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On the Cover

Prince Twins Seven-Seven (b. 1944)
The Lazy Hunters, and the Poisonous Wrestlers, Lizard Ghost and the Cobra (1967)
Ink, paint, and chalk on plywood (124.5 cm × 78.1 cm)

National Museum of African Art
Gift of Mr. and Mrs. Sean Kelly, 75-28-3

Photo by Franko Khoury

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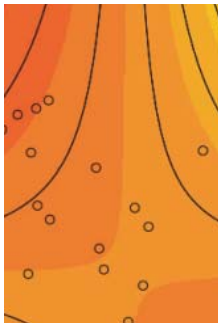

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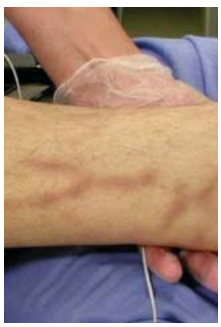

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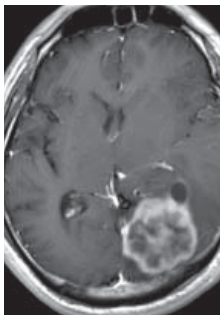

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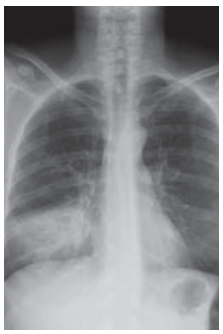

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Conference Summary

Persistence of Highly Pathogenic Avian Influenza Viruses in Natural Ecosystems

Camille Lebarbenchon,[1] Chris J. Feare, François Renaud, Frédéric Thomas, and Michel Gauthier-Clerc

Understanding of ecologic factors favoring emergence and maintenance of highly pathogenic avian influenza (HPAI) viruses is limited. Although low pathogenic avian influenza viruses persist and evolve in wild populations, HPAI viruses evolve in domestic birds and cause economically serious epizootics that only occasionally infect wild populations. We propose that evolutionary ecology considerations can explain this apparent paradox. Host structure and transmission possibilities differ considerably between wild and domestic birds and are likely to be major determinants of virulence. Because viral fitness is highly dependent on host survival and dispersal in nature, virulent forms are unlikely to persist in wild populations if they kill hosts quickly or affect predation risk or migratory performance. Interhost transmission in water has evolved in low pathogenic influenza viruses in wild waterfowl populations. However, oropharyngeal shedding and transmission by aerosols appear more efficient for HPAI viruses among domestic birds.

Wild birds, especially waterbirds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders), are natural hosts for influenza A (avian influenza) viruses. Avian influenza viruses are classified on the basis of genetic, antigenic, and structural characteristics of hemagglutinin and neuraminidase proteins. These proteins are involved in binding of virus to host cells and release of new virions from these cells, respectively. Sixteen hemagglutinins (H1–H16) and 9 neuraminidases (N1–N9) have been described. For avian influenza viruses of subtypes H5 and H7, there are 2 types of virulence: low pathogenic avian influenza (LPAI) virus generally produces benign intestinal tract or respiratory infections; highly pathogenic avian influenza (HPAI) virus generally produces multiorgan systemic infections.

LPAI viruses naturally infect wild waterbirds according to host species, age, immune status, feeding behavior, premigration aggregation, and aquatic survival of the virus. Long-term studies in Europe and North America also identified seasonal variation in prevalences of infection of LPAI virus and circulating subtypes. HPAI viruses primarily infect poultry in which viruses of subtypes H5 and H7, presumably from wild birds or contact with their derivatives, sporadically switch to highly virulent strains.

At the end of the 19th century, a disease that caused high mortality rates and spread rapidly was described in domestic birds in Italy. This fowl plague spread through Europe in the early 20th century, most likely through trading of domestic birds. In 1955, the pathogen responsible for the disease was classified as an influenza A virus, and its relationship to human influenza viruses was recognized. Domestic birds have been affected by recurrent outbreaks of HPAI viruses, generally limited to localized geographic areas but responsible for high mortality rates and substantial economic losses. In contrast, wild birds have rarely been involved in HPAI virus infections. Before 1996, only 1 HPAI virus outbreak was documented in the wild, resulting in the death of ≈1,300 common terns (*Sterna hirundo*) in South Africa (*1*). Since then, emergence and spread of the HPAI virus lineage from Asia (H5N1), first discovered in domestic geese in southern People's Republic of China in 1996, has been responsible

Author affiliations: Centre de Recherche de la Tour du Valat, Arles, France (C. Lebarbenchon, M. Gauthier-Clerc); Institut de Recherche pour le Développement, Montpellier, France (C. Lebarbenchon, F. Renaud, F. Thomas); WildWings Bird Management, Haslemere, United Kingdom (C.J. Feare); and Université de Montréal, Montréal, Québec, Canada (F. Thomas)

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[1]Current affiliation: The University of Georgia, Athens, Georgia, USA.

for the death of thousands of wild birds, occasionally through mass mortality events (e.g., Lake Quinghai, People's Republic of China, in May–June 2005). Extensive surveillance of apparently healthy wild populations has rarely detected HPAI virus (H5N1), even in areas where the virus is endemic in domestic birds (*2*). In addition, some reports of asymptomatic infection by HPAI virus (H5N1) in apparently healthy free-living wild birds lack important substantiating information and such cases of infection have yet to be convincingly demonstrated (*3*).

Although recent studies have focused on environmental factors that contributed to the persistence and spread of HPAI virus (H5N1) in southeastern Asia, Europe, and Africa (*4*–*6*), general knowledge concerning mechanisms of emergence and persistence of HPAI viruses is limited. We propose that because the ecologic landscape in which avian influenza viruses evolve differs markedly between natural (i.e., wild birds) and artificial (e.g., intensive poultry farming, free-grazing ducks, and live bird markets) conditions, selective pressures differ. These phenomena are likely to explain virulence heterogeneity among avian influenza viruses and why HPAI viruses do not naturally emerge or persist in natural ecosystems.

Natural Selection

The avian influenza virus genome is composed of 8 segments of negative single-stranded RNA coding for 11 proteins. Replication by these viruses is termed low fidelity because RNA mutations, due to imprecision in the replication processes, lead to a wide diversity of genetic variations in progeny. Genetic reassortment between segments of different virus subtypes during co-infection of a host cell further contributes to progeny diversity, providing a basis for rapid evolution and emergence of new avian influenza viruses in the wild (*7*). The switch from an LPAI virus to an HPAI virus phenotype is achieved mainly by introduction of multiple basic amino acid residues into the hemagglutinin cleavage site. This introduction generally occurs in poultry, as has been demonstrated experimentally (*8*,*9*).

