical specimens (*12*). Recently, a novel cardiovirus, Saffold virus (SAFV; genus *Theilovirus*), was reported in association with fever of unknown origin in a child 8 months of age (*13*) and in children with symptoms of respiratory or gastrointestinal illness (*14*–*16*). This finding suggests that additional cardioviruses may be pathogenic for humans. Serologic studies also indicate that humans have been infected by EMCV or immunologically related viruses (*17*–*22*). Antibody prevalence varied somewhat from study to study, but seropositivity rates tended to increase in persons of advancing age, consistent with a continuous risk for in-

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fection throughout life. In early 2004, we identified EMCV infection in serum samples from 2 febrile patients in Peru.

Materials and Methods

Surveillance System

In collaboration with the Peruvian Ministry of Health, the Naval Medical Research Center Detachment, Lima, Peru, has established a febrile illness surveillance program designed to identify the causes of febrile diseases in the country. The program carries out passive surveillance in Ministry of Health posts, where febrile patients are identified, signs and symptoms are recorded, and acute- and convalescent-phase blood samples are collected for diagnostic studies. Tests routinely performed in support of the surveillance program include immunoglobulin (Ig) M ELISA for flaviviruses, alphaviruses, bunyaviruses, arenaviruses, and rickettsia, as well as virus isolation, reverse transcription–PCR (RT-PCR), and sequencing. The study protocol was approved by the Naval Medical Research Center Institutional Review Board (Protocol NMRCD.2000.0006, Department of Defense 31535), in compliance with all federal regulations governing the protection of human patients; the protocol also was reviewed by the Peruvian Ministry of Health.

Case Definitions

Clinical diagnoses were considered confirmed if laboratory testing resulted in isolation of virus from the specimen, virus detection by RT-PCR, or a 4-fold or greater increase in IgM antibody titers. Diagnoses were considered presumptive if the IgM titer was elevated in the acute-phase sample only or a 4-fold or greater increase was discerned upon comparison of acute- and convalescent-phase titers. In the absence of laboratory evidence of a specific pathogen, cases were classified as negative.

Virus Isolation

Viruses were isolated by using a modification of a published protocol (*23*). Acute-phase serum samples, obtained no more than 5 days after disease onset, were transported on dry ice to the Naval Medical Research Center Detached laboratory in Lima and stored at –80°C. Serum samples were thawed and diluted 1:5 in minimum essential medium containing 2% heat-inactivated fetal bovine serum and antimicrobial agents. African green monkey (Vero) (37°C) and/or mosquito C6/36 (28°C) cell cultures were each injected with 200 μL of the diluted serum into 25-cm^2 flasks. Upon observation of viral cytopathic effect, or 10 days postinoculation (dpi) if no cytopathic effect was observed, cells were removed from the flasks and placed on 12-well glass spot-slides for examination by immunofluorescence assay using group- and virus-specific polyclonal and monoclonal antibodies. The antibodies used were reactive with dengue viruses 1–4, yellow fever virus, St. Louis encephalitis virus, Rocio virus, Ilheus virus, West Nile virus, Venezuelan equine encephalitis virus, eastern equine encephalitis virus, Mayaro virus, Trocara virus, Oropouche virus, Caraparu virus, Murtucu virus, Guaroa virus, hantavirus, Machupo virus, and Tacaribe virus; after the cardiovirus isolation, EMCV antibody also was used.

Upon receipt of the viruses at the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA), a master seed stock was prepared from each virus and working stocks were made by passage in Vero E6 cells. Cytopathic effect developed rapidly, and the cultures were harvested at 3–4 dpi; some infected cells were fixed in 2.5% glutaraldehyde for electron microscopy examination. For virus reisolation at CDC, acute-phase serum samples were injected into Vero E6 cells. Virus isolates were obtained in Vero E6 cells from acute-phase serum of both case-patients. The isolates, IQD6726 (Iquitos) and FSC575 (Cusco), were passaged in Vero E6 cells to obtain stocks for further analysis. Litters of suckling mice were inoculated intracerebrally with both strains of virus. Brain, spleen, and liver tissues were harvested and fixed in 2.5% glutaraldehyde for electron microscopic examination.

RNA Extraction, RT-PCR, and Sequencing

RNA was extracted from the virus isolates by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). cDNA reactions consisted of 2 μL of extracted viral RNA, 50 ng of random hexamers (Applied Biosystems, Foster City, CA, USA), 4 μL of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), 200 μmol/L of each dNTP (GE Healthcare, Piscataway, NJ, USA), 40 U of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U of SuperScript II reverse transcriptase (Invitrogen) in a total volume of 20 μL. The cDNA reactions were incubated for 10 min at 25°C, followed by 45 min at 42°C and 4 min at 95°C. For the PCR assays, 3 genome regions were targeted, the 5′-NTR, VP1, and 3D regions (Table). Two sets of PCR primers targeting the viral protein 1 (VP1) gene were used, yielding overlapping PCR fragments. PCR reactions consisted of 2 μL of cDNA, 200 μmol/L of each dNTP (GE Healthcare), 5 μL of 10× buffer with $MgCl_2$ (Roche Molecular Biochemicals, Indianapolis, IN, USA), 2.5 U of Fast Start Taq DNA Polymerase (Roche), and forward and reverse primers (10 pmol of each of AN312 and AN315; 40 pmol each of AN283, AN285, AN393, and AN286; 20 pmol each of P1 and P2; and 60 pmol each of 1C340F and 2B188R), in a total volume of 50 μL. Cycling parameters were 42ºC for 50 min, 50°C for 10 min, and 95ºC for 5 min, followed by 45 cycles of 95ºC for 30 sec, 45ºC for 40 sec, and 60ºC for 1 min. All PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

Table. Oligonucleotide primers used for cardiovirus PCR amplification and sequencing*

Primer	Sequence (5′ → 3′)	Region	Coordinates	Reference
AN312	GARTVWCGYRAAGRAAGCAGT	5′-NTR	455–475	This study
AN315	GGYRCTGGGGTTGYRCCGC	5′-NTR	618–600	This study
AN283	GCAGACGGWTGGGTNACNGTNTGG	VP3	2559–2582	This study
AN285	AGAGTAACCTCTACRTCRCAYTTRTA	VP1	3097–3072	This study
AN393	TTTCCACTCAAGTCTAARCARGAYT	VP1	3015–3049	This study
AN286	AAGAAGACAGTCGGACGNGGRCARAANAC	VP1	3472–3444	This study
P1	CCCTACCTCACGGAATGGGGCAAAG	3D	7655–7631	(*24*)
P2	GGTGAGAGCAAGCCTCGCAAAGACAG	3D	7370–7395	(*24*)

*NTR, nontranslated region; VP1, viral protein 1.

Amplicons were purified from an agarose gel (QIAGEN gel extraction kit) before sequencing. Both strands were sequenced by using the PCR primers, a Prism BigDye Terminator version 1.1 or 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems), and a model 3100 or 3130 Genetic Analyzer (Applied Biosystems). The sequences determined in this study were deposited in the GenBank sequence database, accession nos. EU979543–EU979548.

Sequences were identified in GenBank by using BLAST to compare sequences (www.ncbi.nlm.nih.gov/blast). For phylogenetic reconstruction, sequences were aligned with those of other cardioviruses by using PileUp (Wisconsin Sequence Package, version 11.1; Accelrys, San Diego, CA, USA), and trees were generated with the neighbor-joining method implemented in ClustalX version 1.83 (*25*). Genetic distances were estimated by the Kimura 2-parameter method. To assess the confidence of branching patterns of the neighbor-joining tree, 1,000 bootstrap replicates were performed.

Serologic Testing

Serum specimens were tested for neutralizing antibody against EMCV by a modified microneutralization assay (*26*). Approximately 80–100 median 50% cell culture infectious dose ($CCID_{50}$) of isolate FSC575 and serial dilutions of serum (starting at 1:10 and ending at 1:1,280) were incubated together at 37°C for 2.5 h before Vero E6 cells were added to the wells. After incubation for 5 days at 37°C, each plate was stained with 0.05% crystal violet in 25% ethyl alcohol and, after drying, the optical density in each well was measured at 570 nm. Each specimen was run in triplicate; the final titer was estimated by use of the Spearman-Karber method (*27*). Positive-control anti-EMCV serum was generously provided by Ann Palmenberg.

Pathogen-specific IgM titers were determined by using an adapted IgM-capture ELISA (*28*). Briefly, 96-well plates were coated with antihuman IgM antibody to capture patient IgM molecules. Virus-specific IgM was detected by adding viral antigen, followed by virus-specific mouse hyperimmune ascitic fluid and horseradish peroxidase-labeled antimouse IgG. After the addition of colorimetric substrate, absorbance was read at 490 nm. All acute- and convalescent-phase samples were initially screened at 1:100. Samples exceeding the reference cutoff value, calculated as the median of 7 antibody-negative samples plus 3 standard deviations, were considered IgM positive. Positive samples were subsequently retested by using 4-fold serial dilutions to determine end-point titers. Viruses included in the ELISAs included dengue viruses 1–4, yellow fever virus, St. Louis encephalitis virus, Rocio virus, Ilheus virus, West Nile virus, Venezuelan equine encephalitis virus, eastern equine encephalitis virus, Mayaro virus, Trocara virus, Oropouche virus, Caraparu virus, Murtucu virus, Guaroa virus, hantavirus, Machupo virus, and Tacaribe virus; after the cardiovirus isolation, EMCV was also used.

Results

Clinical Description of Cases

The first patient was identified in the Amazonian city of Iquitos, Loreto Department, Peru. Iquitos (3°44.69′S, 73°15.25′W) is situated at an elevation of 120 m and has a population of ≈350,000. The city is surrounded by the Nanay, Itaya, and Amazon rivers and is accessible only by air or river. The climate is tropical, mean temperature is 25°C, and annual rainfall is 2.7 m. Rodents are common in urban and rural areas; swine and primates are abundant in rural zones.

Case-patient 1 was a 59-year-old housewife from Iquitos. She sought treatment at Hospital Apoyo Iquitos after 3 days of fever, pallor, poor appetite, malaise, nausea, and headache. She recovered completely from fever after 6 days of treatment as an outpatient. Acute-phase (3 days postonset), convalescent-phase (15 days postonset), and late convalescent–phase (8 months later) blood samples were obtained. She had no identifiable focus of infection, negative thick and thin smears for malaria, and a negative tourniquet test. She reported no yellow fever or hepatitis B vaccination and no travel outside of Iquitos within 2 weeks before onset. The patient was interviewed 8 months after seeking treatment when virus isolation results were completed. She reported only limited contact with rats and cats in her house and a neighbor's house where she played

bingo frequently. After 7 days and nights of trapping in both houses and in a sewer adjacent to the neighbor's house, no rodents were collected.

The second case-patient was identified in the Cusco Regional Hospital but had traveled from Quebrada, a small rural village on the Yanatile River, about 8 hours travel time from the city of Cusco. Quebrada (approximately 13°55′S, 71°40′W) is located in Yanatile district, Calca Province, in the Department of Cusco. The weather in Quebrada is warmer than that in Cusco (temperate climate) at an altitude of 2,926 m. Malaria and leishmaniasis are endemic to the area. The city is accessible by air and land from Cusco.

Case-patient 2 was a 39-year-old male farmer from Cusco-Acomayo/Quebrada Calca. He sought treatment at the local hospital after 7 days of fever. Additional symptoms were headache, malaise, retro-ocular pain, sweats, weight loss, arthralgia, photophobia, poor appetite, myalgia, chills, pallor, nausea, vomiting, and abdominal pain. He was hospitalized with a diagnosis of febrile syndrome and urinary tract infection (UTI). Urinalysis showed 20–25 leukocytes per high-power field. The patient had elevated values for alkaline phosphatase (680 U/L; reference range 68–240 U/L), total bilirubin (1.68 mg/dL; reference value <1.0 mg/dL), and direct bilirubin (1.06 mg/dL; reference value <0.2 mg/dL). Thick and thin smears for malaria and *Bartonella* spp. were negative, as were urine culture and a tourniquet test for dengue hemorrhagic fever. The patient was treated with oral ciprofloxacin (750 mg 2×/d for 7 days), oral paracetamol (acetaminophen; 500 mg as needed for fever >38.5ºC), and intravenous fluids. He recovered completely after 7 days as an inpatient. At the time of his convalescent-phase sample (15 days postonset), his physical examination was within normal limits. He reported tuberculosis in a family member contact (treated for 5 months), yellow fever vaccination in 1994, and no vaccination against hepatitis B. No case investigation was carried out for this patient to determine risk factors for disease. However, the typically rural populations near Cusco have contact with a variety of animal species such as mules, dogs, cats, swine, rodents, rabbits, llamas, alpacas, and vicuñas.

Virus Isolation and Identification

Virus isolates were obtained from acute-phase serum specimens of both patients by inoculation of Vero E6 cell cultures. Electron microscopic studies of infected Vero E6 cells and mouse tissues demonstrated cytoplasmic accumulations of particles consistent with the features of picornaviruses. Virions averaged 24 nm in diameter and were occasionally found in paracrystalline arrays (Figure 1, panel A). The infected cells were notable for areas of vesiculation and membrane proliferation (Figure 1, panel C), consistent with the replication complexes, which have been described for picornavirus-infected cells (*29*). Newborn mice inoculated intracerebrally all died at 3 dpi. Rapid death in neonatal mice, coupled with ultrastructural evidence of picornavirus infection, was consistent with the presence of a cardiovirus.

To confirm the diagnosis, we used cardiovirus-specific RT-PCR assays targeting the 5′-nontranslated region and the VP1 and 3D coding regions (Table). Each of the cardiovirus-specific primer sets amplified a specific DNA product of the expected size; in each instance, the identity of the amplicon was confirmed by sequencing. In a phylogenetic reconstruction done on the basis of sequences from the VP1 capsid region, the Peru viruses clustered with sequences of EMCV strains derived from pigs from Belgium, Cyprus, and Italy; the EMCV reference strains and field strains from pigs and orangutans formed separate subgroups within the EMCV species group (Figure 2, panel A). The VP1 nucleotide sequences of the Peru strains were 91%–100% identical to those of other strains within their phylogenetic subgroup. VP1 nucleotide identities of the Peru cardioviruses to the other EMCV subgroups ranged from 71% to 80% and from 47% to 51% to the rodent and human viruses in the *Theilovirus* genus, respectively. The Peru strains were also most closely related to the European pig strains in the 5′-NTR and 3D regions (Figure 2, panels B, C). Within the larger EMCV 5′-NTR group, containing the Peru cardioviruses, pairwise nucleotide identities ranged from 95.8% to 100.0% (Figure 2, panel B). Relatedness of the Peru cardioviruses' 5′-NTR to the EMCV and Theiler's viruses outside of this group ranged from 30.3% to 58.4%. Within the large 3D clade containing the Peru cardioviruses and European

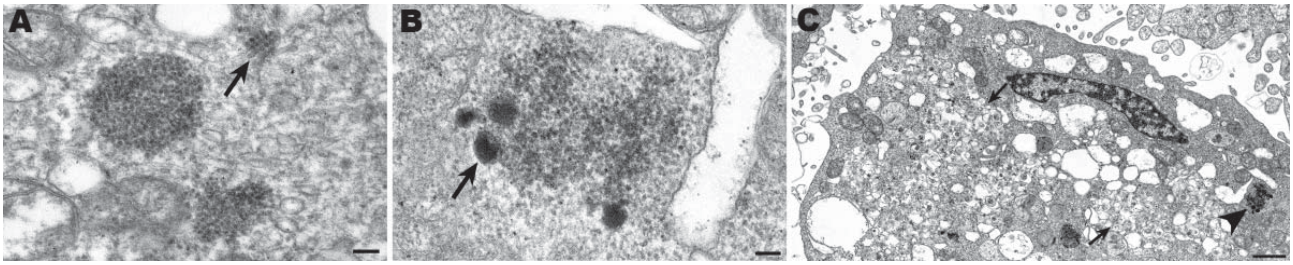

Figure 1. Ultrastructural morphologic features of cardiovirus-infected Vero E6 cells. A) Collections of picornavirus particles, some arranged in a paracrystalline array (arrow). Scale bar = 100 nm. B) Higher magnification of area pointed to by arrowhead in panel C showing condensed material (arrow) at periphery of a viral cluster. Scale bar = 100 nm. C) Cardiovirus-infected cell, showing membrane proliferation and vesiculation (arrows). Scale bar = 1 μm.

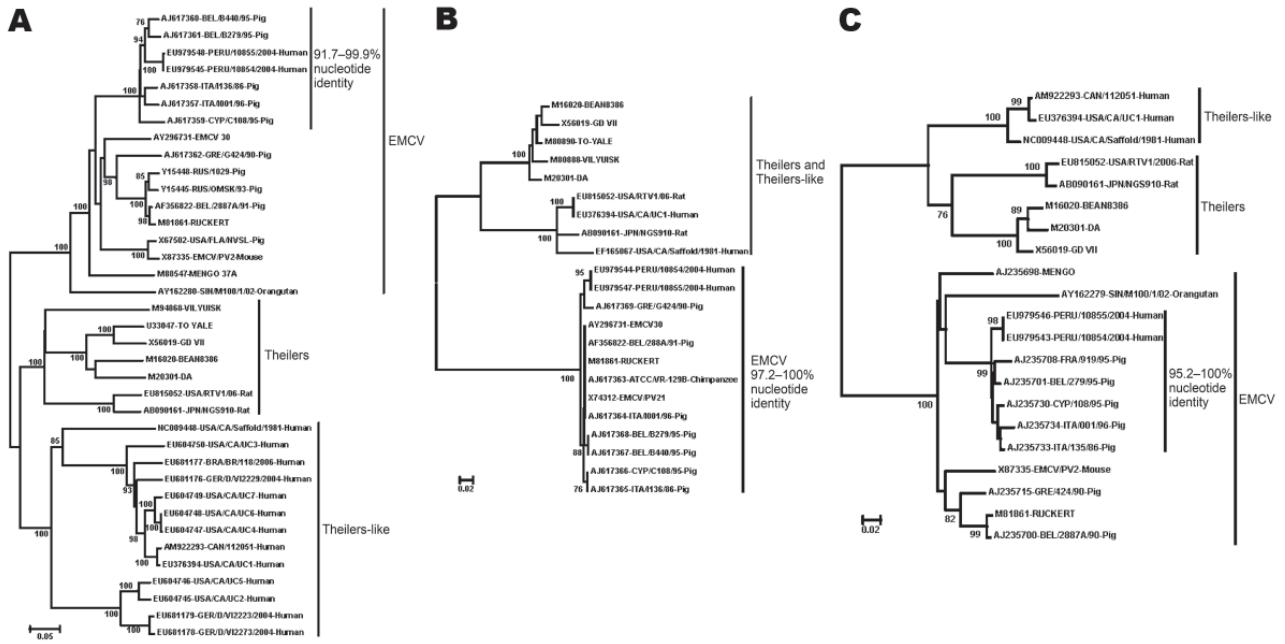

Figure 2. Phylogenetic relationships among viruses detected in Peru and other encephalomyocarditis viruses (EMCVs), and their relationship to the Theiler and Theiler-like cardioviruses. A) Viral protein 1 (VP1); 737 nucleotides (90% of the VP1 gene). The missing portion of the VP1 gene is at the 3′ end. B) 5′ nontranslated region; 145 nucleotides consisting of a highly conserved portion of the internal ribosome entry site, sequence coordinates 558 to 699 relative to EMCV GenBank accession no. AX786477. C) 3D; 210 nucleotides (15% of the 3D gene). The use of this portion of the 3D gene for phylogenetic analysis has been described elsewhere (*25*). Phylogenies were reconstructed with the neighbor-joining method implemented in ClustalX version 1.83 (*27*). Genetic distances were estimated by the Kimura 2-parameter method. To assess the confidence of branching patterns, 1,000 bootstrap replicates were performed. Cardioviruses are identified by GenBank accession number and strain name; when available, complete virus information is given, using the following convention: GenBank accession number, country of origin/strain name/year of isolation-host species. Country abbreviations: BEL, Belgium; BRA, Brazil; CAN, Canada; CYP, Cyprus; FRA, France; GER, Germany; GRE, Greece; ITA, Italy; JPN, Japan; RUS, Russia; SIN, Singapore; USA, United States. Scale bars indicate number of nucleotide substitutions per site.

pig strains (Figure 2, panel C), the nucleotide identities ranged from 95.2% to 100.0%. 3D nucleotide identities of the Peru cardioviruses to the EMCVs outside the Peru clade ranged from 80.0% to 87.6%. Relatedness of the Peru cardioviruses' 3D region to the Theiler's viruses ranged from 56.7% to 61.4%.

Serologic Confirmation of Infection

Serum from case-patient 1 was seropositive for cardiovirus by IgM monoclonal antibody-capture ELISA; titer was high. Seroconversion was documented by an increase in titer from <8 in the acute-phase sample (day 3) to >1,024 in the convalescent-phase sample (day 15). The neutralization assay yielded similar results; acute-phase titer was <10 and a convalescent-phase titer was >1,280. Serum samples from case-patient 2, collected on days 7 and 15, were negative in both assays (titers <8 and <10, respectively), despite the presence of virus in the day 7 serum sample.

Discussion

Our ongoing febrile surveillance studies identified and documented 2 cases of human EMCV disease, each of which was diagnosed by virus isolation from acute-phase serum. One patient had an undifferentiated fever with complete recovery after 6 days of fever. The second patient's disease was complicated by concomitant UTI, which presumably prolonged the course of disease (11 days of fever). Even though EMCV was isolated from this patient, we cannot be certain whether specific symptoms resulted from the viral infection or from the concurrent UTI. At the time of hospitalization, the urine culture was negative, but the patient had received a course of antimicrobial drugs before his hospitalization. However, the urinary tract symptoms also could be consistent with EMCV infection because orchitis has been observed in male laboratory male mice and hamsters (*30*,*31*). Hyperbilirubinemia, associated with EMCV infection in humans, is consistent with the observation of liver compromise that has been well documented in laboratory studies of EMCV-infected mice, swine, and primates (*5*,*32*). The 2 case-patients probably had contact with infected animals. The proposed routes of transmission to humans are contamination of wounds and contact with domestic animals, such as cats, that frequently contact EMCV-infected ro-

dents. The virus identified was distinct from EMCV reference strains but closely related to EMCV strains from pigs in Europe in all 3 genomic regions analyzed.

Few cases of human EMCV disease have been documented; however, in the older literature, virus isolation was reported from cerebrospinal fluid, blood, feces, and throat washings of patients (particularly children) with aseptic meningitis, poliomyelitis-like paralysis, encephalomyelitis, Guilláin-Barré syndrome, and fever of unknown origin (*10*–*12*). Human disease characterized by chills, fever, severe headache, stiff neck, pleocytosis, delirium, delusions, vomiting, photophobia, and fever also has been reported (*10*). A novel cardiovirus, SAFV, isolated from the stool of an infant with fever of unknown origin, recently was reported (*15*). SAFV and SAFV-like viruses also have been detected in nasopharyngeal aspirates from children with respiratory illness (*16*) or gastroenteritis (*17*,*18*). In all of these cases, however, virus was isolated only from specimens obtained from nonsterile sites, making the patients' symptoms impossible to associate unequivocally with the cardiovirus infection. In other human picornavirus infections, virus can sometimes be isolated from whole blood, serum, or plasma, but viremia is usually of short duration and relatively low titer (*33*). For enterovirus infections, for example, blood is not considered a reliable source of virus, except in very young children (*34*). In infants, prolonged enterovirus viremia may lead to multiple organ involvement and more serious disease. In the 2 EMCV-infected patients described here, virus was isolated from serum collected 3 and 7 days after onset of symptoms, respectively, suggesting that viremia level was high and of long duration. Together, virus isolation from serum and, for 1 case-patient, documentation of >4-fold rise in antibody titer, provide conclusive evidence for causality.

Because few clinical or public health laboratories are capable of identifying cardiovirus infection, the effect on human health is unknown. The original detection and identification of SAFV used a combination of traditional virus isolation in cell culture and creation and characterization of a cDNA library by DNase sequence–independent single-primer amplification (*15*,*16*). A panviral DNA microarray also has been used to detect SAFV and SAFV-like human cardioviruses (*18*). Such laborious methods are not practical for routine diagnostic testing. Direct detection of human cardioviruses by RT-PCR, as described here, or by SAFV-specific RT-PCR (*17*), can be adapted for use in the clinical laboratory, and sequencing of PCR products can be used for confirmation and more detailed molecular epidemiologic analysis. On the basis of an analysis of SAFV sequences, the PCR assays described here should efficiently amplify and detect SAFV and all other known cardioviruses (data not shown), providing a valuable tool to detect cardioviruses in human specimens. However, before routine testing of human specimens can be justified, studies are needed to assess the prevalence of cardiovirus infection in human populations, to associate specific disease syndromes with cardiovirus infection, and to identify reservoir species involved in zoonotic transmission. These studies will help estimate the impact of disease caused by cardiovirus infection and identify prevention and intervention strategies.

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Rapid Point-of-Care Diagnostic Test for Syphilis in High-Risk Populations, Manaus, Brazil

Meritxell Sabidó, Adele S. Benzaken, Ênio José de Andrade Rodrigues, and Philippe Mayaud

We assessed the acceptability and operational suitability of a rapid point-of-care syphilis test and identified barriers to testing among high-risk groups and healthcare professionals in a sexually transmitted infections clinic in Manaus, Brazil. Use of this test could considerably alleviate the impact of syphilis in hard-to-reach populations in the Amazon region of Brazil.

The new generation of rapid point-of-care (POC) syphilis diagnostic tests has shown good reliability and can be performed in any clinical setting. These tests can provide fast results during a patient's initial visit (*1*).

Implementation of syphilis screening programs can be hampered by operational and technical difficulties (*2*–*4*) such as inadequate training, poor supervision, inconsistent quality control, disruptions in receiving medical supplies, and erratic electricity or refrigeration facilities needed to perform the test or store its reagents (*5*). Patients' barriers to testing are often structural (accessibility and clinic hours) or financial (*4*). Further, test-seeking behavior can be negatively affected by the silent nature of the infection, the patient's limited syphilis-related knowledge, and the perceived quality of healthcare provided. Overcoming any of these barriers would result in increased accessibility of services to those most in need and effective implementation of testing within often fragile healthcare systems located in resource-limited countries.

The Study

The study was undertaken within a larger field evaluation of a novel POC test for the detection of treponemal antibodies (VisiTect Syphilis, Omega Diagnostics, Alloa, Scotland) (Figure 1) in a sexually transmitted infections clinic located in a "red-light" area near the harbor of Manaus, Brazil (*6*). Before the evaluation, all staff were trained in the use of the test. One month after the start of syphilis screening, 10 clinical staff and 2 laboratory technicians were interviewed to identify factors that facilitated or impeded performance of the test.

Over a 6-week period, 60 patients, who had given informed oral consent, were interviewed while awaiting test results. The questionnaire was designed to examine reasons and potential barriers for syphilis testing, participant satisfaction with the information and attention received, and syphilis knowledge.

A separate subsample of consecutive patients, who were not interviewed, participated in a time-flow analysis. At all stages of the consultation, staff recorded, on forms given to the patient, the exact start and finish time of contact with the patient and the number of minutes required to perform each task with the patient (Figure 2). Time difference between tasks is the waiting time.

Descriptive analysis of quantitative data was done by using STATA version 9.0 (StataCorp, College Station, TX, USA). Detailed notes on qualitative items were analyzed thematically, coded, and categorized according to underlying themes included in the questionnaire. Categorized information was classified into 5 themes (confidence in test results, syphilis knowledge, test-seeking behavior, test preference, and evaluation of health services).

Most staff (10/12) thought training was satisfactory, and 9/12 reported test instructions as "perfectly easy" or "very easy" to follow. Laboratory technicians (2/2) found the test easy to use and interpret, requiring only ≈2 minutes to perform. In contrast, 2/10 physicians and nurses found interpretation of the test results "complex" or "not easy" because the test sometimes yielded a blurred result line difficult to assess and because the test could react and turn positive after the expected reading time (15 minutes). Most physicians and nurses (6/10) lacked confidence in the POC test result. They correctly pointed out that the POC test did not differentiate between past-treated and recent syphilis.

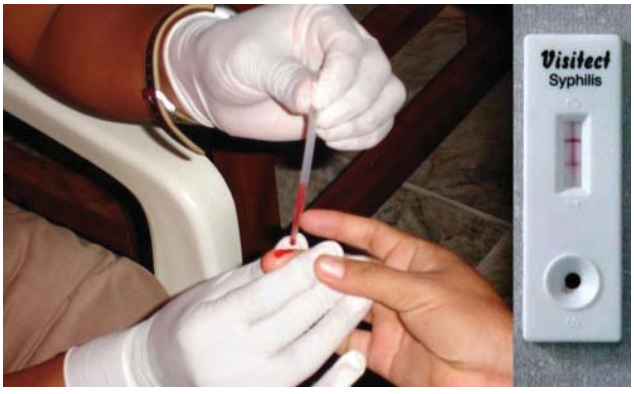

Figure 1. Rapid point-of-care syphilis test. Finger prick (left); diagnostic cassette with test bands results (right).

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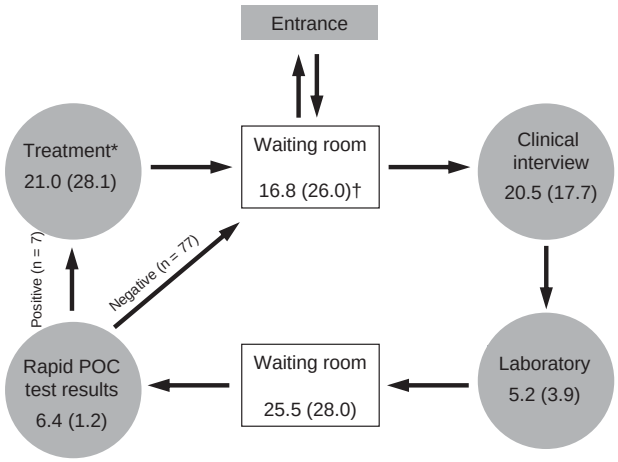

Figure 2. Time-flow analysis for point-of-care (POC) syphilis testing and treatment during a visit to a sexually transmitted infections clinic in a red-light area of Manaus, Brazil, 2006 (N = 84). Total time in minutes (SD) spent by patients completing all stages is shown, regardless of treatment. Average duration time spent at the health facility in mean (SD) minutes: 88.9 (37.1). *Only 7/84 (8.33%) of patients required to complete this stage; †includes time required to get into and to leave the health facility.

Some staff reported finding discrepancies between the results of the POC test and conventional treponemal assays (*7*). Conventional treponemal assays, which rely on testing venous blood samples, were preferred by 6/10 clinical staff, were sometimes perceived to be less painful for the patient (4/10 clinicians responding), and provided more relevant information for patient care.

Sixty patients (36 women, 60%) were interviewed: 25 female sex workers (42%), mean age 31 years (SD, 10.5); 20 male clients of female sex workers (33%), mean age 44 years (SD, 15.0); and 15 (25%) other patients living or working in the Manaus harbor area, mean age 40.2 years (SD, 15.7). After an information campaign began (posters, street banners, flyers, and peer communication), patients took a median of 3.5 days (range 0–30) to attend the clinic; 20 (33%) participants sought testing on the same day they received the information.

Participants cited perceived risk for infection and knowledge of people who had already been tested as the primary motivators for testing. As one male harbor worker said, "I came for testing because some people said I was sick." For three quarters of the participants (45/60), work schedules were not a limitation to seeking testing. Most respondents (69%) found that the time required for testing was short or very short. Almost half (48%) did not incur any costs in coming to the clinic for testing; others incurred only transportation costs. The rapid POC test did not cause any discomfort to 41 (68%) persons, but others found the fingerprick more painful and frightening than venipuncture. As one female sex worker remarked, "It (the fingerprick) is really itching!" Half (52%) of the respondents stated that they would choose the conventional test because this test was less painful, and they were accustomed to blood tests by venipuncture. Among patients who preferred rapid testing, the main reasons given were the rapidity of knowing their syphilis status and, for some, fear of needles. One female sex worker explained, "I am afraid of needles, and the fingerprick is better and much quicker!"

All respondents, with the exception of 2, trusted test results mainly because of their respect for the organization that ran the clinic. One male client commented, "I trust them because Fundação Alfredo da Matta is a serious organization that takes care of the human person and doesn't care about the money."

Almost all participants classified the attention received as satisfactory and indicated the general caring attitude of staff and lack of stigmatization as remarkable qualities of the service (Table). Total mean time at the health facility in minutes was 88.9 (SD 37.1). Results of the time-flow analysis conducted among 84 patients showed that, excluding time spent receiving treatment for 7 (8.3%) patients, average time spent at the clinic was 51 minutes (SD 32) (Figure 2).

Table. Responses to selected questions on a questionnaire administered to 60 patients undergoing POC syphilis testing in an STI clinic, Manaus, Brazil, 2006*

Questions	No. (%) responses
Were you satisfied with services received?†	
5 out of 5 factors	56 (93)
4 out of 4 factors	4 (7)
Would you recommend the syphilis rapid test to friends?	
Yes	57 (95)
No	2 (5)
How would you rate the information received from clinical staff?	
Satisfactory	36 (60)
Difficult to understand	8 (13)
Did not receive information	16 (27)
How much do you know about syphilis?‡	
Could identify STI	12 (20)
Could explain some or all of its symptoms	12 (20)
Could explain some of its complications	5 (8)
Do you know how syphilis is transmitted?‡	
Unprotected sex	12 (20)
Sex regardless of condom use	19 (32)
Mother to child	6 (10)
Contaminated blood	13 (22)
Kissing	6 (10)
Sitting in the same place	3 (5)
Skin lesions	2 (3)
Do you know whether syphilis can be cured?‡	
Yes	53 (89)
No/don't know	6 (11)

*POC, point of care; STI, sexually transmitted infection.
†Measured on a scale from 0 (totally unsatisfactory) to 5 (totally satisfactory).
‡Open questions.

Conclusions

From patient and laboratory technician perspectives, the rapid POC test was acceptable and operationally appropriate as a screening tool for diagnosis of syphilis and was performed within a reasonable waiting time for patients. However, of concern was the staff's lack of trust in test results, which was correctly attributed to the test's failure to differentiate between past-treated infections and active cases. Tests that could overcome this main technological handicap would be welcomed. Barriers to testing that need to be addressed are the pain caused by the finger puncture and poor knowledge of syphilis in a clearly high-risk population (*6*). The main study limitation is the possible selection bias of the target population who sought testing at the clinic.

The performance of the POC test combined with the advantage of on-site testing and same-day treatment are operational characteristics likely to improve coverage of syphilis screening in hard-to-reach populations such as highly stigmatized groups or those living in remote rural areas (*8*). Immediate, on-site testing is especially important for extending syphilis screening programs in the Amazon Region, a region characterized by long distances to most of its settlements, the need for river transportation, and the lack of well-equipped laboratories and trained technicians.

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Spatial Distribution of Leprosy in the Amazon Region of Brazil

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To detect areas with increased case-detection rates, we used spatial scan statistics to identify 5 of 10 clusters of leprosy in the Amazon region of Brazil. Despite increasing economic development, population growth, and road infrastructure, leprosy is endemic to this region, which is a source of case exportation to other parts of Brazil.

Leprosy is a public health problem in Brazil. Despite economic development, expansion of public healthcare, and efforts of the leprosy control program in the past 30 years, this disease has not been eliminated, and new cases are still being detected.

Leprosy has been a notifiable disease in Brazil since 1980. In 1999, a leprosy surveillance system was adopted throughout the country. Each reported case is recorded by the municipal health authority into its database. This information is then reported to the Ministry of Heath. The leprosy control program in Brazil distributes free dapsone, rifampin, and clofazimine as part of the World Health Organization multidrug regimen for treatment of leprosy. The need for treatment is determined on the basis of reported data. Brazil has 5,560 municipalities, 26 states, and 1 Federal District, and an area of 8,514,205 km^2. Knowledge of the spatial distribution of leprosy will increase the efficiency of the leprosy control program in this country.

Leprosy has been highly endemic to the Amazon region of Brazil for >100 years. In the 19th century, leprosy incidence was high among Native Americans in the state of Pará (*1*). In 1913, Oswaldo Cruz, then head of the Brazilian Public Health Division, recognized the high frequency of leprosy in the Amazon River Basin (*2*). In 1975, Agrícola (*3*) reported that state of Acre, in the western Amazon region of Brazil, had the highest seroprevalence rate for leprosy. Conversely, states in northeastern Brazil, which have a semiarid climate, had the lowest seroprevalence rates. In 2007, the state of Mato Grosso in the southern Amazon region of Brazil, reported the highest case-detection rate (100.27/100,000 inhabitants), and the state of Rio Grande do Sul, the southernmost state, reported the lowest case-detection rate (1.74/100,000 inhabitants) (*4*). These findings suggest that the spatial distribution of leprosy has changed in the past 30 years.