Transmission–virulence trade-off models proposing that high rates of pathogen transmission indirectly select for higher levels of virulence have long dominated scientific thinking. Along with recent criticisms of these simplistic models (*10*), we consider that differences in host conditions and environments are also major determinants of virulence evolution. In many host-pathogen interactions, evolution toward an optimal virulence can occur, more or less rapidly, after successful introduction into a new host species (*11*). Virulence evolution involving the occasional switch from an LPAI virus to an HPAI virus, after introduction into domestic birds (*12*), does not solely result from host species switch but is probably driven by a larger set of ecologic parameters encountered in artificial ecosystems. These ecosystems include poultry farming (especially when intensive), free-grazing duck production, and live bird markets. Thus, it is likely that LPAI viruses and HPAI viruses are adapted respectively to natural and artificial ecosystems in which they face different ecologic constraints such as host population structure, density and genetic diversity, and optimal opportunities for virus transmission (Figure).

Under the conditions in which domestic birds are maintained, the range of host species available for infection, compared with natural ecosystems, is considerably reduced and limited mainly to galliform birds and waterfowl, often in monospecific flocks. Poultry host density is often considerably higher than the virus would encounter in the wild, and in intensive systems the high density is maintained throughout the life of the flock. Age structure is generally more uniform and environmental conditions are frequently kept more equable and constant. In addition, wider opportunities for viral transmission exist in the form of multiple physical transport mechanisms for living poultry and their products. The latter mechanisms include feces, feathers, and meat, and physical transport can include cages, packaging, farm workers and their clothes, and vehicles used on farms and over long distances. During these physical modes of transport, the ability of the virus to survive in the various environments encountered is likely to be subject to selection, but at this stage pathogenicity per se to potential hosts will assume no major role, unless this also affects environmental survival. Under these circumstances, selective pressures differ greatly from those encountered by the virus in their natural, primarily aquatic, ecosystems. Avian influenza virus strains that have evolved to survive under these domestic conditions are highly likely to be maladapted to natural ecosystems and hosts. In particular, HPAI viruses, which often induce high and rapid lethality in their hosts, require high and sustained host contact rates that are rare under natural conditions, being restricted to extreme weather conditions or to particular stages of the life cycle.

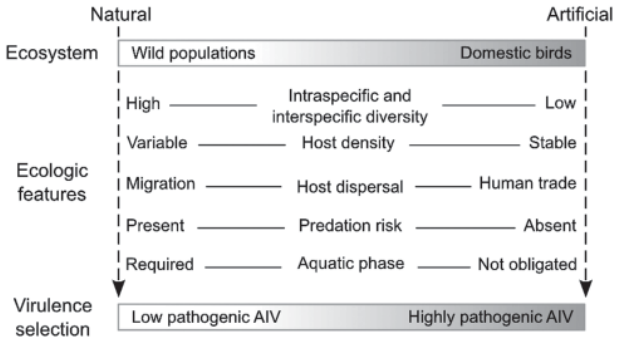

Figure. Comparison of natural versus artificial ecosystems showing different ecologic constraints for evolution of avian influenza virus (AIV).

HPAI viruses in wild birds appear to retain their high virulence, leading to infections being discovered almost entirely in sick or dead birds (*13*). After HPAI virus outbreaks in wild birds, LPAI viruses with genetic affinities to HPAI virus lineages have not been reported. Although there have been examples of mutual host–pathogen coevolution when new highly virulent viruses enter new wild hosts, e.g., amelioration of myxomatosis virulence in wild rabbit (*Oryctolagus cuniculus*) populations (*14*), there is no indication that co-evolution occurs when HPAI viruses gain access to wild bird populations.

Host Density and Diversity

The influence of host population structure in selection for virulence is often critical (*15*). Levels of virulence are, in part, determined by and proportional to the frequency with which interhost transmission opportunities occur (*10*). Low virulence can be selected for when host–host contacts are infrequent, and high virulence can be selected when the host contact rate is high (*16*). In wild bird populations, contact rates between individuals differ markedly between seasons (e.g., reproductive period, molt, migration, wintering), species (e.g., colonial birds), and age. High densities of birds are reached by some species in molting and wintering areas. However, these seasonal aggregations do not lead to selection and emergence of HPAI viruses. Compared with conditions in the wild, host densities in farming conditions are not only high (even extreme under intensive poultry production), but consistently so, which may be a major determinant for selection of high virulence.

In the wild, in addition to variable host contact and thus transmission rates, avian influenza viruses encounter high host-species diversity, as in multispecies waterfowl aggregations. In this context, generalist pathogens are probably favored because they can infect a large spectrum of host species, thereby maximizing replication and dispersal opportunities (*17*). LPAI viruses have been isolated from >105 bird species in 26 families (*13*), suggesting that they are able to infect a large diversity of host species. Under farming conditions, the low (sometimes null) host species diversity is likely to select for species-specific or ecosystem-specific pathogens.

Recently, some studies investigated species-related variation in susceptibility (from ducks to passerine birds) and clinical signs generated by infection with HPAI virus (H5N1). Among the bird species artificially challenged with virus, some laboratory-maintained mallards (*Anas platyrhynchos*) did not show clinical or pathologic signs related to HPAI virus (H5N1) infections (*18*,*19*). Variations related to virus excretion have been reported between studies, but 1 study reported that the domestic mallard can excrete HPAI virus (H5N1) for long periods (≤17 days depending on the virus strain) (*20*). Such a long duration contrasts with LPAI virus excretion recently reported in wild mallard populations, in which virus was shed for ≤8 days with a mean minimum duration of only 3 days (*21*). The domestic mallard could act as an efficient host reservoir in domestic birds and favor viral transmission during an extended period, without clinical evidence of infection. Such evolution of host specificity is believed to have contributed to the spread and endemicity of HPAI viruses (H5N1) in Asia through domestic ducks (*20*,*22*,*23*).

Similarly, LPAI viruses must evolve in the face of intraspecific variability between individuals of a host population. In the wild, immune response varies among birds of the same species, depending on their genetic backgrounds, age, breeding, molting, migration, health status, and exposure to previous infections with pathogens and other parasites (*24*). LPAI viruses are adapted to this diversity in wild birds. Conversely, under many modern farming conditions, potential hosts have low genetic diversity and highly structured age distribution and are regularly protected from some pathogens through vaccination and antimicrobial drugs. Such artificial conditions are likely to select for specialist pathogens. Knowledge on the effects of intraspecific diversity on the evolution of avian influenza viruses is limited to extrapolations from experimental studies. However, these experimental conditions do not reflect intraspecies diversity encountered in the wild and avoid detection of potential individual variation in response to avian influenza virus infection, according to host life history traits. Development of experimental and theoretical studies focusing on the effects of host density and diversity at the community and species levels and in different environments (ranging from totally enclosed to open air) should provide useful information regarding evolution of virulence of avian influenza viruses in natural and artificial ecosystems.

Should I Kill My Host?

In many host–pathogen associations, the pathogenesis related to infection leads to an overall weakening of infected individuals. Although LPAI virus infections in wild birds are generally considered to be benign, in poultry they can cause mild disease, depression, and problems with egg production (*25*). However, physiologic and behavioral effects in wild birds may have been overlooked. van Gils et al. (*26*) reported impaired foraging and migration efficiencies in infected Bewick swans (*Cygnus columbianus bewickii*), suggesting that host behavior might be affected by LPAI viruses in subtle ways not previously envisaged. Latorre-Margalef et al. (*21*) also reported that body mass was lower in infected wild mallards than in uninfected wild mallards and that the amount of virus shed by infected juveniles was negatively correlated with body mass. These recently discovered effects of LPAI virus infection, although mild, could nevertheless have implications for host fitness.

For example, delayed migration and impaired foraging of Bewick swans likely retarded their arrival on their arctic breeding grounds, reducing the chances of these birds reproducing successfully in the year of infection. The implications of such mild symptoms for transmission and evolution of avian influenza viruses remain to be determined.

Given that predators sometimes prey preferentially on sick animals, differential susceptibility between several host species (or individuals) can lead to greater predation on the more susceptible ones (*27*). Adverse physiologic or behavioral effects of infection might decrease host antipredator performance, favor predation on infected birds, and thus decrease transmission of avian influenza viruses among target hosts of the pathogen (i.e., immunologically unexposed waterbirds). Conversely, host antipredator performance reduction can favor infection of predators, and host fatality can lead to infection in scavengers. Such virus transmission between prey and predators and scavengers has been shown for HPAI virus (H5N1), with reported deaths in crows, birds of prey, and mammals (e.g., Felidae and Mustelidae). However, transmission of avian influenza viruses has not been sustained within predator and scavenger populations, indicating that these host infections represent a dead-end for virus transmission. Viruses inducing strong physiologic effects, even if they do not directly lead to death, are therefore unlikely to be selected in wild waterbird populations if they affect host antipredator performance. Studies focusing on differential fitness and predation rates on avian influenza virus–infected wild bird species, considering the effects of the infection itself, but also differences in behavior induced by infection, would help to clarify these aspects. In terms of fitness, experiments on artificially infected captive waterfowl, examining egg mass, clutch size, incubation behavior, hatching success, chick mass, and chick survival could also be illuminating.

Pathogen distributions and abundances in the environment can alter the decisions hosts make with regard to whether to stay and resist or to disperse from 1 place to another with lower pathogen risk (*28*). Pathogen transmission and dispersal are intricately linked, and it is widely acknowledged that pathogens may benefit from investing in dispersal strategies. Dispersal of avian influenza viruses is poorly documented in natural conditions. For HPAI viruses, current knowledge of the potential for virus dispersal through long-distance migration is mainly limited to extrapolations from experiments on captive-reared ducks performed under laboratory conditions (*19*). These birds are not subjected to sustained high-energy expenditure and unlikely to experience immunosuppression, for which there is increasing evidence in birds undertaking migration (*29*). Although infected birds might be able to disperse virus over short distances, e.g., during periods of cold weather (*4*), experiments in which birds are subjected to physiologic stresses associated with migration are needed to determine their capacity to spread virus over long distances. Experimental studies with captive wild waterbirds could test responses to infection during exposure to realistic physiologic or nutritional stresses that replicate long-distance migration or winter food shortage. In addition to monitoring the extent of virus shedding, effects on physical activity, response to stimuli, or time spent feeding should be investigated. Indirect estimations of virus dispersal derived from knowledge of bird migrations could also provide complementary information related to the spread of avian influenza viruses.

Life Outside the Host

Transmission of LPAI viruses among wild waterbirds is considered to be mainly by the fecal–oral route, with virus particles excreted from infected birds directly from feces into water and contracted by potential hosts by ingestion of virions in water or on food therein (*30*). Although no evidence has been provided, potential fecal–fecal transmission through fecal drinking could also favor infection of the cloaca and the lower part of the digestive system. Recent studies have highlighted that HPAI virus (H5N1) replicates more (and for longer periods) in the host bird trachea than in the digestive tract (*18*,*19*). In addition, severe lung congestion and alveolar and bronchiolar edema, together with virus isolation from tracheal swab specimens, suggested that oropharyngeal excretion occurred in infected wild birds (*31*). Preponderance of oropharyngeal excretion is associated with systemic infections caused by HPAI viruses, in contrast to the propensity for cloacal virus excretion associated with digestive tract infections of LPAI viruses. Thus, oropharyngeal and fecal excretion represent 2 strategies that may be selected according to ecosystem characteristics. Production of viral particles in aerosols is probably the most efficient transmission strategy in confined environments with high densities of birds, high ambient temperature and humidity, and forced air circulation, as under intensive farming conditions (*32*). Selection for systemic infection, accompanied by oropharyngeal excretion and airborne transmission, could potentially be favored under these circumstances (*33*). Experimental studies focusing on the evolution of wild-origin LPAI viruses in domestic birds in confined environments could provide interesting insights regarding selection for oropharyngeal excretion and airborne transmission.

However, after excretion, virus must survive in the environment long enough to be able to contact and infect susceptible hosts. Although persistence of avian influenza viruses in water appears to be the natural mechanism to maintain and transmit influenza viruses in wild bird populations, Brown et al. (*34*) compared 2 strains of HPAI virus (H5N1) with several wild bird–origin LPAI viruses and found that HPAI virus (H5N1) does not persist in water as long as LPAI vi-

ruses, at least under experimental conditions. This finding suggests that HPAI viruses could be less adapted than LPAI viruses to spread by the fecal–oral route in water. Inactivation processes of avian influenza viruses in the environment are far from being well understood, but abiotic factors such as salinity, temperature, relative humidity, or ultraviolet radiation are likely to play a key role (*33*,*35*).