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The Study

To detect areas with an increased case-detection rate for leprosy, we used spatial scan statistics (*5*). This method scans an area for leprosy clusters without a priori knowledge of their location or size. A circular window moves though a map with its center at the coordinates of municipal councils. At each position, the radius of the circular window varies from 0 km to 500 km, and each window includes different groups of neighboring municipalities. A Poisson model defines the presence of spatial clusters. Under the null hypothesis, the expected number of cases in each area is proportional to the person-years in that area. All possible clusters are tested for statistical significance by a log likelihood ratio test, which accounts for multiple testing (*6*). The log likelihood ratio defines cluster order.

We used Satscan software (*7*) to obtain statistical estimates on the basis of the number of new cases diagnosed during 2005–2007 by municipality of residence (obtained from the national database available on July 5, 2008) and population estimates by municipality for the same period (obtained from the Brazilian Institute of Geography and Statistics). The 10 most probable nonoverlapping clusters of leprosy (Table 1) were located between latitudes 21°S and 4°N. These clusters comprised 1,173 municipalities with 65,357 cases diagnosed during 2005–2007, 53.5% of all cases in Brazil, and 17% of the person-years in this period (33,080,363 inhabitants in 2007). These clusters covered a wide but sparsely populated area (Figure). The leprosy case-detection rate for the clusters was 66.80/100,000 inhabitants and 12.30/100,000 for the area outside these clusters (rate ratio 5.43). These findings indicate that lep-

Table 1. Ten most probable clusters of leprosy defined by using spatial scan statistics, Brazil, 2005–2007*

Cluster order	No. cases		RR	LLR
	Observed	Expected		
1	24,564	6,345.04	4.59	16,545.44
2	9,735	2,224.77	4.67	7,099.49
3	4,136	928.37	4.58	3,014.57
4	6,944	2,912.92	2.47	2,070.23
5	5,778	2,424.91	2.45	1,711.11
6	5,891	2,674.40	2.26	1,479.21
7	2,223	1,039.11	2.16	512.49
8	1,325	476.37	2.80	509.78
9	3,288	1,799.11	1.85	502.97
10	1,473	581.84	2.55	480.32

*RR, relative risk for the cluster compared with the rest of the country; LLR, log likelihood ratio. p<0.001 for all comparisons.

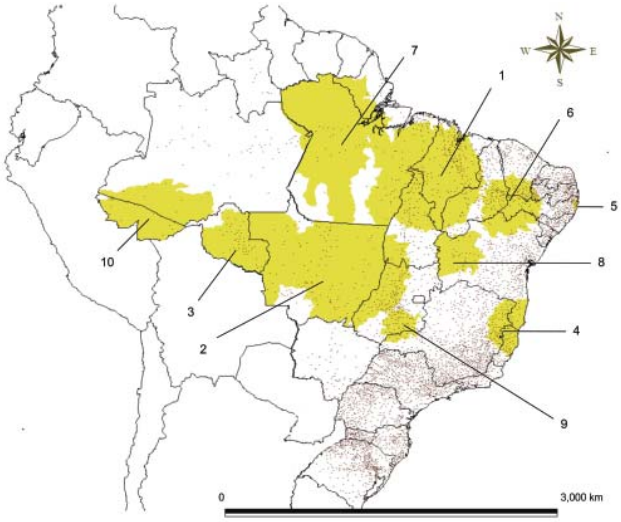

Figure. Locations of the 10 most probable leprosy clusters (yellow regions) and municipal councils (dots), Brazil, 2005–2007.

rosy is endemic to concentrated in a small portion of the population in Brazil.

Case-detection rates for each cluster are shown in Table 2. By comparison, the highest case-detection rates reported to the World Health Organization in 2006 were from Micronesia (136.04/100,000, population 602,000) and Papua New Guinea (63.95/100,000, population 111,000) (*8*). Cluster 1, which contained 20.1% of cases detected during 2005–2007, was located in an area that had a population of 9,592,600 in 2007.

Some municipalities within these clusters had lower case-detection rates than the average case-detection rate in Brazil. A probable explanation is that fewer cases were detected because of failures in the healthcare system, such as low population coverage and the inability of healthcare workers to diagnosis leprosy.

Of the 10 most likely clusters, 5 (1, 2, 3, 7, and 10) were located in the Amazon region of Brazil, and 3 (6, 8, and 9) were contiguous to 1 of these 5 Amazon clusters and located in dry savannah areas. Cluster 5, which was spatially isolated from the others, corresponds to the Recife metropolitan region, the third largest metropolitan area in Brazil. This area is populated by poor immigrants from dry rural areas of northeastern Brazil. Cluster 6, also spatially isolated, was in a region that has a hot, humid climate and contains remnants of the Atlantic rainforest.

Conclusions

Leprosy is still highly endemic in the Amazon region of Brazil. This fact cannot be of explained by socioeconomic conditions of the population because the populations of northeastern states (semiarid climate) and of large metropolitan areas have a much higher risk for malnutrition than the population in the Amazon region of Brazil.

A hypothesis to explain the clusters in the Amazon region of Brazil is that when leprosy was introduced into this region, it probably caused an epidemic among the indigenous population because of their lack of exposure to infection (*9*). The disease likely spread slowly throughout the area because of the isolated population and large distances. Until 1970, the most heavily populated areas of the Amazon region of Brazil could be described as population islands because the only means of transportation to them were riverboats or small airplanes. For this reason, the epidemiologic pattern of leprosy in this region was similar to that in the Pacific islands, where similar case-detection rates have been noted.

The first highway (length 2,039 km) to cross the Amazon rainforest was completed in 1974. Today, there are 25,900 km of federal highways in the Amazon region of Brazil (*10*). Development projects and small-scale strip mining, mostly for gold, resulted in an increase in the population (*11*). Although agricultural development led to relatively stable settlements in the area, small-scale mining was undertaken by an extremely mobile population. Although the population increase was largely caused by an influx of immigrants from regions of Brazil that have fewer cases of leprosy, this disease remains highly endemic in the Amazon region of Brazil and is now concentrated in the areas of greatest population increase.

Strip miners return seasonally to their homes, and persons involved in unsuccessful land settlement projects often sell their plots and move to new agrarian development projects (*12*). Families who succeeded in land cultivation periodically returned to their city of origin as a sign of success (*13*). This population movement results in leprosy cases in other regions and raises the question whether leprosy can reemerge in other parts of Brazil.

Clusters 6, 8, and 9 are located in dry savannah lands and are adjacent to the Amazon clusters. Since 1980, case-detection rate for leprosy increased in Brazil (*14*), and the

Table 2. Leprosy case-detection rates and proportion of all cases in the 10 spatial clusters, Brazil, 2005–2007

Cluster order	Case detection rate/ 100,000 inhabitants	% Cases
1	85.36	20.10
2	95.42	7.97
3	97.15	3.39
4	51.99	5.68
5	51.96	4.73
6	48.04	4.82
7	46.65	1.82
8	60.66	1.08
9	39.85	2.69
10	55.21	1.21
Brazil	21.82	100.00

northeastern region had the highest increase of any region (*4*). In addition, clusters 6 and 8 must be addressed by the leprosy control program because they overlap current agrarian development initiatives in northeastern Brazil.

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Oral Transmission of Chagas Disease by Consumption of Açaí Palm Fruit, Brazil

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In 2006, a total of 178 cases of acute Chagas disease were reported from the Amazonian state of Pará, Brazil. Eleven occurred in Barcarena and were confirmed by visualization of parasites on blood smears. Using cohort and case–control studies, we implicated oral transmission by consumption of açaí palm fruit.

Chagas disease (American trypanosomiasis) chronically infects ≈10 million persons in Latin America (*1*). The etiologic agent is *Trypanosoma cruzi*, which is transmitted by bloodsucking triatomine insects. Other modes of transmission are transfusional, congenital, and oral (foodborne) (*2*). Oral transmission occurs by consumption of foods contaminated with triatomines or their feces or by consumption of raw meat from infected mammalian sylvatic hosts (*3*). The precise stage of food handling at which contamination occurs is unknown. The first outbreak of orally transmitted Chagas disease in Brazil was reported in 1965 (*4*). Two outbreaks were associated with consumption of sugar cane juice (*5*,*6*). In these outbreaks, the incubation period was ≈22 days, compared with 4–15 days for vectorial transmission and 30–40 days for transfusional transmission (*7*).

Chagas disease has not been considered endemic in the Brazilian Amazon region. The first Amazonian outbreak of acute Chagas disease was reported in 1968; oral transmission was suspected (*8*). During 1968–2005, a total of 437 cases of acute Chagas disease were reported in this region. Of these cases, 311 were related to 62 outbreaks in which the suspected mode of transmission was consumption of açaí (*9*).

Açaí is the fruit of a palm of the family *Aracaceae* (Figure 1, panel A); it is crushed to produce a paste or beverage. Most of the Amazonian population consumes açaí juice daily. Contamination is believed to be caused by triatomine stools on the fruit or insects inadvertently crushed during processing (*10*). There are no reports of collection of açaí for laboratory testing during an outbreak of acute Chagas disease. Because outbreaks with high attack rates occur in small groups whose members all consume the same foods, açaí has not been epidemiologically implicated in transmission of this disease.

During January–November 2006, a total of 178 cases of acute Chagas disease were reported in Pará State, Brazil, in the Amazon basin (Ministry of Health, unpub. data). Eleven of these cases occurred in Barcarena (population 63,268) (*11*) (Figure 1, panel B). All patients had symptom onset in September and October. Of the 11 case-patients, 5 were staff members at a health post who shared a meal at a staff meeting on September 15. We attempted to identify risk factors for illness.

The Study

We conducted a retrospective cohort study of staff members at the health post who participated in the meeting on September 15. A case-patient was any person who participated in the meeting and had a positive direct parasitologic examination for *T. cruzi* or positive serologic results and clinical evidence of acute Chagas disease. A noncase was any person who participated in the meeting and had negative test results for *T. cruzi*. We also conducted a 1:3 case–control study (11 case-patients and 34 controls matched by sex and age) that included patients with laboratory confirmed cases from Barcarena. A case-patient was any person in whom during September 1–October 15 *T. cruzi* was found by direct parasitologic examination, irrespective of signs or symptoms of disease, or who had positive serologic results and clinical evidence of disease. This interval was based on date of symptom onset of the first and last case-patient and a reported incubation period of 3–22 days for orally transmitted disease. Controls were age- and sex-matched residents of case-patient neighborhoods who had negative serologic results for *T. cruzi*.

Parasitologic examinations were conducted for case-patients by using quantitative buffy coat test, thick blood smear, or buffy coat test (the latter 2 tests included Giemsa staining). Serologic tests were conducted by using indirect hemagglutination test, ELISA, or indirect immunofluorescent test. An immunoglobulin (Ig) M titer ≥40 was considered positive. Controls had nonreactive IgM and IgG titers. We ruled out leishmaniasis in all persons with positive serologic results for *T. cruzi* by using an immunofluorescent test for IgM to *Leishmania* spp. (*12*).

We conducted an entomologic investigation during December 11–16, 2006, at the homes of 5 case-patients and in forested areas near the homes of 2 case-patients; at

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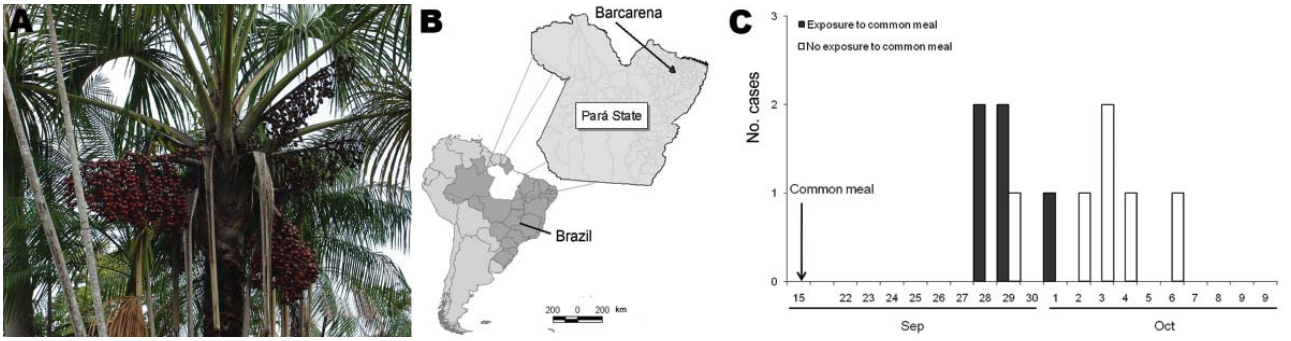

Figure 1. A) Açaí palm and açaí fruit. B) Location of Barcarena in Pará State, Brazil. C) Epidemic curve for 11 case-patients with acute Chagas disease, Barcarena, Brazil, September–October 2006.

the commercial establishment where açaí consumed by the case-patients linked to the health post was prepared and served; at an açaí juice production and sale establishment reported to be frequented by other case-patients; and at the river dock market where açaí delivered to Barcarena is unloaded. At this market, we searched baskets used to transport açaí in river boats. We applied an insect-displacing compound (piridine; Pirisa, Taquara, Brazil) to the interior and exterior of buildings at investigation sites and placed traps (*13*) to obtain triatomines.

Data were analyzed by using Epi Info version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). We measured relative risk in the cohort study and matched odds ratios in the matched case–control study, with 95% confidence intervals and $\alpha = 5\%$. Fisher exact, McNemar, Mantel-Haenszel, and Kruskall-Wallis tests were used as needed. Study power $(1 - \beta)$ was 5%.

All case-patients had positive results for *T. cruzi* by direct examination of blood (Figure 2). Nine (82%) patients were female; median age was 39 years (range 7–70 years).

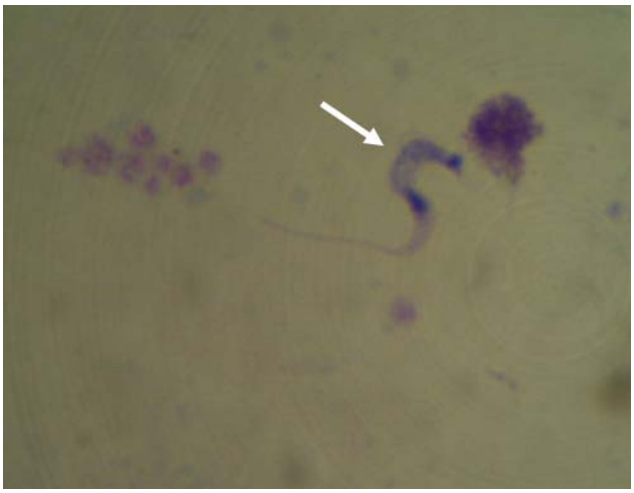

Figure 2. *Trypanosoma cruzi* (arrow) in a peripheral blood smear of a patient at a local health facility in a rural area of Pará State, Brazil (Giemsa stain, magnification ×100). Image provided by Adriana A. Oliveira, Brazilian Field Epidemiology Training Program, Brasilia, Brazil.

Eight (73%) patients resided in urban areas, 7 (64%) in brick dwellings, and 3 (27%) in mixed brick and wooden dwellings. All patients denied having had blood transfusions or organ transplants, having slept in rural or sylvatic areas, and having been bitten by triatomines.

The epidemic curve for the 11 patients is shown in Figure 1, panel C. Main signs and symptoms were fever, weakness, facial edema, myalgia, arthralgia, and peripheral edema (Table 1). No deaths occurred, and median time from symptom onset to treatment initiation was 22 days.

The cohort consisted of 12 persons who attended the staff meeting. Of these persons, 6 shared a meal, 5 (83%) of whom were case-patients. The remaining persons were seronegative for *T. cruzi*. Exposures associated with infection were consumption of thick açaí paste and drinking açaí juice at the health post; consumption of chilled açaí was protective (Table 2). This shared meal was the only common exposure among cohort members. No other foods consumed at the meal were associated with illness (Table 2). Among exposures tested, drinking açaí juice on September 15 and at the health post were significantly associated with illness ($p<0.02$ and $p<0.001$, respectively; matched odds ratio not determined). Other exposures were not associated with illness. No triatomine insects were identified at any sites of the entomologic investigation.

Table 1. Signs and symptoms in 11 patients with laboratory-confirmed acute Chagas disease, Barcarena, Brazil, 2006

Sign or symptom	No. (%) patients
Fever	11 (100)
Fatigue	11 (100)
Facial edema	11 (100)
Headache	10 (91)
Myalgia	9 (82)
Arthralgia	9 (82)
Peripheral edema	9 (82)
Shortness of breath	7 (64)
Tachycardia	7 (64)
Nausea/vomiting	7 (64)
Jaundice	5 (46)
Epigastric pain	5 (46)
Retroorbital pain	5 (46)

Table 2. Food exposures in a cohort study of 5 case-patients with acute Chagas disease, Barcarena, Brazil, 2006*

Exposure†	Ill, no. (%)	Not ill, no. (%)	RR	95% CI	p value‡
Açaí, thick paste	3 (100)	0	4.5	1.3–15.3	0.04
Açaí juice at health post	3 (100)	0	4.5	1.3–15.3	0.04
Chilled açaí juice	1 (12)	7 (88)	0.1	0.02–0.8	0.02
Charque	3 (75)	2 (25)	5.3	0.8–35.1	0.09
Cupuaçu	2 (100)	0	3.3	1.3–8.6	0.15
Biribá	1 (50)	1 (50)	1.3	0.3–6.1	0.68
Muruci	1 (100)	0	2.3	1.3–6.0	0.42
Any raw food	4 (67)	2 (33)	4.0	0.6–26.1	0.12

*RR, relative risk; CI, confidence interval.
†Charque is dried, salted meat; cupuaçu, biribá, and muruci are fruits.
‡By Fisher exact test.

Conclusions

Our study findings implicated açaí in an outbreak of acute Chagas disease. Oral transmission of this disease in the Amazon region has been reported since the 1960s. Açaí has long been the principal suspected food vehicle, but characteristics of outbreaks, small groups with universal exposure and high attack rates, have precluded epidemiologic implication of this food. There are no reports of timely collection of açaí for laboratory testing in an outbreak.