Avian Influenza Virus in a Human-Made World

Technologic and cultural changes in human populations open new ecologic niches for pathogens, which differ from niches available in the wild, and inevitably influence their evolution (*36*). Networks of poultry production are likely to favor persistence of virulent strains, with continuous circulation of avian influenza viruses between connected farms or markets. Such networks probably favor the endemicity of HPAI virus (H5N1) in Southeast Asia (*6*). Multispecies live-animal markets are good examples of how humans have artificially created a dynamic system in which a large variety of avian influenza viruses can be generated and maintained, thus offering enhanced opportunities for genetic reassortments (*37*). Connectivity in modern human populations through transportation has increased during the past century, especially during the past few decades, in volume and, with regard to virus spread, in speed and geographic extent. The past 2 decades have seen a huge increase in poultry production and associated national and international trade in Southeast Asia. After adapting to intensive farming processes, avian influenza viruses can be spread intercontinentally among domestic bird populations by human activities. This finding appears to be the most likely scenario for spread of HPAI virus (H5N1) from Asia to Europe and Africa, in which the poultry trade (legal, unregulated, and illegal) seems to have been the predominant mechanism (*38*).

Thus, human activities are likely to artificially shape evolutionary ecology of avian influenza viruses and select for traits (e.g., virulence, oropharyngeal excretion, host specialization) that confer optimal viral fitness under the artificial conditions of poultry production, distribution, and processing. Evolution of this host–pathogen system created by humans might represent one of the main threats to human health. Because of an increasing number of studies focused on genetic characteristics of avian influenza viruses, we are aware of the mechanistic basis of high pathogenesis. However, our efforts to predict and control emergence of these viruses through this complex host–pathogen system must consider host ecology and ecosystem characteristics (natural or linked with human activities) in which these viruses evolve.

If application of evolutionary theory to medical sciences enables a predictive framework for long-term host–pathogen interactions, it also provides interesting possibilities for design of medical and public health protection measures (*39*). Integration of ecologic and evolutionary theory in epidemiology and human diseases has shown increased interest (*40*). These theories could provide useful information for long-term disease management. However, such approaches and their possible applications for avian influenza viruses are lacking.

Do we have to favor developments of poorly diversified farming conditions with high densities of genetically impoverished birds? What are the long-term effects of mass vaccination? Can we avoid virus exchanges between wild and domestic birds? Answering such key questions first requires sound understanding of natural mechanisms of virulence selection and, from that knowledge, taking account of ecologic features that may select for HPAI viruses in artificial ecosystems.

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Dr Lebarbenchon is a postdoctoral research associate at The University of Georgia, Athens, GA. His research interests are interactions between pathogens and ecosystems, in particular, the ecology and epidemiology of avian influenza viruses in wild birds.

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Address for correspondence: Camille Lebarbenchon, Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, 589 D.W. Brooks Dr, Athens, GA 30602, USA; email: cleb@uga.edu

Extensive Drug Resistance in Malaria and Tuberculosis

Chansuda Wongsrichanalai, Jay K. Varma, Jonathan J. Juliano, Michael E. Kimerling, and John R. MacArthur

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Learning Objectives

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

- Define multidrug-resistant tuberculosis and describe effective management strategies.
- Identify multidrug-resistant malaria and discuss management issues.

Editor

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CME Author

Charles P. Vega, MD, Associate Professor; Residency Director, Department of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships..*

Authors

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Drug resistance in malaria and in tuberculosis (TB) are major global health problems. Although the terms multidrug-resistant TB and extensively drug-resistant TB are precisely defined, the term multidrug resistance is often loosely used when discussing malaria. Recent declines in the clinical effectiveness of antimalarial drugs, including artemisinin-based combination therapy, have prompted the need to revise the definitions of and/or to recategorize antimalarial drug resistance to include extensively drug-resistant malaria. Applying precise case definitions to different levels of drug resistance in malaria and TB is useful for individual patient care and for public health.

Author affiliations: US Agency for International Development, Bangkok, Thailand (C. Wongsrichanalai, J.R. MacArthur); US Centers for Disease Control and Prevention, Beijing, People's Republic of China (J.K. Varma); US Centers for Disease Control and Prevention, Atlanta, Georgia, USA (J.K. Varma, J.R. MacArthur); University of North Carolina School of Medicine, Chapel Hill, North Carolina, USA (J.J. Juliano); and The Bill and Melinda Gates Foundation, Seattle, Washington, USA (M.E. Kimerling)

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Malaria and tuberculosis (TB) are 2 of the most common infectious diseases in resource-limited countries. Each year, >1.7 million persons die of TB (*1*) and almost 1 million die of malaria (*2*). For each disease, emergence of resistance to common first-line therapies has been a major challenge to disease control. Drug resistance in TB and malaria arises from inadequate or inappropriate use of antimicrobial agents; however, the definitions used to classify drug resistance, as well as the public health control measures, vary. These differences sometimes lead to confusion and misinterpretation by those unfamiliar with either or both diseases. This confusion is compounded by the fact that each pathogen is increasingly resistant to more drugs, and new descriptive terms such as multidrug resistant (MDR) and, for TB, extensively drug-resistant (XDR) have been introduced to describe these changes. For TB,

definitions of these new terms are now widely accepted. For malaria, recent changes in clinical effectiveness of antimalarial drugs, in particular the emergence of artemisinin resistance, are forcing malaria experts to consider revising the definitions of drug resistance for *Plasmodium falciparum.* Without clear definitions, prioritization of resources to treat and control malaria will be difficult.

Categories of Drug Resistance

Tuberculosis

Drug-resistant TB was identified shortly after the first anti-TB drugs were introduced in the 1940s (*3*); the term refers to TB strains resistant to at least 1 anti-TB drug, usually determined by in vitro phenotypic methods (e.g., mycobacterial culture). Globally, resistance to a single anti-TB drug is the most common pattern of drug resistance. Recognition of the relatively rapid onset of resistance to anti-TB monotherapy, usually within months, led to the development of multidrug therapy as the standard of care in the 1960s (*4*).