In this outbreak, vectorborne, transfusional, transplant-associated, and transplacental transmission were excluded. Incubation periods of cohort case-patients were compatible with those of previous reports. A shared meal was the only event linking case-patients, and cohort and case–control studies demonstrated an association between açaí consumption at this meal and infection. These findings indicate an outbreak of orally transmitted disease from contaminated açaí.

Limitations of this study are possible recall bias caused by delay between illness and investigation and failure to collect food samples for testing. Studies are needed to determine viability of *T. cruzi* in açaí, along with the tree-to-bowl continuum of açaí, to identify sources of contamination. Because açaí is a major dietary component in the Amazon region and a component of the local economy, identifying practical prevention measures is essential.

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Severe Acquired Toxoplasmosis Caused by Wild Cycle of *Toxoplasma gondii*, French Guiana

Bernard Carme, Magalie Demar, Daniel Ajzenberg, and Marie Laure Dardé

From 1998 through 2006, 44 cases of severe primary toxoplasmosis were observed in French Guiana in immunocompetent adults. *Toxoplasma gondii* isolates exhibited an atypical multilocus genotype. Severe disease in humans may result from poor host adaptation to neotropical zoonotic strains of *T. gondii* circulating in a forest-based cycle.

French Guiana is a French territory in South America; the Amazon rain forest covers 92% of the territory. Toxoplasmosis is a cosmopolitan parasitic disease. It is usually benign in patients without immunosuppression, but in French Guiana, it is a major public health problem, mainly because of the high prevalence of AIDS and because of an emerging severe form of acquired toxoplasmosis in immunocompetent patients.

The Cases

Until 1998, only 2 cases of severe primary toxoplasmosis had been reported in French Guiana, in 1992 (*1*) and 1997 (*2*). However, from 1998 through 2006, 44 cases occurred (*3*–*5*). All patients were immunocompetent (not HIV-infected) adults who had been hospitalized because of a marked, nonspecific, infectious syndrome. All patients had elevated and prolonged fever; most had weight loss, hepatic cytolysis, lymphadenopathy, headache, and pneumopathy. Other signs such as rash, retinochoroiditis, myocarditis, myositis, and neurologic disorders, may occur with toxoplasmosis, albeit infrequently. Prognosis is linked to lung involvement, which typically occurs 10–15 days after onset of fever and requires hospitalization. Approximately one third of patients with this severe form of disease experience respiratory distress and need to be in an intensive care unit. Unless specific treatment (sulfadiazine and pyrimethamine) is initiated quickly, death may occur.

In these patients, acute toxoplasmosis was diagnosed by serologic tests, which suggested recent primary infection (substantial and rapid increase in immunoglobulin [Ig] G, associated with specific IgM, in 2 separate samples taken 1–2 weeks apart tested at the same time); by blood or bronchoalveolar lavage samples positive by PCR; and by absence of an alternative cause. *Toxoplasma gondii* strains, virulent in mice, were isolated in some cases. Microsatellite analysis performed on isolated strains or on toxoplasma DNA extracts showed that all isolates exhibited an atypical multilocus genotype, in contrast with strains usually described in Europe or North America. Most patients reported forest-related activities such as ingestion of surface water, consumption of undercooked game meat, and hunting. Of the 44 patients, 1 died; the others recovered after standard treatment.

We recently described an outbreak of toxoplasmosis from late December 2003 through mid January 2004 involving 11 cases among the 38 inhabitants of a village in Suriname near the French Guiana border (*6*). Using 8 microsatellite markers with a high power of discrimination, we described a unique multilocus genotype for 5 patients and demonstrated that only 1 strain was responsible for this outbreak (in at least 5 of 11 patients). However, the same strain was responsible for different clinical outcomes in each of the 11 patients: 2 cases were congenital and lethal, 9 cases occurred in immunocompetent adults (5 patients, 1 of whom died, had disseminated toxoplasmosis and needed hospitalization; and 4 had less severe disease with no life-threatening signs or need for hospitalization). Genetic susceptibility of the host to this unusually severe form of toxoplasmosis may explain in part the severity of symptoms, although we observed this disease in the different ethnic groups of French Guiana (Caucasians from inland France, Creoles, Amerindians, Bushinengé, and Brazilians).

Conclusions

Such a severe outcome in humans may be explained by poor host adaptation to emerging and highly virulent strains of *T. gondii* circulating in a forest-based cycle involving wild felids (definitive hosts) and their prey (intermediate hosts) (Figure). The high seroprevalence for noncarnivorous wild mammals in French Guiana, especially terrestrial animals such as deer, armadillos, pacas, and peccaries (*7*), suggests oral exposure to oocysts eliminated by wild felids in the absence of domestic cats (*8*). Wild felids are still numerous in French Guiana. Isolation of 1 genetically atypical *Toxoplasma* strain in a free-living jaguar (*Panthera onca*) is a further argument for the existence of a *T. gondii* wildlife cycle (*9*). Sources of contamination are uncooked

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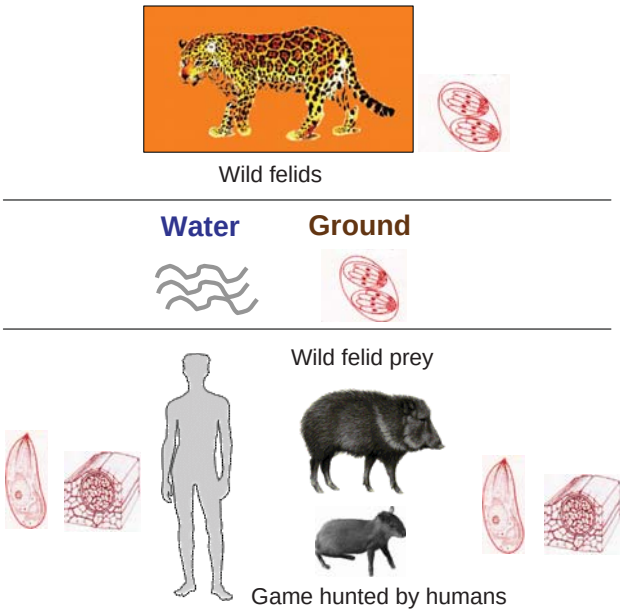

Figure. Transmission cycle of highly virulent strains of *Toxoplasma gondii* involving wild felids (definitive hosts) and their prey (intermediate hosts).

meat from hunted game and river water containing oocysts excreted by wild cats.

In North America and in Western Europe, *T. gondii* has been considered to have a clonal population structure and low genetic diversity; >95% of strains belonged to 3 clonal lineages, types I, II (mainly), and III (*10*). The greater genetic diversity of *T. gondii* in a wild and tropical environment, compared with the domestic environment, could be connected to the considerably higher diversity of hosts in the wild. In the neotropical rain forest, fauna are abundant and highly diverse; 560 species of mammals have been recorded (*11*). A plurality of alleles is needed for *T. gondii* to colonize a maximum of ecological niches in such a diverse environment.

Because little molecular assessment has been performed for *T. gondii* from asymptomatic infected persons in French Guiana, it is difficult to know what proportion of persons infected with the atypical strains found there have severe disease. In French Guiana, a wild cycle of *T. gondii* coexists with a domestic cycle, in which cats take a central role. Both cycles, wild and domestic, can even merge with the anthropization of the Amazonian forest. For the domestic cycle, there are 11 DNA samples (6 urban isolates from animals raised in captivity [3 cats and 3 monkeys] and 5 human isolates from patients with classical disease [2 AIDS patients with cerebral toxoplasmosis, 2 patients with congenital infections, and 1 patient with lymphadenopathy]). These samples have less allelic diversity than wild isolates.

Recent studies that used multiple markers and collected samples from other countries have shown that, at least in the tropical part of South America, *T. gondii* strains have higher genetic diversity (*12*,*13*). Few data concerning the genetic diversity and population structure of this parasite are available in Africa (*14*) and Asia (*15*). The number of cases of this severe form of primary *T. gondii* infection (>50 well-documented observations in the past decade) is particularly high in French Guiana, given the small size of the population (≈200,000 inhabitants), compared with the low number of published cases.

Although such a wild *T. gondii* cycle with severe human clinical consequences has not been described in Brazil or in other countries of the Guyana Shield, it seems unlikely that it is a disease specific to French Guiana. Amazonian areas, and likely other rain forest areas where felids live, could be affected by this form of toxoplasmosis, which could be designated "Amazonian toxoplasmosis" or "wild rain forest toxoplasmosis."

From a healthcare perspective worldwide, physicians should consider a diagnosis of acute toxoplasmosis as soon as possible after the onset of signs and symptoms in patients who live in or have recently visited the Amazonian region and who have a severe infectious syndrome with visceral, especially lung, involvement. Serologic tests should be promptly submitted for such patients. If recent infection with *T. gondii* is diagnosed, a potent antitoxoplasmosis treatment (sulfadiazine plus pyrimethamine) must be prescribed without delay.

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Links between Climate, Malaria, and Wetlands in the Amazon Basin

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Climate changes are altering patterns of temperature and precipitation, potentially affecting regions of malaria transmission. We show that areas of the Amazon Basin with few wetlands show a variable relationship between precipitation and malaria, while areas with extensive wetlands show a negative relationship with malaria incidence.

Global models of malaria can be used to forecast the impact of climate change on malaria, a highly climate-sensitive disease that causes >1 million deaths worldwide each year, mostly in children. However, a limitation of these models is the application of a uniform malaria–precipitation relationship to geographically diverse regions (*1*–*3*). Moreover, the Millennium Ecosystem Assessment has recognized a lack of knowledge about climate-sensitive diseases such as malaria and has called for a "more systematic inventory, by region and country, of current and likely population health impacts of ecosystem change" (*4*). Understanding malaria–precipitation relationships at regional levels will enhance predictability of ecosystem or climate change impact on population health.

Precipitation and surface hydrology are key factors in determining the abundance of *Anopheles* mosquito vectors for malaria. Mosquitoes require pools of water to complete their life cycle, and malaria models have estimated changing transmission by setting minimum levels of precipitation below which mosquito populations are (theoretically) suppressed. However, using a uniform hydrologic threshold for malaria does not capture critical characteristics of landscape, soil, and rainfall (i.e., intensity, frequency), all known contributors to the abundance, persistence, and spatial distribution of mosquito habitats.

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In the Amazon Basin, the predominant malaria vector is *Anopheles darlingi*. Short longitudinal studies show that human-landing catches of *An. darlingi*, which breeds along the edges and in debris of clear, partially sunlit pools, are closely associated with local malaria rates (*5*,*6*). These observations establish that biting rates are elevated in regions of elevated malaria risk. Likewise, biting rates correlate with abundance of larvae and larval habitats and proximity of humans to larval habitats (*7*,*8*).

Local observations demonstrate the existence of different seasonal patterns for malaria. In a 3-year study in Roraima, 8 municipalities showed increased risk for malaria during the middle of the dry season or shortly after the wet season (*9*). Other literature on seasonal patterns is limited to local and short (<3 years) longitudinal studies that lack statistical analysis. Although different seasonal patterns emerge in graphs, the collage of different data sources makes formulating a cohesive picture of these patterns in the Amazon region difficult.

At the regional level, interannual climatologic cycles provide insight into low-frequency malaria patterns. In Columbia, El Niño events (caused by warming sea surface temperatures in the central tropical Pacific) are associated with warmer temperatures, higher dew points, and less precipitation and river discharge. These climate changes have been associated with increases in malaria during the second half of El Niño years and during the following year (*10*). Similarly, malaria incidence has increased during the year after an El Niño event in Venezuela and Guyana (*11*).

Using monthly reports of malaria and precipitation from across the Brazilian Amazon Basin, we demonstrate that malaria incidence and precipitation patterns vary throughout this large region and are influenced by the extent of wetlands.

The Study

We used monthly reports of slide-confirmed malaria and annual census–based population data from 434 counties (municípios) in the Brazilian Amazon region for 1996–1999, during which no coordinated national malaria interventions occurred (*12*). To study the relationship of reported malaria cases to climate, we used monthly precipitation and temperature from the CRU TS 2.1 gridded climate data set for selected states (*13*) (Figure 1). To consider how the precipitation–malaria relationship depends on surface water conditions, including the extent of open water and wetlands, we used 100 m × 100 m maps from the JERS-1 Synthetic Aperture Radar satellite and calculated the percentage of maximum inundatable open water and wetland coverage for each county (Figure 2, panel A) (*14*). In this region, monthly temperatures were between 24.6°C and 29.4°C (well within the range for optimal malaria transmission) for 95% of the observations (18,416 of

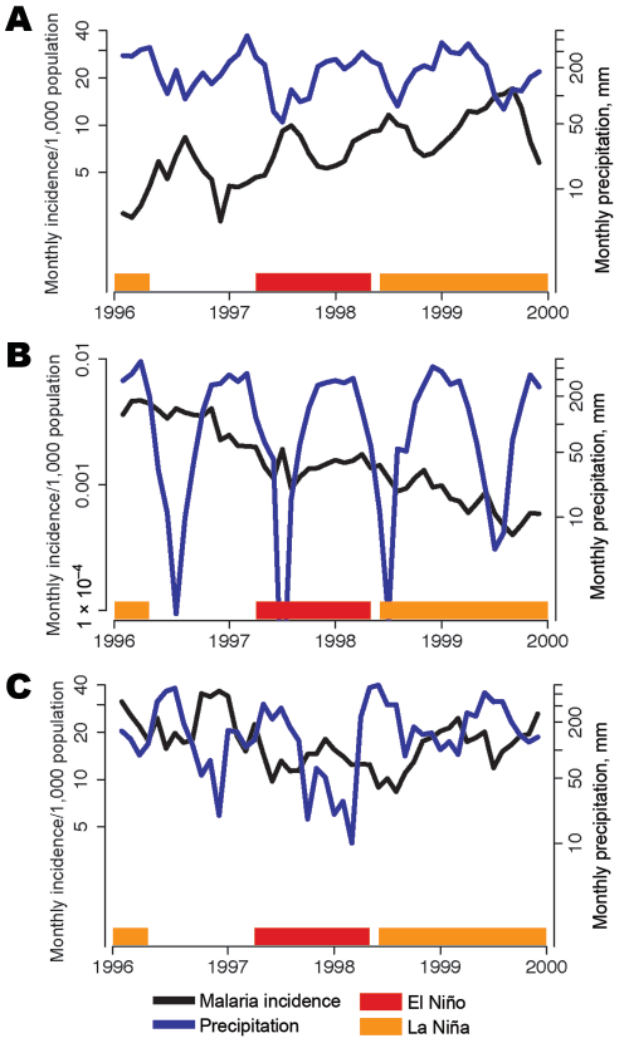

Figure 1. Malaria incidence per 1,000 population (black lines) and mean monthly precipitation (blue lines) during La Niña (orange bars) and El Niño (red bars) events for the states of A) Amazonas, B) Mato Grosso, and C) Roraima.

19,364) included in the analysis (temperature relationships not shown).

Evaluation of seasonal patterns requires comparability of the models across regions. If the lag and the rainfall coefficient vary across regions, meaningful geographic comparisons would be difficult to achieve because neither the lag nor coefficient have consistent meanings across models. To interpret results, either the coefficient must be fixed and the lags varied (difficult to do) or the lags must be fixed and the coefficients varied (relatively easy to do). The aim is to describe the variable patterns of malaria incidence and precipitation, not create a highly predictive model. We chose to fix the lag and vary the coefficients.

To assess the association between malaria incidence and precipitation data, we estimated the rate ratio of malaria incidence associated with 1 SD-increase in monthly precipitation (≈14 cm) for each county by using the following Poisson regression model, which includes a flexible temporal trend represented as a natural cubic spline with 6 degrees of freedom (Figure 2, panel B):

$$\text{malaria}_{it} \approx \text{Poisson}(\mu_{it})$$
$$\log \mu_{it} = \log \text{pop}_{it} + \alpha_i + \beta_i \text{precip}_{it} + f_i(t)$$
$$\beta_i \approx \text{Normal}(g(\text{lat}_i, \text{lon}_i), \sigma^2)$$

The (estimated) regression coefficients from the county-specific models were then modeled as a spatially smooth surface, a thin-plate spline. Degrees of freedom for the thin-plate spline were selected using generalized cross-validation (Figure 2, panel C).

The relationships between precipitation and malaria incidence in the Amazon Basin are spatially varied and change signs, depending on the region. Positive correlations between monthly precipitation and malaria incidence (rate ratios >1) occur in the upland regions of the southwest and central Amazon Basin, whereas negative correlations between precipitation and incidence (rate ratios <1) occur in the north, largely along the main waterways of the Amazon River and the major wetland regions of the Basin (Figure 2). For a ≈14-cm increase in monthly rainfall, the malaria rate can double in the upland area, yet decrease by up to 80% along the main Amazon channel. The p values of the precipitation coefficient are 0.0002–0.0009 along the main waterways and 0.004–0.10 in uplands areas.

We hypothesize that this reversal of the malaria–precipitation relationship from positive to negative is related to the extent of open water and wetlands in the Basin. Mosquito habitats in wetlands or along large rivers may wash out or become too deep during months with high precipitation, but in areas with fewer wetlands, mosquito habitats are limited by precipitation.

To test this hypothesis, we compared the malaria–precipitation association for 338 counties that reported ≥80 cases of malaria over the 48 months against the estimated percentage of open water and wetland cover for each county (Figure 2, panel D). As expected, the precipitation-linked risk for malaria fell as the percentage of wetland in each county increased, but the risk for malaria varied in counties with low percentages of wetlands. The central-east region had the lowest level of malaria incidence, which may explain why this region also lacked a malaria-precipitation relationship.

Conclusions

Explanations similar to our wetlands hypothesis have been reported. Studies have proposed that flooding created

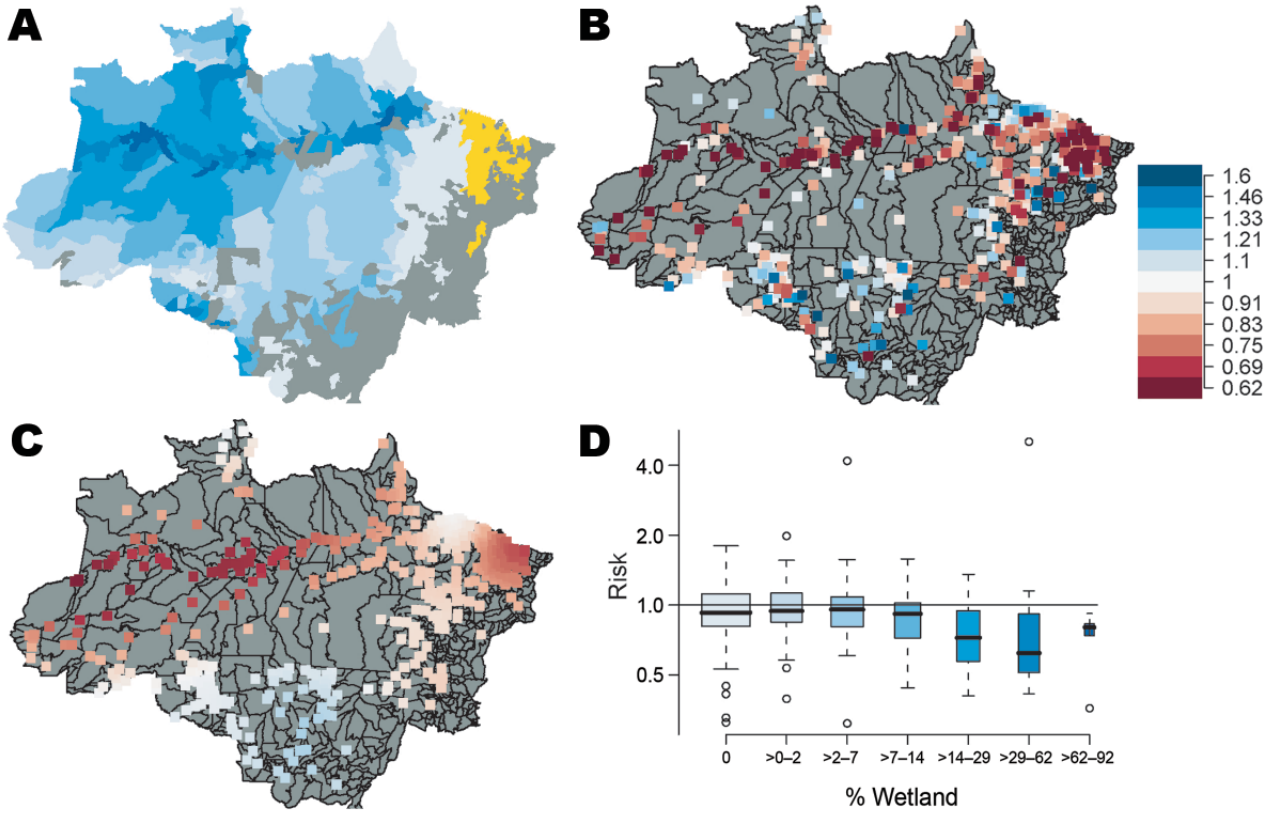

Figure 2. Connection of malaria incidence and precipitation risk ratios to wetlands. A) Percentage of wetlands in Amazon Basin counties (shades of blue), counties without wetlands data (orange), and counties with <80 total malaria cases (gray). Wetland colors correspond to percentage wetland values in panel D. B) Risk ratios for malaria incidence for 1 SD (≈14 cm) change in monthly precipitation (January 1996–December 1999), plotted at each county seat of government; C) spatially smoothed risk ratios for ≈14-cm changes in monthly precipitation. In both panels, red shaded squares show reduced risk for ≈14-cm increase in monthly precipitation; blue shaded squares show increased risk for malaria with increased precipitation. D) Boxplot of risk ratios for malaria incidence for ≈14-cm changes in monthly precipitation, by percentage wetland cover. Box width is proportional to the number of counties in each box. Error bars indicate interquartile ranges, and thick horizontal bars indicate the median.

new pools of water suitable for mosquito larvae as the water levels slowly receded from alluvial forests along the Rio Branco River in Roraima and the Maroni River on the frontiers of Suriname and French Guiana (*6*,*15*). Our results suggest that monthly precipitation along the Amazon Basin can have both strong positive and negative associations with malaria incidence.

Further research is needed to address the limitations of our study, including its short time frame and the crude countywide approximation of percentage wetlands as an exposure. The quality and reliability of the health data were concerns, but we verified that the distribution of null reporting was unbiased temporally and spatially. Also, our study did not quantify increasing malaria incidence in response to increasing or decreasing precipitation or the impact of lag factors. Instead, we focused on the seasonality of these patterns until longer data series of malaria incidence and climate data are available.

Our evidence suggests that precipitation drives malaria risk in the Amazon Basin, but the relationship varies in the uplands (more precipitation, more/less malaria) and is negative in areas dominated by wetlands and large rivers (more precipitation, less malaria). Our findings show the need to account for local landscape characteristics, especially the extent of wetlands and open water, in regional to global projections of the effects of climate change on malaria. Better understanding the impact of climate and landscape on malaria will improve our ability to assess health risks.

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etymologia

Kaposi **[kah′po-she, kap′o-sē]** ***sarcoma***

First described by dermatologist Moritz Kaposi (1837–1902) at the University of Vienna in 1872. Dr Kaposi's last name was originally Kohn, but to distinguish himself from other physicians of the same name, he chose a new name in honor of the Kapos River, near his birthplace, Kaposvár, Hungary. The condition he described, Kaposi sarcoma, is a malignant tumor of the lymphatic endothelium, characterized by bluish-red cutaneous nodules. Human herpesvirus 8 has been implicated in its etiology.

Source: Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders; 2007; http://www.hemoctoday.com/article.aspx?rid=31545

Seroprevalence of Kaposi Sarcoma–associated Herpesvirus and Other Serologic Markers in the Brazilian Amazon

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To determine the presence of Kaposi sarcoma–associated herpesvirus (KSHV) and other serologic markers, we tested serum specimens of 339 Amerindians, 181 rural non-Amerindians, and 1,133 urban blood donors (13 Amerindians) in the Brazilian Amazon. High KSHV seroprevalence in children and inverse association with herpes simplex virus type 2 indicates predominant nonsexual transmission among Amerindians.

Kaposi sarcoma–associated herpesvirus (KSHV) is the cause of Kaposi sarcoma (KS) and certain lymphoproliferative diseases (*1*). KSHV seroprevalence is low (<5%) in most Western populations (*1*) and reaches 50% in some African populations (*2*), mirroring KS incidence rates (*3*). However, the highest KSHV seroprevalences worldwide (>80% in adults) have been reported in Amerindian tribes from the Amazon regions of Brazil (*4*,*5*) and Ecuador (*6*), despite the apparently low KS incidence in these populations (*7*). KSHV is thought to be transmitted through saliva between young siblings in disease-endemic areas such as French Guiana (*8*) or Africa (*9*), whereas sexual transmission in low-prevalence countries occurs within risk groups such as men who have sex with men (*10*). Modes of transmission have not been clearly determined in Amerindian populations.

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The Study

We conducted a cross-sectional study during February 2003–April 2004 to investigate the seroprevalence and factors associated with KSHV infection in Amerindian and non-Amerindian populations living in 2 regions of the Brazilian Amazon: a remote rural region of Para State (Mapuera, on the banks of the Trombetas River) and Manaus, the capital city of Amazonas State (Figure). Serologic markers of fecal–oral (hepatitis A virus [HAV]), blood-borne (hepatitis B and C viruses [HBV, HCV]) and sexually transmitted infections (*Treponema pallidum* [syphilis] and herpes simplex virus type 2 [HSV-2]) were used as proxies to identify possible routes of KSHV transmission in these populations.

A convenience sample of unselected Amerindians and non-Amerindians living in the Mapuera area and a consecutive sample of nonpaid first-time blood donors from the Manaus blood bank (HemoAm) consented to collection of blood samples, as previously reported (*4*,*11*) Ethical approval was obtained from the institutional review board of HemoAm, the ethical board of the Brazilian Ministry of Health, and the ethics committee of the London School of Hygiene and Tropical Medicine.

In the absence of a definitive test to determine KSHV infection, all serum specimens were tested by using a previously validated in-house whole-virus KSHV ELISA (*12*) and 2 immunofluorescence assays (IFAs) that detected antibodies against lytic (IFA-lytic) and latent-associated nuclear antigens (IFA-LANA) (*12*). KSHV infection was defined as positivity by any of these serologic assays. Serum specimens were also tested for the agent of syphilis by us-

Figure. Map of Brazil showing study area (black box) in Amazonas (Manaus) and Para (Mapeura region) States. Printed with permission of the Instituto Brasileiro de Geografia e Estatística.

ing a *T. pallidum*–specific assay (Enzygnost Syphilis; Dade Behring, Marburg, Germany); for HSV-2 antibodies by using the type-specific HerpeSelect gG2 ELISA (Focus Technologies, Cypress Hill, CA, USA), with a higher cut-off (>3.5) to increase specificity (*13*); and for HAV antibodies by using BioELISA HAV (Biokit, Barcelona, Spain). Presence of HBV anti-core antibodies was determined by using Ortho HBc ELISA (Ortho Diagnostics, Raritan, NJ, USA) in Mapuera serum specimens and Hepanostika anti-HBc Uni-Form (Organon-Teknika, Boxtel, the Netherlands) in Manaus serum specimens. HCV antibodies were detected by using Ortho HCV 3.0 ELISA (Ortho Diagnostics) in Mapuera serum specimens and Murex Anti-HCV version 4.0 ELISA (Murex Biotech S.A., Kyalami, South Africa) in Manaus serum specimens.

KSHV seroprevalence was calculated separately for men and women and directly age-standardized to the Mapuera Amerindian population. The risk associated with KSHV infection was estimated with prevalence ratios (PRs) and 95% confidence intervals (CIs), adjusted for sex and age group (18–24 years, 25–34 years, and ≥35 years for the blood donor population; 0–9 years, 10–17 years, 18–24 years, 25–34 years, and ≥35 years for both Mapuera populations). The associations of KSHV with sociodemographic variables, indicators of socioeconomic status, and other serologic markers were estimated with odds ratios (ORs) and 95% CIs. Variables associated with a significant increased risk for KSHV ($p<0.05$) in univariable analysis were included in a multivariable logistic regression model adjusted for age and sex.

We recruited 339 Amerindians (median age 22 years, interquartile range [IQR] 13–37 years; 57.5% female) and 181 non-Amerindians (median age 17 years, IQR 9–35 years; 58.6% female) in the Mapuera communities and 1,133 blood donors (median age 25 years, IQR 21–32 years; 22.9% female) in Manaus. The blood donor population had a similar age distribution to that of the adult population in Manaus in the 2000 regional census (*14*).

Among Mapuera Amerindians, KSHV seroprevalence was 65.0% in those 0–9 years, increasing to 92.9% in those ≥35 years. In contrast, among Mapuera non-Amerindians, KSHV seroprevalence was 9.8% in those 0–9 years of age, increasing to 50.0% in those ≥35 years of age. Among blood donors, KSHV seroprevalence was 31.3% in those ≥35 years of age and 53.8% in the 13 who were of Amerindian descent. After age standardization, KSHV seroprevalence remained lower among Mapuera non-Amerindians (30% and 27% among men and women, respectively) and blood donors (16% and 23%, respectively) than among Mapuera Amerindians. When results were compared with those of the Mapuera Amerindians, the age-and sex-adjusted PRs were 0.35 (95% CI 0.28–0.45) and 0.59 (95% CI 0.56–0.63) in Mapuera non-Amerindians and blood donors, respectively.

In each population, KSHV seroprevalence was slightly higher among females, and increased with age (p for trend <0.001) in Mapuera Amerindians and non-Amerindians, but not among (adult) blood donors (Table 1). KSHV seroprevalence varied little with house crowding (socioeconomic indicator), and hepatitis infections, but was associated with HSV-2 infection in non-Amerindians (OR 4.2, 95% CI 2.1–8.5) and blood donors (OR 1.3, 95% CI 1.0–1.7). In Amerindians, KSHV infection was not associated with HSV-2 in univariable analysis (OR 0.7, 95% CI 0.3–1.9).

In multivariable analysis (Table 2), KSHV infection remained associated with female sex among blood donors (age- and sex-adjusted OR [aOR] 1.3, 95% CI 1.0–1.7), and increased significantly with age in both Mapuera populations (p for trend <0.001). KSHV infection was associated with HSV-2 infection among Mapuera non-Amerindians (aOR 2.7, 95% CI 1.2–6.5) and Manaus blood donors (aOR 1.3, 95% CI 1.0–1.6), but was inversely associated with HSV-2 infection in Mapuera Amerindians (aOR 0.3, 95% CI 0.1–0.9).

Conclusions

Our data confirm the high KSHV seroprevalence observed among Amazonian Amerindian populations (*5*,*7*). However, the inclusion of convenience samples of remote populations and first-time blood donors, who may not necessarily be representative of the adult general population and notably exclude persons who report a range of potentially high-risk behavior for sexually transmitted and bloodborne infections, may have limited the generalizibility of our findings. High KSHV seroprevalence combined with an apparent lack of KS development among Amerindian populations support the theory of genetic predisposition to KSHV acquisition, as hypothesized for other Amazonian populations, in whom segregation genetic analysis has suggested that an unidentified recessive gene may influence KSHV serostatus (*15*).

The high KSHV seroprevalence (65%) among Mapuera Amerindians <10 years of age contrasts with the low (9.8%) seroprevalence among non-Amerindians of the same age group living in the same area, which suggests different transmission modes in these neighboring populations. Although we did not collect data on the age of initial sexual experience in either population, the high prevalence in childhood and inverse association with HSV-2 supports nonsexual transmission of KSHV in Amerindians. Conversely, the association of KSHV infection with HSV-2 among Mapuera non-Amerindians and blood donors supports a role for sexual transmission in these groups, although saliva transmission in younger urban inhabitants cannot be ruled out. Universal HAV infection status and low rates of HBV and HCV in all populations precluded any meaningful analysis of transmission routes associated with hepatitis viruses.

In summary, this study contributes data on the epidemiology of KSHV infection and transmission in some Brazilian Amazonian populations. Irrespective of urban or rural setting, our data are consistent with a predominant nonsexual transmission of KSHV (most likely through saliva) in Amerindian tribes compared with a probable combination of sexual and nonsexual modes of transmission among non-Amerindian populations living in the same region.

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Table 1. Seroprevalence of KSHV among 3 populations in the Brazilian Amazon*†

	Mapuera Amerindians, n = 339†		Mapuera non-Amerindians, n = 181†		Manaus blood donors, n = 1,133†	
Variables	% KSHV positive (no. tested)	OR (95% CI)	% KSHV positive (no. tested)	OR (95% CI)	% KSHV positive (no. tested)	OR (95% CI)
Sex						
Male	79.2 (144)	1	26.7 (75)	1	28.6 (874)	1
Female	82.6 (195)	1.2 (0.7–2.1)	27.4 (106)	1.0 (0.5–2.0)	34.4 (259)	1.3 (1.0–1.7)
p value		0.4		0.1		0.08
Age group, y						
0–9	65.0 (43)	0.1 (0.05–0.4)	9.8 (51)	0.1 (0.03–0.3)	–	–
10–17	70.0 (93)	0.2 (0.07–0.4)	22.5 (40)	0.3 (0.1–0.7)	–	–
18–34	86.5 (104)	0.5 (0.2–1.3)	27.3 (44)	0.4 (0.1–0.9)	29.6 (916)	0.9 (0.7–1.3)
≥35	92.9 (99)	1	50.0 (46)	1	31.3 (217)	1
p for trend		<0.001		<0.001		
Crowding‡						
1–2	93.7 (16)	1	55.6 (9)	1	32.6 (175)	1
3	91.3 (23)	0.7 (0.06–8.4)	33.3 (15)	0.4 (0.07–2.2)	29.9 (941)	0.9 (0.6–1.2)
≥4	79.7 (300)	0.3 (0.03–2.0)	24.8 (145)	0.3 (0.07–1.3)	6.2 (16)	0.4 (0.2–1.0)
p value		0.1		0.1		0.1
Ethnicity						
African	–	–	–	–	29.6 (743)	1
Caucasian	–	–	–	–	30.5 (308)	1.0 (0.8–1.4)
Indigenous	100 (339)	–	–	–	53.8 (13	2.8 (0.9–8.3)
Other	–	–	100 (181)	–	25.8 (66)	0.8 (0. 5–1.5)
p value						0.08
Hepatitis A virus						
Negative	83.3 (6)	1	12.5 (16)	1	42.9 (7)§	1
Positive	81.1 (333)	0.9 (0.1–7.5)	28.5 (165)	2.8 (0.6–12.7)	28.6 (154)§	0.5 (0.1–2.5)
p value		0.9		0.2		0.4
Hepatitis B virus						
Negative	81.6 (315)	1	32.0 (75)§	1	30.2 (1,075)	1
Positive	73.9 (23)	0.6 (0.2–1.7)	53.3 (15)§	2.4 (0.8–7.5)	25.0 (56)	0.8 (0.4–1.4)
p value		0.4		0.1		0.4
Hepatitis C virus						
Negative	81.0 (338)		36.0 (90)†		29.9 (1,129)	1
Positive	0		0		25.0 (4)	0.8 (0.1–7.5)
p value						0.8
HSV-2						
Negative	81.5 (314)	1	18.1 (127)	1	27.8 (715)	1
Positive	76.0 (25)	0.7 (0.3–1.9)	48.1 (54)	4.2 (2.1–8.5)	33.2 (406)	1.3 (1.0–1.7)
p value		0.5		<0.001		0.06
Trepomena pallidum			26.3 (171)			
Negative	81.0 (338)	–	40.0 (10)	1	29.9 (1,122)	1
Positive	0			1.9 (0.5–6.9)	36.4 (11)	1.2 (0.6–2.3)
p value				0.3		0.7

*Seroreactivity by any serologic assay, whole virus. KSHV, Kaposi sarcoma–associated herpesvirus; OR, odds ratio; CI, confidence interval; HSV-2, herpes simplex virus type 2.
†Some figures do not add up to the total because of missing data.
‡Number of residents living in the house.
§Only a random subsample tested.