Although TB can be treated with many drugs and a strain of TB can be resistant to any or all of these, an international consensus evolved to define MDR TB as resistance to at least isoniazid and rifampin. Resistance to other anti-TB drugs, without resistance to both isoniazid and rifampin, is defined as polydrug resistance. This consensus definition of what level of resistance constitutes MDR was based on data showing that anti-TB chemotherapy was most likely to fail if the TB strain was resistant to isoniazid and rifampin at the beginning of treatment (*5*). The recognition that MDR strains further evolved with resistance to a selected group of reserve, or second-line, anti-TB drugs led to creation of the term XDR TB in 2006 (*6*). The definition of XDR TB is resistance to isoniazid and rifampin plus resistance to any fluoroquinolone and at least 1 of the 3 injectable second-line drugs used in TB treatment (amikacin, kanamicin, or capreomycin) (*7*).

Malaria

Although the decreased sensitivity of malaria parasites to an antimalarial drug was first reported about a century ago in association with quinine, the term drug-resistant malaria was rarely used; resistance was not considered a major problem until the late 1950s, after chloroquine resistance emerged. Historically, chloroquine was widely used as the standard first-line drug against *P. falciparum*. Resistance was first detected on the Thailand–Cambodia and the Venezuela–Colombia borders, near areas where chloroquinated salt was used for malaria control, forcing the affected countries to begin switching to sulfadoxine–pyrimethamine (SP) in the 1970s. Resistance to SP developed quickly, again on the Thailand–Cambodia border. The spread of chloroquine and SP resistance to other parts of Asia and as far as Africa is well documented (*8*). A review of the development of drug-resistant malaria is available elsewhere (*9*). Only drug resistance in *P. falciparum* will be discussed in this article.

The term drug-resistant malaria originally referred to *P. falciparum* strains resistant to chloroquine, SP, or both. Multidrug resistance of *P. falciparum* is strictly defined as resistance to >2 antimalarial compounds of different chemical classes, recommended by the National Malaria Control Program (NMCP) (*10*). The Thailand–Cambodia border was the first area to be recognized as a multidrug resistant zone because of the successive failure of chloroquine, SP, and then mefloquine in the late 1980s. These antimalarial drugs belong to different chemical classes; all were designated as the first-line drugs against falciparum malaria by the Thai NMCP and were also used by the Cambodian NMCP.

Relationship between Drug Resistance and Treatment

For new cases of TB, treatment is usually a 4-drug regimen of isoniazid, rifampin, pyrazinamide, and ethambutol for 2 months (intensive phase), followed by 4–6 months of only isoniazid and rifampin or isoniazid and ethambutol (continuation phase). The duration of this standardized regimen may vary, depending on a number of factors, including economic considerations and availability of culture-based diagnosis and monitoring. For example, United States guidelines, in contrast to those of most resource-constrained and developing countries, emphasize mycobacterial culture for TB diagnosis and recommend extending TB treatment for patients with cavities visible on chest radiograph and persistence of positive sputum cultures after 2 months of treatment.

To prevent inadequate drug ingestion and thereby resistance, staff in TB programs often directly observe patients ingesting their medications. Because directly observed therapy requires substantial human resources, the World Health Organization (WHO) recommends that directly observed therapy should be used any time that rifampin is administered. Many countries began using a combination of isoniazid and ethambutol, rather than isoniazid and rifampin, in the continuation phase, because of the cost of rifampin and an inability to provide directly observed therapy for the entire duration of this phase. The most common reason to vary the treatment regimen is documented drug resistance or a history of previous TB treatment, which is a risk factor for resistance development. In most developing countries, drug resistance data are sparse because confirmation of infection with *Mycobacterium tuberculosis* followed by drug susceptibility testing requires use of advanced molecular diagnostics and/or slower and more laborious culture methods. Therefore, patients are usually treated on the assump-

tion that they are infected with a drug-susceptible strain. The drug regimen is usually changed only if the patient's condition does not clinically improve, including having persistently positive sputum smears, after months of treatment. Retreatment protocols in most countries require prolonged therapy with essentially the same basic drugs before the patient is eligible to receive a drug regimen containing second-line drugs specifically for treatment of MDR TB.

Although multidrug regimens to prevent and treat drug-resistant TB were first evaluated in the 1950s, use of true combination therapy for malaria, the simultaneous use of ≥2 drugs (with independent modes of action and different chemical targets) to kill asexual blood-stage parasites, did not arise until much later. However, during the past decade, combination therapy has become the norm, intended to improve effectiveness and reduce the spread of resistance.

In uncomplicated malaria, an outpatient is usually treated with the first-line antimalarial drugs recommended by the local health authority for the malaria-endemic region in which the patient became infected. For example, a patient infected with *P. falciparum* on the eastern Thailand–Laos border would be treated with an artesunate–mefloquine combination plus primaquine at a government malaria clinic on the Thailand side of the border, or with an artemether–lumefantrine combination (Coartem; Novartis AG, Basel, Switzerland) on the Laos side of the border. For travelers returning to their home country outside a malaria-endemic area, different drugs may be prescribed. In none of these situations would a physician expect any laboratory tests to determine drug susceptibility before making a treatment decision. As an acute, potentially fatal disease, falciparum malaria requires effective treatment promptly.

For malaria, the geographic location in which infection is acquired is the primary determinant of the risk for a drug-resistant infection. Unlike MDR TB, the decision to treat and the treatment of MDR malaria do not require complex clinical and laboratory assessment of an individual patient's isolate, except for severe malaria, which requires critical care capacity. For TB, the geographic area in which infection is acquired is not as reliable a determinant of the treatment choice. Despite wide differences in MDR TB prevalence across countries, the most reliable predictors of MDR risk for a TB patient are a history of prior treatment or known exposure to another case-patient (i.e., contact with an index MDR TB case-patient), not geography (*11*).

Public Health Implications

Knowing the drug susceptibility pattern of a TB strain or whether a malaria infection was acquired in a specific malaria-endemic area helps not only with therapeutic decision making but also with predicting the patient's prognosis. From the public health perspective, information on drug resistance is useful for strategic planning.

The proportion of TB case-patients infected with MDR strains, when stratified by previous treatment status, helps public health officials evaluate the intensity of community transmission and the strength of the TB program in curing patients. Unfortunately, the absence of continuous, systematic, representative, and timely drug susceptibility data, especially for second-line anti-TB drugs, is a major obstacle for the control of drug-resistant TB. Consequently, a large number of infectious MDR- and XDR TB cases globally may go undiagnosed.