Table 2. Multivariable analysis of risk factors for KSHV infection among 3 populations in the Brazilian Amazon*

Variables	aOR (95% CI)		
	Mapuera Amerindians, n = 339	Mapuera non-Amerindians, n = 181	Manaus blood donors, n = 1,133
Sex			
Male	1	1	1
Female	1.2 (0.7–2.2)	1.0 (0.5–2.1)	1.3 (1.0–1.7)
p value	0.5	0.9	0.08
Age group, y			
0–9	0.1 (0.05–0.4)	0.1 (0.04–0.3)	
10–17	0.2 (0.07–0.4)	0.3 (0.1–0.7)	
18–34	0.5 (0.2–1.2)	0.4 (0.1–0.9)	0.9 (0.7–1.3)
≥35	1	1	1
p value	<0.001	<0.001	0.6
HSV-2			
Negative	1	1	1
Positive	0.3 (0.1–0.9)	2.7 (1.2–6.5)	1.3 (1.0–1.6)
p value	0.03	0.02	0.09

*Seroreactivity by any serologic assay (whole virus ELISA, IFA-LANA, or IFA-lytic) in multivariable analysis. KSHV, Kaposi sarcoma–associated herpesvirus; IFA-LANA, immunofluorescence assay that detected latent-associated nuclear antigens; IFA-lytic, IFA that detected lytic-associated nuclear antigens; aOR, age- and sex-adjusted odds ratio; CI, confidence interval; HSV-2, herpes simplex virus type-2.

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Concurrent Dengue and Malaria in Cayenne Hospital, French Guiana

Bernard Carme, Severine Matheus, Gerd Donutil, Olivia Raulin, Mathieu Nacher, and Jacques Morvan

Dengue–malaria co-infection reports are scarce. Of 1,723 consecutive febrile patients in Cayenne Hospital, 238 had dengue (174 early dengue fever cases) and 393 had malaria (371 acute malaria); 17 had both. Diagnosis of 1 of these 2 infections should not rule out testing for the other infection.

Despite a wide overlap between malaria- and dengue-endemic areas, published reports of co-infections are scarce in the literature. The first 2 patients with concurrent malaria (*Plasmodium falciparum*) and dengue were identified in July 2005 (*1*) and November 2006 (*P. vivax*) (*2*). Since then, a few publications described proven or suspected associations, but always as isolated cases (*3*–*6*).

In French Guiana, a French territory in South America that is 92% covered by Amazon rain forest, malaria and dengue fever represent 2 major public health concerns. The annual number of *P. falciparum* and *P. vivax* malaria cases ranges from 3,500 to 4,500. In addition, all 4 dengue virus serotypes have been isolated in the country (*7*). To determine the frequency of concurrent infection with dengue and malaria in French Guiana, we conducted a 1-year study of patients evaluated in the emergency department of Cayenne Hospital.

The Study

We carried out a retrospective study by testing blood and serum samples on 1,740 patients who consulted the emergency department of Cayenne Hospital seeking treatment for fever compatible with malaria and/or dengue during a 1-year period, July 2004–June 2005 (Figure). Diagnosis of malaria has always been quick; dengue diagnosis was initially conducted only in malaria-negative patients. In our study, dengue investigations were conducted retrospectively at the Pasteur Institute of French Guiana for 99% of patients (1,723/1,740) by using serum samples obtained at admission and frozen at –80°C. Medical records of case-patients with dengue–malaria co-infections were consulted retrospectively to look for severe or abnormal features.

Malaria diagnosis was based on the identification of hematozoa on a thin blood film and/or on a thick blood film stained with Giemsa. The screening sensitivity was ≈6 plasmodia/μL (1/1,000 leukocytes). The asexual parasite load (PL) was quantified in percent parasitized erythrocytes for values ≥0.1 %. For lower values, classes were created using thick smears: class 1, <0.00125% but positive; class 2, ≥0.00125% but <0.0125%; and class 3, ≥0.0125% but <0.125%. Asymptomatic *Plasmodium* spp. carriage was considered for classes 1 and 2 (in the absence of prior antimalarial treatment and for case-patients residing >1 year in an area of malaria transmission). Virus isolation or reverse transcription–PCR (RT-PCR) according to Lanciotti et al. (*8*) was performed on all serum samples obtained during the acute phase of infection, between day 0 and day 4 (n = 264). For malaria-positive samples, virus isolation was conducted on all samples without a date of onset of disease (n = 163).

Serologic immunoglobulin (Ig) M testing was performed on all serum samples (n = 1,723). Dengue was detected in 238 case-patients (13.8%); among these, 73% (174/238) were confirmed by positive virologic diagnosis (isolation or RT-PCR), whereas 27% were probable dengue cases (positive IgM serology only). The first group was named early dengue cases (EDC) and the second group late dengue cases (LDC).

Of the 1,723 patients, 393 (22.8%) had smear-positive malaria; of those, 251 (63.9%) were *P. vivax,* 133 (33.8%)

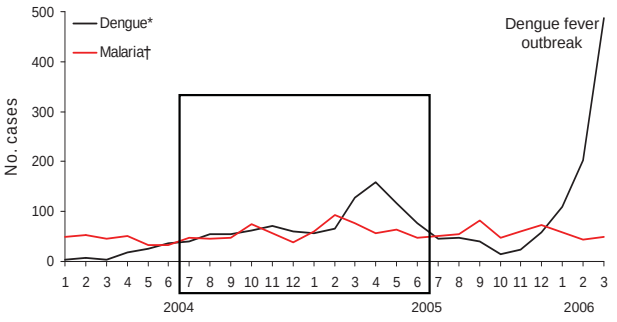

Figure. Comparison of confirmed cases of dengue fever and of symptomatic malaria in patients examined at the emergency department of Cayenne Hospital, Cayenne, French Guiana, January 2004–March 2006. The black frame corresponds to the period of the retrospective study (July 2004–June 2005). *Cases confirmed by positive test results from reverse transcription–PCR or virus isolation (Pasteur Institute, Cayenne). †Cases diagnosed based on recorded fever or history of fever in the previous 24 h associated with microscopic detection of asexual forms of *Plasmodium* spp. by blood smear.

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were *P. falciparum*, 2 were *P. malariae*, and 7 were mixed *P. vivax* and *P. falciparum*. Most malaria-positive case-patients had a parasite count above class 2 (371/393 [94.4 %]), indicating acute malaria.

Concurrent dengue (EDC and LDC) and malaria were confirmed in 17 of the 1,723 patients (1%), corresponding to 7.1% (17/238) of dengue cases and 4.1% (16/393) of malaria cases, respectively (Table). When considering acute malaria associated with EDC, the percentages of confirmed associations were 3.4% for dengue (6/174, 95% confidence interval [CI] 0.7–6.2) and 1.6% for malaria (6/371, 95% CI 0.3–2.9). All 17 associations were considered clinically as malaria, including the 2 case-patients with low parasite counts. Antimalarial drugs were administered promptly in every case. Dengue serology and virology reports were available after the initial episode; however, these results did not influence patient management. Among the 6 acute concurrent infections, none was severe. The clinical evolution was always favorable. Three patients were hospitalized, all in the IgM-seropositive group, i.e., LDC; only 1 was severely ill. This patient, who had *P. vivax* malaria infection, was hospitalized for interstitial pneumonia with severe anemia. Intubation, blood transfusion, and antimicrobial drugs were required, but he was discharged from the intensive care unit after 11 days. No causative agent was identified for this pneumonia. The second patient was hospitalized for diabetes,

Table. Clinical course and diagnosis in 17 case-patients with confirmed or suspected concurrent dengue and malaria, Cayenne Hospital, Cayenne, French Guiana, July 2004–June 2005*

Patient no./birth year	Malaria diagnosis			Hospitalized†	Initial diagnosis and clinical signs	Dengue diagnosis			Conclusion
	Species	Parasitemia %	Parasitemia Class			IgM	RT-PCR	Iso	
1/1983	*Pv*	<0.1	C3	No	Malaria	–	+ DEN-1	ND	Confirmed acute concurrent disease
2/1984	*Pv*	0.15	C4	No	Malaria, Tp 51,000	–	+ DEN-3	ND	Confirmed acute concurrent disease
3/1973	*Pv*	0.8	C4	No	Malaria	–	+ DEN-3	ND	Confirmed acute concurrent disease
4/1975	*Pf*	0.1	C3	No	Malaria	–	+ DEN-3	ND	Confirmed acute concurrent disease
5/1983	*Pv*	0.1	C3	No	Malaria	–	+ DEN-3	ND	Confirmed acute concurrent disease
6/1980	*Pv*	0.2	C4	No	Malaria, Tp 52,000, Hb 9.4	SeroC	–	ND	Confirmed acute concurrent disease
7/1981	*Pf*	<0.1	C1	No	Malaria‡	–	ND	DEN-3	Confirmed concurrent infection
8/1953	*Pv*	<0.1	C3	Yes§	Malaria, Tp 71,000	+	–	–	Suspected concurrent infection
9/1952	*Pv*	0.8	C4	No	Malaria, Tp 30,000, BP 80/50	+	–	–	Suspected concurrent infection
10/1948	*Pv*	0.3	C4	No	Malaria, Tp 70,000	+	–	–	Suspected concurrent infection
11/1982	*Pv*	0.25	C4	No	Malaria	+	–	–	Suspected concurrent infection
12/1979	*Pf*	0.4	C4	Yes¶	Malaria	+	–	–	Suspected concurrent infection
13/1956	*Pv*	0.5	C4	Yes#	Malaria, Tp 63,000, Hb 5.9, Sat 83%	+	–	–	Suspected concurrent infection
14/1976	*Pv*	0.7	C4	No	Malaria	+	–	–	Suspected concurrent infection
15/1988	*Pv*	0.5	C4	No	Malaria, Tp 29,000, BP 90/50	+	–	–	Suspected concurrent infection
16/1974	*Pv*	0.1	C3	No	Malaria, HR 146 bpm	+	–	–	Suspected concurrent infection
17/1961	*Pv*	<0.1	C2	No	Malaria‡	+	–	–	Suspected concurrent infection

*IgM, immunoglobulin M; RT-PCR, reverse transcription–PCR; Iso, isolate; *Pv*, *Plasmodium vivax*; – , negative; +, positive; DEN, dengue; ND, not done; *Pf*, *P. falciparum*; Tp, thrombocytopenia++ (<100,000 platelets/μL); Hb, hemoglobin (reference range <10 g/L); BP, arterial blood pressure (systolic/diastolic); Sat, blood oxygen saturation; HR, heart rate; bpm, beats per minute.
†Hospitalization >8 hours.
‡Possible asymptomatic carrier of *Plasmodium* spp.
§For diabetes requiring insulin.
¶For treatment with Riamet (arthemether + lumefantrine).
#For interstitial pneumonia.

the third because treatment with Riamet (arthemether + lumefantrine) was only available to inpatients.

Conclusions

Malaria and dengue must be suspected in febrile patients living in or returning from areas endemic for these infections. Although the usual places of contamination by malaria and dengue viruses are quite different in French Guiana, considering that the incubation phase is longer for malaria than for dengue and that the population's mobility is high, a simultaneous clinical expression of the 2 diseases is plausible. Moreover, in French Guiana, dengue viruses have spread to malaria-endemic rural areas (*9*).

The confirmation of malaria is rapid, and after malaria is confirmed, dengue is usually ruled out without screening for it. Two methods can confirm dengue: dengue-specific IgM seroconversion or detection of dengue virus particles during the acute phase (day 0 to day 4 after onset of fever) by RT-PCR, which is faster and more specific. In published case reports (*1*–*7*), the diagnosis of dengue infection is usually made based on positive dengue IgM; however, this cannot confirm recent dengue, because IgM can persist for months and cross-react with other arboviruses (*10*). If RT-PCR requires a specific laboratory and cannot be performed on site, a new test, the Platelia, is now easily included in any laboratory and is indicated particularly for early-acute phase samples (*11*). To investigate the frequency of dengue and malaria co-infection, the Platelia test should be used in all cases of dengue-like or malaria-like syndrome, even when malaria diagnosis was positive, in regions where both infections may overlap.

Of the 1,723 patients investigated in this study, 17 had concurrent dengue and malaria. In 10 of these patients, recent acute dengue fever could not be confirmed (LDC). Two patients, 1 of whom was part of the EDC group, could have been asymptomatic carriers of *Plasmodium* spp. (1 patient with *P. falciparum* and 1 with *P. vivax*) because of low parasitemia (*12*). A true acute concurrent infection (strictly defined diagnosis) was confirmed in 6 case-patients. Concurrent acute malaria and recent dengue fever had a lower frequency than predicted by the multiplication of both prevalences, but such reasoning implies the same overlapping contamination areas for malaria and dengue, which it is not the situation in French Guiana. The greater prevalence of LDC than EDC associated with acute malaria infection illustrates the prolonged persistence of specific IgM or IgM cross-reaction, which increases the probability of a malaria case when comparing the short 4–5 day period corresponding to EDC. Virologic investigations using isolation or RT-PCR techniques were not performed on samples taken after the 4th day because of the usual disappearance of viremia. Additional associations where fever was initially caused by malaria and followed by dengue after the 4th day of malaria fever could have been undiagnosed.

EDC were diagnosed on average after 4 days of fever, never 5. Thus, delayed complications of dengue or malaria may not be detected using this definition. Such complications could be observed in patients considered LDC. One of these patients had pneumonia, which has recently been described as a complication of *P. vivax* (*13*).

Although acute concurrent infections were benign in our study, special attention should be given to the possibility of co-infection with malaria and dengue, especially when *P. falciparum* is implicated. The distinction between severe dengue and severe malaria must be made in an emergency department or hospital setting because in both situations, early diagnosis is essential for patient care.

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Lobomycosis in Inshore and Estuarine Dolphins

To the Editor: Lobomycosis is a chronic dermal infectious disease affecting humans and some species of dolphins but not, to date, freshwater dolphins. Because this disease is still considered rare despite the increasing number of reported cases in humans and cetaceans, clinical and epidemiologic information must be accurately reported to help clarify many of the unknown aspects of this disease.

We address this point because after carefully reading the excellent report by Elsayed et al. on the first human case of lobomycosis in Canada, we noticed that the authors describe the natural disease as occurring in humans and marine and freshwater dolphins only (*1*). However, this information is only partially correct because to date lobomycosis has not been described in freshwater dolphins. What is more worrisome is that this information is beginning to be referenced in other published articles (*2*). So far, lobomycosis has been confirmed in 2 species of inshore and estuarine Delphinidae: 1) the common bottlenose dolphin (*Tursiops truncatus*) from Brazil, the Atlantic coast of the United States, and Europe and 2) the Guiana dolphin (*Sotalia guianensis*) from the Surinam River estuary (*3*–*8*).

The fact that lobomycosis is endemic in humans in the Amazon basin could logically raise the suspicion that other animal species in this area may act as reservoirs or even be affected by the disease. However, the infection has never, to our knowledge, been reported in botos (*Inia geoffrensis*) or tucuxis (*Sotalia fluviatilis*) from the Amazon and Orinoco Rivers. Preliminary field studies, like the one carried out by da Silva et al., failed to demonstrate the disease in any of the 385 live-captured *I. geoffrensis* boto specimens from the Mamirauá Reserve in the central Amazon region of Brazil (*9*); similarly, our observational studies in the Venezuelan Orinoco River failed to detect the disease. On the other hand, despite the absence of indigenous cases of lobomycosis in humans reported in the United States, the disease is endemic in dolphins from the Indian River Lagoon in Florida (*7*), suggesting that no apparent epidemiologic link may exist between humans and cetaceans. Unfortunately, the etiologic agent of lobomycosis, *Lacazia loboi* (Figure), has not been cultured in vitro (*10*) despite exhaustive attempts, making its isolation from probable and suspected environmental sources impossible.

Dolphin-to-human transmission of lobomycosis has been reported only 1 time; the case-patient was an aquarium attendant who had had close physical contact with an affected dolphin (*5*). However, because the possibility of zoonotic transmission of this disease remains latent and because many pathologic and clinical aspects of the disease remain poorly understood, it is imperative to clarify these ecological concepts. Up-to-date molecular epidemiology studies to compare the strains affecting humans and dolphins and their possible phylogenetic relationship are needed.

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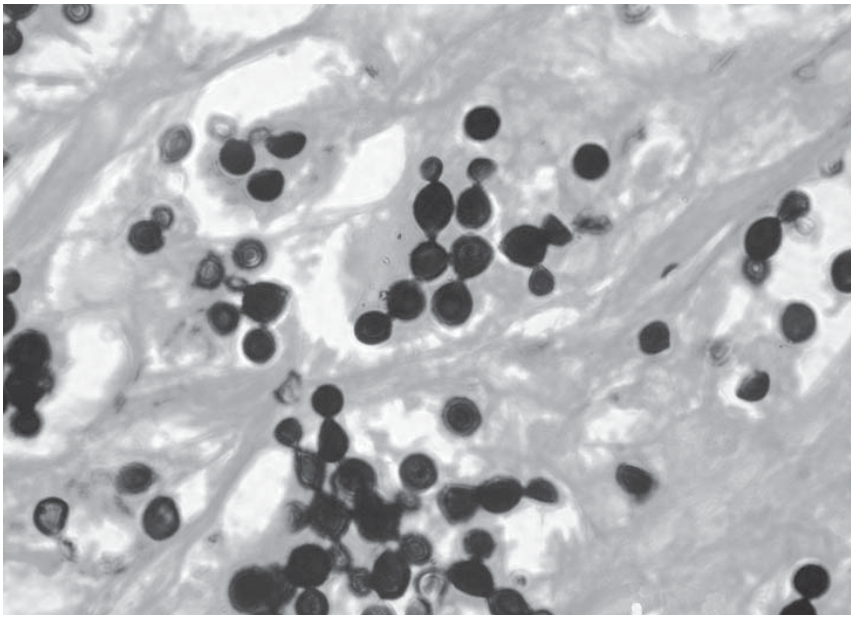

Figure. Grocott methamine silver–stained section from a skin biopsy specimen of a bottlenose dolphin (*Tursiops truncatus*) showing abundant *Lacazia loboi* yeast cells individually and in chains connected by thin tubular bridges. Magnification ×400.

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Variations in Leprosy Manifestations among HIV-Positive Patients, Manaus, Brazil

To the Editor: Contrary to early expectations, the co-occurrence of leprosy and HIV has not increased globally (*1*). However, most of the larger studies on the subject were conducted in the early to mid-1990s in African countries, and the research designs had limited power to describe the true effects of co-infection (*1*). Moreover, the introduction of highly active antiretroviral therapy (HAART), which has been used routinely in Brazil since 1996, altered the clinical evolution of HIV infection (*2*) and led to increasing reports of immune restoration inflammatory syndrome (IRIS) associated with leprosy (*3*,*4*). Although some researchers have argued that this association may not affect public health (*2*), its true importance remains to be clarified. Finally, leprosy has a wide range of clinical manifestations, which sometimes imposes a clinical challenge and may lead to misdiagnosis (*5*). Together, these factors may have helped mask the true scenario of leprosy and HIV co-infection, particularly in areas where these conditions are highly endemic. In this context, case reports from referral centers that reflect the broad clinical aspects of leprosy and HIV co-occurrence are important to increase clinicians' awareness of both diseases.

We report 3 HIV-positive/AIDS patients who showed different clinical manifestations of leprosy; their conditions were diagnosed before and after HAART initiation. All patients lived in Manaus, the capital of the state of Amazonas in Brazil, an area where both leprosy and HIV infection are endemic. The 3 patients represent a sample from our 11-year experience with 21 patients with leprosy and HIV co-infection.

Patient 1 was a 29-year-old woman whose HIV-1 infection was diagnosed in May 2002 at antenatal examination. Her CD4 cell count in 2002 was 513 cells/μL. In November 2007, she sought treatment at the Institute of Tropical Medicine of Amazonas with a 3-month history of a single erythematous plaque on her left arm, which was clinically diagnosed as borderline tuberculoid (BT) leprosy. The patient's sensitivity to pain was decreased. There was no nerve enlargement. Histopathologic examination confirmed the diagnosis, showing a granulomatous dermatitis with no acid-fast bacilli on Wade stain. At this time, her CD4 cell count was 342 cells/μL. HAART and multidrug therapy (MDT) for paucibacillary leprosy were initiated. The leprosy resolved, and the lesion disappeared within 2 months of therapy.

Patient 2 was a 22-year-old man who had neurocryptococcosis and HIV infection diagnosed in September 2007. At that time, he exhibited disseminated, infiltrated lesions on the trunk and upper and lower limbs. Borderline lepromatous (BL) leprosy was clinically diagnosed. Skin biopsy confirmed the diagnosis; the biopsy specimen showed a granulomatous dermatitis, foamy cells, and multiple acid-fast bacilli. His CD4 cell count was 6 cells/μL. HAART and MDT for multibacillary leprosy were prescribed. In February 2008, the patient was readmitted to the Institute of Tropical Medicine of Amazonas and died of nonspecified bacterial pneumonia and sepsis.

Patient 3 was a 23-year-old woman who had HIV-1 infection (CD4 cell count 435 cells/μL) diagnosed in November 2006 at antenatal examination. HAART was begun 3 months later. In August 2008, she sought treatment with a 3-month history of a single patch on the left leg with erythematous papules on its border (Figure). There was decreased pain sensitivity in the

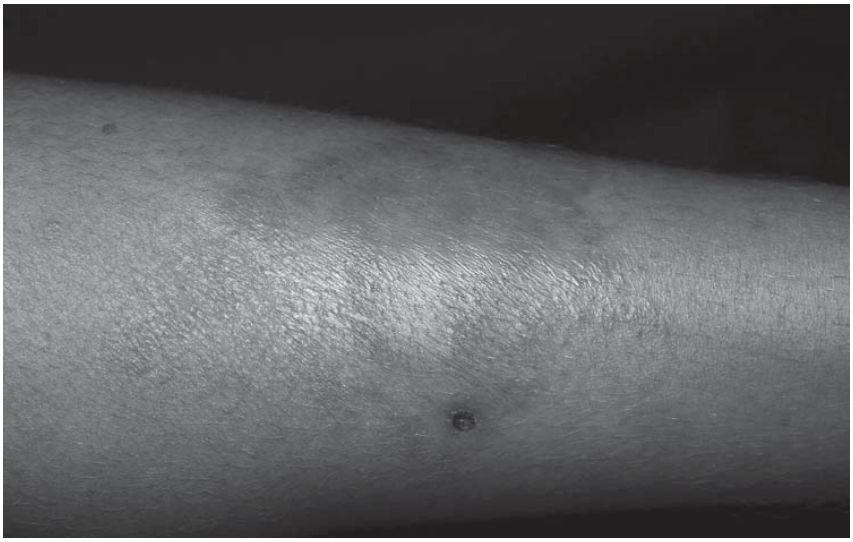

Figure. Skin lesion of patient 3, a solitary patch on the left leg with erythematous papules on the border.

lesion and no nerve enlargement. At that time, her CD4 cell count was 372 cells/μL. Histopathologic examination showed tuberculoid granulomas consisting of lymphocytes and epithelioid cells. Wade staining showed no acid-fast bacilli. Histopathologic findings led to a diagnosis of BT leprosy. MDT for paucibacillary leprosy was promptly started, and HAART was continued. When she was last seen, in December 2008, the skin lesion had disappeared and she was still receiving MDT.

The reliability of the cardinal signs of leprosy (hypopigmented or reddish patches with definite loss of sensation, thickened peripheral nerves, and positive skin smears or biopsy material) has been widely accepted (*5*). However, in some difficult cases, the definitive diagnosis relies solely on the histopathologic examination, which often depends on the experience of the pathologists working in referral centers. According to most pre-HAART studies, the clinical spectrum of leprosy seems to be preserved in HIV-positive and AIDS patients (*1*). This is in agreement with the course of disease in patient 1 (a typical BT lesion before initiating HAART) and patient 2 (a typical multibacillary leprosy in a full-blown AIDS background). For patient 3, a distinct outcome was observed: the appearance of an atypical BT lesion during HAART. Recently, we reported 3 cases of IRIS associated with leprosy in which BL leprosy shifted unexpectedly to BT leprosy (*4*,*6*). Host genetic make-up and unknown consequences of HIV-infection over specific leprosy immune mechanism may be implicated in these unusual outcomes. Further prospective studies should be performed to elucidate these findings.

Although previous studies have shown that HIV infection is not a risk factor for leprosy (*1*), clinicians should be aware of this potential co-infection, which may mimic different skin diseases. Moreover, reports of leprosy after HAART initiation have been described from countries where leprosy is not endemic (*7*). Precise diagnosis and prompt treatment of leprosy in co-infected persons are mandatory.

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Suspected Brazilian Purpuric Fever, Brazilian Amazon Region

To the Editor: Brazilian purpuric fever (BPF), a *Haemophilus aegyptius*–caused febrile hemorrhagic illness of children that begins with conjunctivitis and has a case-fatality rate of 40%–90% (*1*,*2*), was first recognized during a 1984 outbreak. Before June 2007, 69 cases were reported worldwide; 65 were from Brazil (*1*–*3*). To our knowledge, the disease had not been reported in the Amazon region until this investigation, which was precipitated by the report of 5 cases of a compatible syndrome in Anajás, Pará State, Brazil, in August 2007.

To determine whether recent reports of BPF were accurate, we reviewed medical records of the hospital in Anajás. We identified cases by using the following definition: fever >38.5ºC , abdominal pain, vomiting, purpura, and antecedent conjunctivitis during July 1–September 30, 2007, in a child 3 months–10 years of age; absence of signs or symptoms of meningitis in those children; and laboratory exclusion of meningococcal infection. In addition, we searched retrospectively and prospectively for conjunctivitis among pupils of the elementary schools of Anajás during July–September 2007. We found 7 children with illnesses that met our case definition.

From 2 children with nonfatal illness, we collected blood, serum, conjunctival swabs, and cerebrospinal fluid (CSF). All specimens were submitted for bacterial culture in half agar chocolate without bacitracin; serum and CSF were also subjected to real-time PCR for detection of *Neisseria meningitides, Streptococcus pneumoniae,* and *Haemophilus influenzae* serotypes a, b, c, and d and to conventional PCR for the *ompP2* gene of *H. influenzae*. All serum samples were also tested by hemagglutination inhibition for Flavivirus, Oropouche, Catu, Caraparu, Tacaiuma, Mayaro, Mucambo, western equine encephalitis, eastern equine encephalitis, Guaroa, Maguari, Ilhéus, Rocio, and St. Louis encephalitis; by immunoglobulin M antibody-capture ELISA for dengue and yellow fever; and, when reactive for dengue, by reverse transcription–PCR for dengue types 1, 2, 3, and 4.

Because of the remoteness of the outbreak site, samples for bacterial culture were collected on locally available blood agar enriched with rabbit serum without antimicrobial drug–selective agents, rather than on the recommended chocolate agar enriched with horse serum and bacitracin (*1*). Samples were transported over several days by open boat at ambient temperature (≈35 ºC) in improvised containers without an incubator. Serum and CSF samples were thawed and refrozen repeatedly for removal of aliquots before testing. Microbiologic and virologic testing was conducted at the Pará State Health Laboratory and Evandro Chagas Institute. Serum and CSF samples were tested by PCR at Adolfo Lutz Institute.

We identified 7 case-patients (median age 4 years, range 2–8 years): 6 from review of charts at the local hospital and 1 from active search in the rural community. Onset of illness was August 1 for the first case-patient and August 31 for the last. Five (71%) did not receive antimicrobial drugs and died within 24 hours after fever onset; 2 were treated with amoxicillin within 24 hours after fever onset and survived (Table).

Laboratory tests showed leukopenia on the day of hospital admission (Table). All case-patients had antecedent conjunctivitis. All except the first case-patient had had physical contact with a previous case-patient through school or family; 5 were related (siblings or cousins). The period from exposure to onset of fever was 8–21 days.

Of 1,598 elementary school pupils investigated for conjunctivitis, 111 (7%) reported symptoms of conjunc-

Table. Characteristics of 7 case-patients with suspected Brazilian purpuric fever, Amazon region, Brazil, August 2007*

Case-patient no.	Sex/age, y	Date of conjunctivitis onset	Date admitted to hospital	Hospitalization, d	Antimicrobial drug treatment	Date of death	Type of sample(s) collected	WBC/mL
1	M/2	Aug 1	Aug 5	<1	No	Aug 5	NC	NT
2	F/3	Aug 10	Aug 13	<1	No	Aug 14	Blood	3,700
3	M/3	Aug 14	Unknown	Unknown	No	Aug 21	NC	NT
4	M/6	Jul 19	Aug 22	<1	No	Aug 23	Blood	2,300
5†	M/8	Unknown	Sep 3	7	Yes	–	CSF, conjunctival swab	9,060
6	F/4	Aug 23	Aug 26	<1	No	Aug 26	CSF, blood‡	2,000
7	F/4	Aug 31	Sept 3	12	Yes	–	Oropharyngeal and conjunctival swabs, CSF, blood, serum	4,800

*Case-patients 5 and 7 underwent testing for dengue and yellow fever by immunoglobulin M antibody-capture ELISA, for dengue by reverse transcription–PCR, and for *Neisseria meningitidis*, *Streptococcus pneumoniae,* and *Haemophilus influenzae* serotypes a, b, c, and d and conventional PCR for detecting the *ompP2* gene. All tests were negative. WBC, white blood cells; NC, not collected; NT, not tested; CSF, cerebrospinal fluid.
†Sample collected 20 days after admission.
‡Non-standard collection, transport, storage, or processing.

tivitis during July–September 2007. Reported treatment included home remedies and a nonprescription, locally available, cream containing a sulfa drug. Review of municipal hospital records during June 1–September 30, 2007, identified no additional cases of conjunctivitis. After the last case-patient died, 17 other persons were identified with purulent conjunctivitis: 4 at the municipal hospital and 13 during active case-finding in schools and the community. All were treated with oral amoxicillin and chloramphenicol optic solution, and 76 contacts were treated prophylactically with oral rifampin. No further suspected BPF cases were detected. Test results for acute arbovirus infection and PCR were negative for all patients (Table).

This outbreak of highly fatal illness is clinically compatible with BPF. Compatible features included young age, antecedent purulent conjunctivitis, signs and symptoms (i.e., antecedent conjunctivitis, fever 39.5Cº–41.0Cº, abdominal pain, nausea, vomiting, petechiae or ecchymoses), and high case-fatality rate. Epstein-Barr infection has reportedly produced similar symptoms (*4*) but with an illness lasting >7 days in contrast to the <24 hours for our case-patients.

We did not detect *H. aegyptius* in peripheral blood by culture or in serum or CSF by PCR in the 2 surviving children and in contacts of case-patients. One reason could be the remoteness of the investigation site, which resulted in improper sample collection, storage, and processing in the field before samples reached reference laboratories. Hemagglutination tests for arboviruses have low specificity. Therefore, another known or novel pathogen could have caused these cases.

Timely treatment with antimicrobial drugs for patients with suspected disease, prophylaxis of contacts, and treatment of children with conjunctivitis resulted in no additional cases. Intensive surveillance for febrile illness preceded by conjunctivitis, immediate treatment, contact prophylaxis, and appropriate prompt laboratory testing are essential for continued control of BPF in this region.

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Hepatitis C Virus in Blood Donors, Brazil

To the Editor: The Fundação de Hematologia e Hemoterapia do Amazonas is a public health service in Manaus, Brazil, that is responsible for serologic screening the serum of all blood donations in the region. In the state of Amazon, 9.0% of donated blood is discarded on the basis of serologic findings; discarding because of hepatitis C virus (HCV) antibodies declined from 1.25% in 1995 to 0.32% in 2007. The aim of this study was to characterize the serologic and molecular profile of HCV-antibody–positive blood donors from the Fundação de Hematologia e Hemoterapia do Amazonas.

For the study, 154 donors were selected from a routine database of voluntary blood donors who had donated from September 2005 through April 2007 (82,851 donations). Fresh plasma samples were sent to the laboratory in Manaus through the usual transportation systems for regular donations; i.e., samples from 27 cities are transported by air for ≈2 hours, and samples from 21 localities are transported by boat or road, all under refrigerated conditions.

An in-house standardized nested-PCR was used to detect HCV RNA (*1*). Genotype assignment was based on type-specific motifs on the sequenced amplicons delimited by primers HC11/HC18 from the 5′ untranslated region

(*2*). Viral load was determined by commercial assay (HCV Monitor, Roche Molecular Systems, Inc., Branchburg, NJ, USA).

An association was observed between HCV RNA and donor age; the same trend was seen in the first-time blood donor group. Associations between HCV-RNA detection and gender (p = 0.875) and place of donation (p = 0.989) were not significant. Using 18–25 years of age as the reference group, we found that odds ratios (ORs) for having HCV viremia were higher for those 45–55 years of age (OR 8.19, p<0.001) and 35–45 years of age (OR 3.49, p = 0.003).

We observed increasing rates of RNA detection according to the signal-to-cutoff (S/CO) ratio. However, some donors had a weak S/CO ratio (between 1 and 2) with positive nested-PCR tests (Figure). Although adopting an S/CO ratio as a criterion for referring for further testing by recombinant immunoblot assay (RIBA) has been advocated by some groups (*3*), our data show that this criterion may be misleading and would deny a confirmatory diagnosis by giving false-negative results for many persons.

A total of 113 samples were analyzed by RIBA; among 48 RIBA-reactive samples, 9 (18.8%) were negative for HCV RNA in plasma. However, because PCR results may sometimes be negative for persons who are actually infected, a single negative PCR result should not be relied on as evidence that virus has cleared from plasma. Such patients must be observed for years before they may be declared cured (*4*).

Among 97 RIBA-positive or -indeterminate samples, viral load was detectable in only 33 samples: 27 (81.8%) RIBA-positive samples and 6 (18.2%) RIBA-indeterminate samples. Only HCV genotypes 1 (87.1%) and 3 (12.9%) were found. Geographic distribution shows genotypes 1 and 3 in Manaus and only genotype 1 in other Amazon cities. This genotype geographic distribution is similar to that found for many Brazilian cities and Eastern countries and may reflect the route of HCV introduction into the Amazon; the virus was probably brought to the Amazon region by European immigrants and blood-derived medicines imported to Brazil. This hypothesis is corroborated by the finding of genotype 3 exclusively in Manaus, suggesting that this city is the point of arrival of HCV and that new strains were disseminated from Manaus to inner localities. Historical reconstruction of HCV in Amazon could be attempted by using these isolates as well as others from hepatitis patients in the region, including genotype 2 (*5*).

We found a higher-than-expected rate of 50% for indeterminate immunoblot results among samples that were HCV-RNA positive by nested PCR. The presence of HCV RNA in plasma samples from 70%–75% of blood donors with indeterminate immunoblot results has also been reported by other groups in Brazil (*6,7*); however, in contrast, other investigators have reported RNA prevalence in such samples to be ≈2.5% (*1,8*). Indeterminate RIBA test results can indicate seroconversion or seroreversion or, occasionally, a chronic infection when RNA HCV is detected in plasma (*9,10*). To provide better understanding of the meaning of these indeterminate results, ongoing follow-up studies are examining the immune status of these persons.

Our data offer insights for counseling of donors who have repeatedly HCV-reactive results. We suggest that Amazon region blood banks screen by enzyme immunoassay and use molecular testing as the first supplemental test and that immunoblot be applied to the remaining HCV-RNA nonreactive samples to distinguish between true and false anti-HCV carriers. This new algorithm would save considerable resources currently spent on immunoblot-indeterminate persons in addition to HCV-RNA reactive persons who do not require further testing for confirmation. Moreover, according to current policy, those with false-positive results may be reinstated as donors if they have negative retesting results after 6 months.

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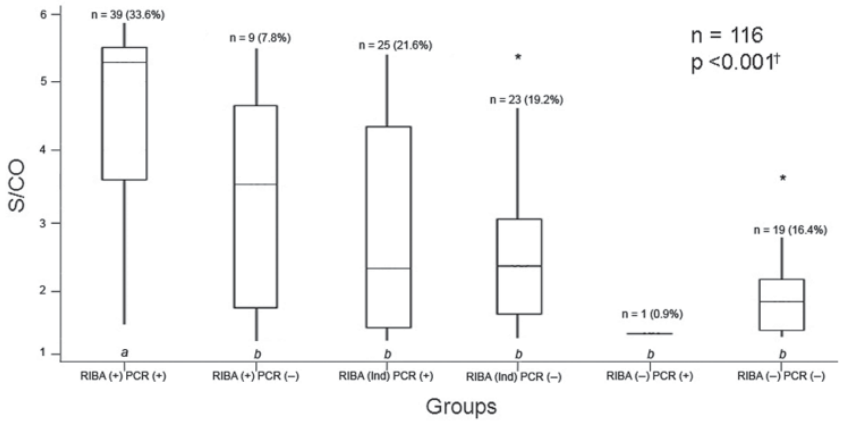

Figure. Distribution of hepatitis C virus (HCV) enzyme immunoassay signal-to-cutoff (S/CO) values by recombinant immunoblot assay (RIBA) interpretations among HCV-RNA–positive [PCR (+)] and HCV-RNA–negative [PCR (–)] donated blood samples. Group *a* differs statistically from all groups *b* with 95% confidence intervals. The Mann-Whitney test was used to compare the 2 groups. (+), positive; (–), negative; (Ind), indeterminate. *S/CO values outside interquartile intervals; †Kruskal-Wallis test.