For malaria, the level of drug resistance in a specific disease-endemic area is usually judged by in vivo therapeutic efficacy monitoring and in vitro drug susceptibility assays of malaria-infected blood specimens (*12*). To determine the trend of drug efficacy over years, each method requires sentinel sites, specially trained staff, and sustained efforts backed by steady public health policy. In fact, the simplest way to monitor the clinical efficacy of a given routine therapeutic regimen against the parasite is universal, comprehensive, posttherapeutic follow-up of the patients for 28–42 days. Such a procedure would draw early attention to the possibility of specific drug resistance, thus prompting appropriate investigations to avoid any possible delay in the confirmation of resistance; delay is an inherent shortcoming when observations are restricted to programmatic efficacy surveillance studies. Unfortunately, in practice this monitoring is sometimes difficult to achieve among some populations, such as mobile migrant populations, who are at high risk along many international borders.

Application of molecular surveys can also be useful for identifying regions at risk for emergence of antimalarial drug resistance and alerting program management of the need to conduct in-depth studies, thus allowing sufficient time for consideration of drug policy change (*8*,*13*). The availability of molecular markers for more drugs, markers with improved accuracy, improvement in the ease of assays, and the lowering of assay-associated costs will enhance the usefulness of molecular mapping of drug resistance.

For TB, the term XDR was created to describe not only TB strains that are resistant to more of the available drugs but also infections that are substantially more difficult to cure. For example, for patients co-infected with HIV, XDR TB is often fatal (*11*). An equivalent term for malaria does not yet exist, although infections with similar characteristics—resistance developed successively to more drugs and the lack of alternative drug choices—will represent identical challenges to control programs.

When the term MDR malaria was first introduced 2 decades ago, it was intended to describe resistance to new drug groups other than the common, standard antimalarial drugs used at that time, namely, chloroquine (a 4-aminoquinoline) and SP (antifolates). The term was first applied to the Thailand–Cambodia border after the emergence of

mefloquine resistance. Artemisinin was introduced, in the form of artemisinin-based combination therapy (ACT), as a replacement for mefloquine monotherapy. Artesunate–mefloquine became the first ACT to be used for the control of MDR malaria. It was adopted as the first-line therapy for falciparum malaria by the Thai NMCP in 1995 and the Cambodian NMCP in 2000. The rapid antimalarial activity of artemisinin compounds means that they are most effective when used together with a partner drug that possesses a longer half-life (e.g., mefloquine), thus the rationale behind the combination. The effectiveness of artemisinin against MDR malaria is always cited as 1 of its advantageous characteristics (*14*). Recent evidence of the failure of artesunate–mefloquine combination therapy on the Thailand–Cambodia border and of resistance to artemisinin (*15*,*16*) has raised concerns about the failure of the last effective antimalarial drugs. The loss of artemisinins could negatively affect global public health because it would jeopardize effective malaria control, leading to increases in illness and eventually deaths. Malaria elimination, which has recently regained much interest, is also threatened by artemisinin failure (*17*).

The Case for Defining XDR Malaria

The emergence of artemisinin resistance creates the need to define a new subgroup of drug-resistant malaria, XDR malaria**.** Such a label should be considered because it signifies the potential loss of artemisinin and highlights the threat of an expanded malaria-endemic area with poor ACT efficacy, similar to prior global spread of antimalarial drug resistance for other chemical classes. From the public health viewpoint, this situation must be handled carefully, beyond the existing WHO recommendations for malaria drug policy revision (*18*). The Cambodian and the Thai NMCPs are implementing special priority control measures against this high level of drug-resistant malaria. They are working together to contain artemisinin-resistant parasites; their ambitious goal is to eliminate falciparum malaria from this epicenter of resistance (*19*). To do so, they face a number of challenges, including the lack of a suitable alternative antimalarial drug for empiric treatment.

Not all malaria-endemic countries have well-documented, successive development of resistance to multiple drugs of different chemical classes, as has been documented on the Thailand–Cambodia border. Although artemisinin resistance has not been described outside Asia, a situation leading to similar development of extensively drug-resistant parasites will likely happen elsewhere. Africa is a primary concern because of its high prevalence of malaria. Chloroquine resistance and high-level SP resistance are highly prevalent in parts of Africa such as Kenya (*20*). A possible scenario of concern is the consolidation of amodiaquine and SP resistance and/or development of a new resistant strain against lumefantrine and/or mefloquine (which is known for its activity correlation with lumefantrine in vitro). In recent years, the combination of artemether and lumefantrine, or Coartem, was introduced in large scale to Africa. High rates of recurrence of *P. falciparum* infection have already been found in Zanzibar after Coartem therapy and include several cases of recrudescence associated with lumefantrine-resistant parasites (*21*). Although an ACT containing mefloquine has never been adopted by any African country, the artesunate–mefloquine combination is already common in some African markets. Antimalarial drugs that are not used, per national treatment guidelines, can be widely available enough, especially in the private sector of low-income countries, to induce selection pressure for resistance or cross-resistance.

After 2 decades of use, the term MDR malaria is not the trigger for action it once was. Designating a malaria-endemic area with artemisinin-resistant falciparum strains as an area with XDR malaria will signal an urgent need for action, such as ongoing public health attention and prioritizing funding and support. A similar sense of complacency with regard to MDR TB, the loss of treatment utility, and the need to instill urgency into global efforts to prevent and treat drug resistance led WHO to establish the term XDR TB in 2006 (*7*).

In most settings, the primary way to control XDR TB is to prevent its emergence through optimal treatment of drug-susceptible TB (*22*). Therefore, from a control program's perspective, knowing that XDR TB exists in the community does not alter the general Stop TB strategy (www.who.int/tb/strategy/en/). However, specific determination of drug resistance is essential for clinical management. The cost of treating MDR or XDR TB cases that do emerge is high and requires extra public health resources, including a greatly expanded and upgraded laboratory network and access to specialized physician and nursing care. Similarly, the development of any drug resistance in malaria introduces an additional set of financial and operational challenges for a malaria control program.

For clinicians dealing with sporadic malaria cases outside a disease-endemic area, the term XDR malaria also deserves special attention. For example, Coartem is often prescribed for drug-resistant malaria. Given its potentially limited efficacy against falciparum malaria in western Cambodia (*23*) and activity correlation between mefloquine and lumefantrine in vitro, its prescription for patients from XDR malaria areas will need to be reconsidered.