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Leishmaniasis in Chaparé, Bolivia

To the Editor: In Bolivia, most cases of leishmaniasis are caused by *Leishmania* (*Viannia*) *braziliensis* (*1*). The parasite is transmitted zoonotically by several sandfly species and, when transmitted to humans, may cause cutaneous leishmaniasis (CL), and potentially, mucosal leishmaniasis (ML) (*2*).

Data on the prevalence and effects of CL in Bolivia have been scarce, even though anecdotal and official reports indicate a dramatic increase in the number of human CL cases in Bolivia in the past decade (*1*,*3*). Also, although CL was originally a sylvatic disease in Bolivia, some evidence indicates that the transmission cycle has adapted to the peridomestic habitat. However, this evidence is largely based on individual case reports. No information is available on parasite species, vectors, and reservoirs in such a peridomestic transmission cycle.

A preliminary study to guide future research focus and assist in immediate leishmaniasis prevention and control policy decision making is underway in Isiboro-Secure National Park, Chaparé, Bolivia. Our objectives were to collect data on the prevalence of leishmaniasis in that area and evidence for peridomestic *Leishmania* transmission.

A survey was carried out during April–July 2007 in 2 communities in Isiboro-Secure National Park, San Gabriel (16°40′31″S and 65°37′38″W) and San Julian (16°41′59″S and 65°38′10″W). These 2 communities were selected because of local knowledge of disease in the community, their moderate degree of urbanization (i.e., ≈50% of the communities' houses are clustered around the main access road), and the accessibility of the sites to the field team. In this area, CL is transmitted from April through October.

Households in both communities were visited by a team of experienced medical staff who interviewed heads of household to collect demographic data (sex, age) and diagnose the clinical condition of all present household members (presence/absence of CL lesions or scars, number of lesions, date of lesion onset) by using a standardized, pretested questionnaire. The study protocol was approved by the Ethical Committee Review Board of the World Health Organization (WHO). All patients with active cases were treated with meglumine antimoniate according to the standard protocol (*2*).

We surveyed 133 and 52 households in San Gabriel and San Julian, which represented 86% and 80% of the total households of the respective communities; 21 and 13 households, respectively, were visited but did not participate because the owners refused or were not present. Of the 965 persons surveyed, 488 (50.6%) were male and 476 (49.3%) were female; 9 (0.9%) had active CL lesions and 62 (6.4%) had CL scars. One person had ML, and 3 had evidence of past ML; all ML patients were male. Of those with CL lesions, all had 1 lesion only. The mean lesion size was 2.3 cm (range 1.5–3 cm), and the mean lesion duration (to survey date) was 5.6 months (range 1–11 months). The clinical CL lesions were parasitologically confirmed by microscopy (n = 4) or PCR (n = 8). Parasite culture was performed on patient isolates (n = 6), and *L.* (*V.*) *braziliensis* was identified and characterized as the etiologic agent of these CL cases.

Active lesion and scar prevalence were associated with male sex (lesions: Fisher exact test, odds ratio [OR] = 7.90 [95% confidence interval (CI) 1.01–169.09], $p<0.05$; scars: Yates-corrected χ^2 test, OR = 3.05 [95% CI 1.65–5.71], $p<0.001$). Children ≤15 years of age were at lower risk of contracting the disease than those >15 years (lesions: Fisher exact test, OR = 0.19 [95% CI 0.01–1.46], $p = 0.094$; scars: Yates-corrected χ^2 test, OR = 0.09 [95% CI 0.03–0.27], $p<0.001$) (Figure). Active lesion and scar prevalence were also associated with prolonged migration into the forest before the survey (lesions: Fisher exact test, OR = 28.10 [95% CI 3.49–184.29], $p<0.01$; scars: Fisher exact test, OR = 35.76 [95% CI 13.49–93.53], $p<0.001$).

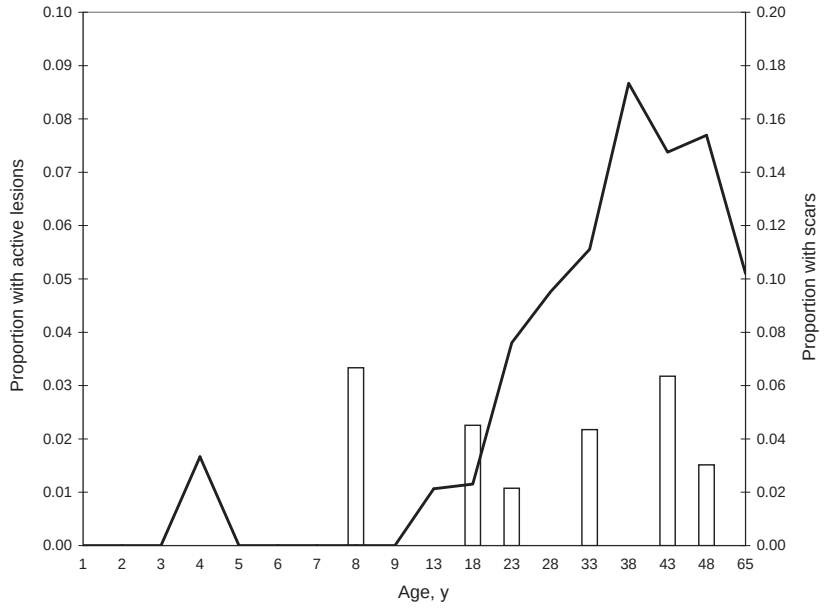

Figure. Age prevalence curve of persons with lesions (white bars) and scars (black line) from cutaneous leishmaniasis, Bolivia, 2007.

Whether the surveyed population is representative of the total population living in the study area is debatable. However on the basis of current population figures (i.e., 16,000) and observed prevalence of CL, we estimate up to 1,440 CL cases in Isiboro-Secure currently. The low prevalence of active disease and scars indicates that *L.* (*V.*) *braziliensis* was introduced into Isiboro-Secure fairly recently, which is corroborated by the short median time since the cure of persons with CL scars (i.e., 7.5 years, range 0.4–30.5 years). Combined with the association of CL with male sex, age, and migration to the forest, we conclude that in Isiboro-Secure, most *L.* (*V.*) *braziliensis* transmission is sylvatic rather than peridomestic. This transmission pattern implies that prevention and control approaches that focus on the person (e.g., use of repellents, early treatment seeking) will most likely be more effective than approaches that focus on the household (e.g., indoor residual spraying with insecticides, insecticide-treated bednets).

Current analyses are underway to establish CL risk factors. Additionally, a prevention and control strategy adapted to the local context is being planned to minimize the population's exposure to sandflies, prepare health professionals for adequate (per protocol) management of cases, and minimize the likelihood that *L.* (*V.*) *braziliensis* transmission becomes peridomestic.

Acknowledgments

We are grateful for the logistical support of local Ministry of Health staff and community leaders in facilitating the survey.

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DOI: 10.3201/eid1504.081257

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Erratum—Vol. 15, No. 2

The name of Vasanthi Thevanesam should have been included in the author list for the article Severe Dengue Epidemics in Sri Lanka, 2003–2006 (N. Kanakaratne et al.). Prof Thevanesam is affiliated with the University of Peradeniya Faculty of Medicine, Peradeniya, Sri Lanka. The article has been corrected online (www.cdc.gov/eid/content/15/2/192.htm).

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.

Travels in Gene Space

Julian W. Tang

The Captain peers over the bow of his computer ship,
Floating down the river of evolution-time,
In the landscape of gene-space.
Gene sequences running parallel on either side,
Spreading, evolving, as far as the eye can see.
Ahead, the future is unclear, sequences shrouded in mist.
But this is no surprise.
Prediction is a risky business.

Looking behind, some sequences shine brightly,
Known samples, from known times.
Others are blurred in the mists of time past.
The Captain taps his keyboard.
Some of the mist dissipates,
And some sequences become visible
In the light of inference.
An epidemic history revealed perhaps?

The Captain sighs and taps his keyboard again.
His environment changes.
He is now surrounded by hills and valleys,
With moving figures all around him.
Markov Chain Monte Carlo robots,
Walking, climbing, up and down.
Some, aimlessly wandering in circles,
In the undulating landscape of tree-space.

He taps his keyboard once more.
The MCMC robots change direction,
Seemingly, becoming more purposeful.
The Captain allows himself a smile.
Suddenly, he feels a tug on his leg.
Looking down, he sees the cherubic face
Of his young son, looking up at him.
"Can we play football, daddy?"

Dr Tang is a clinical and academic virologist with an interest in viral molecular epidemiology and evolution, particularly how sequence analysis with phylogenetic techniques may enhance our understanding of how outbreaks and epidemics may arise and evolve.

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Ray Troll (b. 1954) Fishes of Amazonia (2000) Acrylic on canvas (21.13 m × 4.57 m) Miami Museum of Science, Miami, Florida, USA

When You Were a Tadpole and I Was a Fish

Polyxeni Potter

"All of us with backbones are … the fish group," realized Ray Troll somewhere in his understanding of evolutionary biology. Our hands are but an evolved version of fins. When you know this, "Then you start seeing the world in a different way." This evolutionary connection fueled the artist's imagination as well as his interest in the natural world and its origins, which rivals only his compulsion to paint.

Troll was born in Corning, New York, and spent his early years at various locations where his family lived. He studied art at Bethany College, Kansas, and then moved to Seattle to continue his studies at Washington State University. In 1983, he went to Alaska to visit his sister, "Come on up and visit Ketchikan …," she had written, "Why don't you work up here for the summer?" Enthralled with the beauty of the environment and the pace of the local community, he made this city between a rainforest and the salty water of the Tongass Narrows his home. He incorporated it in his unique style, a bold mixture of naturalist imagery and eccentric humor reminiscent of Brueghel or Bosch that captivates scientist and artist alike.

"At first I didn't notice it," Troll said of indigenous art, another major influence on his development. "But then I fell in love with it." During his travels in the area, he mixed with artists from the coastal clans and watched them work with wood and make totem poles. His iconic representations of fish, which have earned him honorary membership in the Gilbert Ichthyological Society and the Guild of Natural Science Illustrators, reflect the linearity of Northwest Native American design.

A disciplined artist, Troll works out of his waterfront studio, which is filled with fish specimens, fossils, and paleontology texts, "I like to let the art take its time." He travels around the country looking for dinosaur bones and new fish. His drawings and paintings fill science books, anchor multimedia exhibits, or appear online to aid species identification for the National Marine Fisheries Services. One of his murals graces The National Oceanic

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and Atmospheric Administration Fisheries Laboratory in Santa Cruz. And *Hydrolagus trolli*, a species of ratfish, was named after him.

After an autumn 1997 trip down the Amazon River, some 1,600 km, with friends and scientists, Troll produced a mural-sized painting that took a year to complete: Fishes of Amazonia, on this month's cover. The river, home to more than 2,000 known fish species (a fraction of its full inventory), inspired this artistic celebration. Species diversity, due only partly to the river's immense size and abundance of light, food, and favorable temperatures, may have come about from climatic and geographic shifts over millions of years. During these massive changes, species appeared and disappeared, and populations evolved as they adapted to new conditions. Fish recount the long history of the earth.

"The salmon-falls, the mackerel-crowded seas, / Fish, flesh, or fowl, commend all summer long / Whatever is begotten, born, and dies," wrote W.B. Yeats in "Sailing to Byzantium." Awed by the vastness of the sea and the bounty of the world but troubled by their transience, the poet saw permanence only in art as in Byzantium. But the study and recording of species for posterity, left to the scientist and artist, betray a remarkable continuity unseen by the poet. For Troll, these disciplines converge to explore the connection between species diversity and the evolutionary changes that produced it, a connection also pertinent on the microbial level.

Emerging infections are often caused by pathogens present in the environment but brought out of obscurity and given a selective advantage or the opportunity to infect new populations by changing conditions. The presence of malaria, dengue fever, orally transmitted Chagas disease, Kaposi sarcoma–associated herpesvirus infection, adiaspiromycosis, and many other emerging diseases indicates that emergence and epidemiologic change are vigorous and ongoing in the Amazon Basin.

The Amazon River dolphin, adapted for life in turbid waters, has tiny eyes and uses instead advanced echolocation to find prey. Along the same evolutionary lines, the presence of lobomycosis in these dolphins poses the likelihood that the agent of the disease has found ways to infect a broader array of the species than we know in the same ecologic community.

British naturalist Langdon W. Smith in his poem A Tadpole and a Fish (or Evolution, 1909), traced life back to the Paleozoic era, the beginning of life. Threading changes back and forth, between darkness and light, throughout the eons, with humor and romance, he arrived in our times. "Then as we linger at luncheon here, / O'er many a dainty dish," he wrote warning us to be mindful of our origins, "Let us drink anew to the time when you / Were a Tadpole and I was a Fish."

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Unstructured Event-based Reports for Global Infectious Disease Surveillance

Chronic Wasting Disease Prions in Elk Antler Velvet

Hospitalizations and Deaths Related to *Clostridium difficile* Infection, Finland

Virulent Strain of Hepatitis E Virus Genotype 3, Japan

Respiratory Enterovirus and Recombinant Rhinoviruses among Circulating Picornaviruses

CTX-M β-Lactamases in *Escherichia coli* from Urinary Tract Infections, Cambodia

Cross-border Dissemination of Methicillin-Resistant *Staphylococcus aureus* in the Euregio Meuse-Rhin Region

Seroprevalence of Avian Influenza Virus A (H5N1) in 4 Villages with Confirmed Cases, Thailand, 2005

Case–Control Study on the Origin of Atypical Scrapie in Sheep, France

Increased Risk for Severe Malaria in HIV-1–Infected Adults, Zambia

Chloroquine-Resistant Haplotype *Plasmodium falciparum* Parasites, Haiti

Clostridium difficile in Retail Meat Products, Arizona, USA

Clostridium difficile Genotypes in Retail Meat, Canada

Clostridium difficile in Ready-to-Eat Salads, Scotland

Human *Streptococcus agalactiae* Isolate in Nile Tilapia (*Oreochromis niloticus*)

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

Upcoming Infectious Disease Activities

April 2–3, 2009
Exploring the Dynamic Relationship Between Health and the Environment
The Center for Biodiversity and Conservation Milstein Science Symposium
American Museum of Natural History
New York City, NY, USA
http://cbc.amnh.org/health
cbcsymposium@amnh.org

April 16–18, 2009
Emerging Infectious Diseases: Ethics, Law and Professional Obligation
Galveston Island Convention Center
Galveston, TX, USA
http://research.utmb.edu/intramural_funding/sym09

April 27–29, 2009
Twelfth Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/vaccine09

June 4–5, 2009
Drug-resistant and Vaccine-escape Hepatitis B Virus Mutants: Emergence and Surveillance
Atlanta, GA, USA
http://www.cdc.gov/hepatitis/hbvsymposium2009

June 18–21, 2009
26th International Congress of Chemotherapy and Infection
Sheraton Centre Toronto Hotel
Toronto, Canada
http://www.icc-09.com

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Announcements are no longer submitted through Manuscript Central.

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Article Title

Acute Conjunctivitis with Episcleritis and Anterior Uveitis Linked to Adiaspiromycosis and Freshwater Sponges, Amazon Region, Brazil, 2005

CME Questions

1. Which of the following is the *most* likely mechanism for disease associated with adiaspiromycosis?

A. Conidia invasion
B. Immune response
C. Allergy
D. Fungemia

2. Which of the following is the *most* common age group reported to be infected with ocular adiaspiromycosis in the initial case series of 17 patients in this article?

A. Less than 5 years
B. 5 to 15 years
C. 16 to 25 years
D. 26 to 35 years

3. Which of the following is *least* likely to be reported as an ocular-related symptom in patients with ocular disease associated with adiaspiromycosis?

A. Conjunctival hyperemia
B. Photophobia
C. Blurred vision
D. Excessive tearing

4. Which of the following *best* describes the frequency of bilateral corneal opacities in patients with confirmed ocular disease in this case series?

A. 13%
B. 20%
C. 35%
D. 80%

4. Which of the following is *least* likely to be a risk factor associated with ocular adiaspiromycosis in this case series?

A. Diving in the Araguaia River
B. Fishing in the Araguaia River
C. Male gender
D. Drinking Araguaia River water

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

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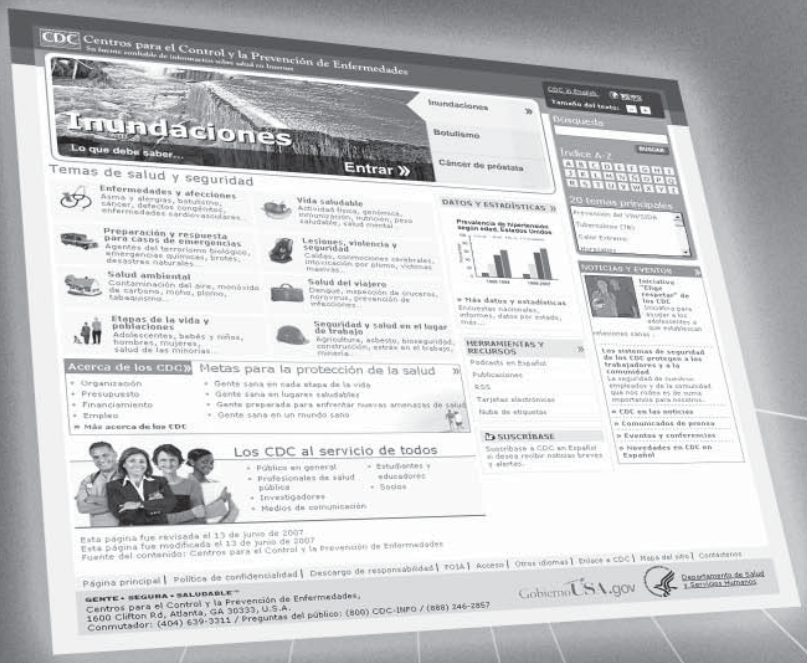

Índice A–Z

A	B	C	D	E	F	G	H	I
J	K	L	M	N	Ñ	O	P	Q
R	S	T	U	V	W	X	Y	Z

Temas de salud y seguridad

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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
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- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

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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

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. 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.