Introducing the term XDR malaria in association with artemisinin resistance should not discourage the deployment of ACTs in Africa, where it is hoped that these regimens will contribute to substantial reduction of malaria incidence and deaths. However, it will help alert countries that have recently adopted ACTs that resistance is pos-

sible and that vigilance in monitoring for resistance and reinforcement of rational drug use are essential. Control programs sometimes strongly strive for universal access to ACTs but are unable to regulate or ensure their rational use, especially when ACTs are widely available in the private and informal healthcare sectors (*24*). Such a balance of concerns should be redressed.

Conclusions

For TB and malaria control, providing specific labels to drug-resistant strains benefits individual patient care and public health. For TB, agreement on specific definitions for MDR and XDR TB facilitated epidemiologic assessment, program planning, laboratory capacity enhancement, and development of standardized treatment regimens. For malaria, the rapid increase in the prevalence of drug-resistant malaria globally and emerging artemisinin resistance in Southeast Asia show that the time to define and combat XDR malaria has arrived.

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Dr Wongsrichanalai is an epidemiologist with the US Agency for International Development Regional Development Mission for Asia, based in Bangkok, Thailand. Her research interests are drug-resistant malaria and the public health control of infectious diseases in developing countries.

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Address for correspondence: Chansuda Wongsrichanalai, Office of Public Health, USAID RDMA, Athenee Tower, 25th Floor, 63 Wireless Rd, Bangkok 10330, Thailand; email: cwongsrichanalai@usaid.gov

Oseltamivir Resistance in Adult Oncology and Hematology Patients Infected with Pandemic (H1N1) 2009 Virus, Australia

Adrian R. Tramontana, Biju George, Aeron C. Hurt, Joseph S. Doyle, Katherine Langan, Alistair B. Reid, Janet M. Harper, Karin Thursky, Leon J. Worth, Dominic E Dwyer, C. Orla Morrissey, Paul D.R. Johnson, Kirsty L. Buising, Simon James Harrison, John F. Seymour, Patricia E. Ferguson, Bin Wang, Justin T. Denholm, Allen C. Cheng, and Monica Slavin

We describe laboratory-confirmed influenza A pandemic (H1N1) 2009 in 17 hospitalized recipients of a hematopoietic stem cell transplant (HSCT) (8 allogeneic) and in 15 patients with malignancy treated at 6 Australian tertiary centers during winter 2009. Ten (31.3%) patients were admitted to intensive care, and 9 of them were HSCT recipients. All recipients of allogeneic HSCT with infection <100 days posttransplantation or severe graft-versus-host disease were admitted to an intensive care unit. In-hospital mortality rate was 21.9% (7/32). The H275Y neuraminidase mutation, which confers oseltamivir resistance developed in 4 of 7 patients with PCR positive for influenza after ≥4 days of oseltamivir therapy. Three of these 4 patients were critically ill. Oseltamivir resistance in 4 (13.3%) of 30 patients who were administered oseltamivir highlights the need for ongoing surveillance of such resistance and further research on optimal antiviral therapy in the immunocompromised.

Author affiliations: Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia (A.R. Tramontana, K. Thursky, L.J. Worth, S.J. Harrison, J.F. Seymour, M. Slavin); Western Hospital, Footscray, Victoria, Australia (A.R. Tramontana); Westmead Hospital, Sydney, New South Wales, Australia (B. George, D.E. Dwyer, B. Wang), World Health Organization Collaborating Centre for Reference and Research on Influenza, North Melbourne, Victoria, Australia (A.C. Hurt); The Alfred Hospital, Prahran, Victoria, Australia (J.S. Doyle, C.O. Morrissey); Austin Health, Heidelberg, Victoria, Australia (K. Langan, P.D.R. Johnson); St. Vincent's Hospital, Melbourne, Victoria, Australia (A.B. Reid, K.L. Buising); Royal Melbourne Hospital, Melbourne (J.M. Harper, J.T. Denholm, M. Slavin); Centre for Infectious Diseases and Microbiology, Sydney (P.E. Ferguson); and Monash University, Prahran (A.C. Cheng)

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Immunocompromised patients are at risk for serious complications from seasonal influenza (*1*). This group of patients has also been disproportionately represented among those with severe infections from influenza A pandemic (H1N1) 2009, comprising 3.4%–19.6% of patients admitted to intensive care units in case series from North America (*2–4*).

The protective effect of seasonal influenza vaccination is reduced in patients with hematologic malignancy and recipients of an allogeneic hematopoietic stem cell transplant (HSCT) (*5*,*6*). Therefore, these patients are likely to remain at increased risk for complications from the pandemic (H1N1) 2009 virus, despite the availability of an effective vaccine. Furthermore, the emergence of resistance to neuraminidase inhibitors may limit the utility of prophylaxis in this population (*7–12*).

During 2009, the predominant strain of influenza in Australia was influenza A pandemic (H1N1) 2009 virus (*13*,*14*). In the state of Victoria, after the initial 3 months of community transmission of pandemic (H1N1) 2009 virus, 50% of patients who died had an underlying hematologic malignancy (*15*). We describe in detail the clinical features, treatment, and outcomes of immunocompromised patients hospitalized in 6 tertiary centers in Australia during winter 2009.

Study Design and Population

Six Australian tertiary centers, 5 in Melbourne, Victoria, and 1 in Sydney, New South Wales (NSW), participated in the study. The 5 centers in Melbourne provide most specialist adult hematology services to the state of Victoria (population 5.4 million), and 2 of these centers perform all

allogeneic HSCTs for the state. The participating Sydney center is the largest of 2 centers that perform adult allogeneic HSCT for the state of NSW (population 7 million). Approval for this study was obtained from human research ethics committees of each center.

Patients were included in the study if they had the following characteristics: 1) ≥18 years of age hospitalized at 1 of the 5 Melbourne study centers during May 1–August 30, 2009, or hospitalized at the Sydney center during May 1–September 15, 2009; 2) recipient of an HSCT or had an underlying malignancy (hematologic or solid tumor); and 3) had laboratory-confirmed pandemic (H1N1) 2009 virus infection identified by nucleic acid testing (NAT) during their hospital stay. At each center, investigators were directly involved in active surveillance and management of patients with pandemic (H1N1) 2009, allowing cases to be identified. Data were retrospectively abstracted onto standardized case record forms.

Influenza Virus Diagnostics

All cases were confirmed in laboratories whose performance is accredited by the National Association of Testing Authorities in Australia. For the Victorian cases, all but 1 case-patient had laboratory confirmation of pandemic (H1N1) 2009 at the Victorian Infectious Diseases Reference Laboratory (VIDRL) with pandemic (H1N1) 2009–specific NAT by using reverse transcriptase–PCR (RT-PCR) as described (*16*). NAT for the other case-patient from Victoria was performed by the laboratory at the hospital where the patient was treated. The NSW cases were all confirmed at the state's reference laboratory with RT-PCR assays as previously described (*17*). Patient specimens had repeat NAT at the discretion of the treating clinicians. Virus isolates or clinical samples from patients whose NAT results were positive after 4 days of oseltamivir therapy were analyzed to determine the presence of the H275Y neuraminidase (NA) mutation (N1 numbering) by using either a pyrosequencing assay (Biotage AB, Uppsala, Sweden) according to the manufacturer's instructions, or rolling circle amplification (*18*). The H275Y mutation confers high-level oseltamivir resistance and has been detected in all of the oseltamivir-resistant pandemic (H1N1) 2009 viruses reported to date, as well as in local circulating seasonal influenza A (H1N1) strains (*8*,*12*). Samples also underwent NAT for other respiratory viruses by using a multiplex PCR.

Definitions and Statistics

Data were stored in Microsoft Access 2003 (Microsoft, Redmond, WA, USA), and descriptive statistics were summarized with proportional outcomes. Nosocomial acquisition was defined as the development of symptoms attributable to pandemic influenza after 48 hours in the hospital that were not present on admission. Steroid-refractory or grade III–IV graft-versus-host-disease (GVHD) was defined as severe GVHD.

Length of stay was calculated as length of hospital admission or period of confinement after onset of symptoms for those with nosocomial acquisition. Epidemic curves for the 2 states were aligned with epidemic curves of community pandemic influenza activity ascertained from surveillance data (*14*,*19*). Daily corticosteroid doses were calculated by using relative glucocorticoid potency to convert to prednisolone equivalents, and mean daily values were determined for patients who received regular intermittent dosing (*20*).

Thirty-two patients fulfilled the inclusion criteria. Patient demographics and clinical features are summarized in Table 1. Seven (21.7%) of the 32 patients died; median length of stay was 6.5 d (interquartile range 4.0–13.5 d).

Patient Demographics and Baseline Features

Eight patients were recipients of an allogeneic HSCT (Table 1). Six patients had a human leukocyte antigen (HLA) matched related, 1 (patient 1) an HLA mismatched related, and 1 (patient 7) an HLA matched unrelated allogeneic HSCT. Patient 6 was the recipient of 2 allogeneic HSCTs. Three patients (5, 6, and 8) were diagnosed with pandemic (H1N1) 2009 within 100 days of the allogeneic HSCT. Six were receiving calcineurin inhibitors. Patient 3 was the only allogeneic HSCT recipient that had documented recurrence of underlying disease posttransplant.

At the time they sought treatment, all 8 recipients of autologous HSCT were ≥2 years posttransplant. These patients had their malignancy diagnosed between 5 and 8 years before pandemic (H1N1) 2009, except patient 11, who had multiple myeloma that had been diagnosed 3 years previously. All autologous HSCT recipients had a relapse of malignancy after transplantation, and 7 of these patients were continuing to receive active treatment for malignancy.

Two patients (26 and 29) who had had recent a diagnosis of malignancy were in remission and continuing on the primary chemotherapy treatment plan. Seven nontransplant recipients had either not shown a response or were deemed by their treating clinicians to be refractory to their current treatments. Patient 17 had an influenza-like illness (ILI) before admission for chemotherapy and autologous HSCT (further details below). Sixteen patients (3 allogeneic HSCT recipients, 6 autologous HSCT recipients, and 10 nontransplant recipients) were receiving corticosteroids before the onset of pandemic (H1N1) 2009 infection. The mean dose in prednisolone equivalents was 40.9 mg/d (range 5.0–156 mg/d).

Findings at Initial Visit

Clinical features at patients' initial visit coincided with peak activity of pandemic (H1N1) 2009 in the Victorian and

NSW communities (Figure; online Technical Appendix, www.cdc.gov/EID/content/16/7/1068-Techapp.pdf). Patient 17 acquired the infection during interstate travel and sought treatment in late August, 2 days after return to Melbourne. Four patients had contact with family members with ILI, and 4 patients had nosocomial acquisition without an identifiable primary or index case (Table 1).

Most patients exhibited fever (94%) and cough (91%). Dyspnea, sore throat, and rhinorrhea were reported in 53%, 50%, and 50% of patients, respectively. Sixteen patients (50%) sought treatment within 48 h of symptom onset. Four patients visited the hospital after experiencing prolonged symptoms: for 12 d (patient 4), 14 d (patients 22 and 25), and 21 d (patient 14). Three of these patients had biphasic illnesses with initial prolonged mild upper respiratory tract illnesses and, on examination, had consolidation shown on chest radiograph. Patient 25 had 2 weeks of dyspnea associated with an exacerbation of chronic obstructive pulmonary disease. Patient 16 sought treatment for shock and respiratory failure.

Diagnosis of pandemic (H1N1) 2009 was confirmed by NAT of bronchial washings of 3 patients (2, 16, 17). One of them, patient 17, had symptoms of a viral respiratory tract infection for 20 d with negative results for pandemic (H1N1) 2009 virus by RT-PCR of nasopharyngeal swab specimens taken 2 and 16 d before bronchoscopy. All

Table 1. Demographics and clinical findings among patients who had malignancy or HSCT and influenza A pandemic (H1N1) 2009 virus, Australia*

Patient no.	Age, y/sex	Underlying malignancy	Coexisting conditions	Acquisition	Radiographic infiltrates	ICU	Death
Allogeneic stem cell transplant recipients							
1	44/F	Prolymphocytic leukemia	GVHD, renal	Community†	None	Yes	Yes
2	53/F	Non-Hodgkin lymphoma	GVHD‡	Nosocomial	Multifocal	Yes	Yes
3	33/M	Hodgkin lymphoma	None	Community	None	No	No
4	57/F	CML	GVHD§	Community	Unifocal	No	No
5	61/M	Myelodysplastic syndrome	None	Nosocomial	Multifocal	Yes	Yes
6	56/M	Myelofibrosis	GVHD‡	Community	Multifocal	Yes	Yes
7	61/M	AML	GVHD,§ cardiac	Community	None	No	No
8	63/F	AML	None	Nosocomial	Unifocal	Yes	No
Autologous stem cell transplant recipients							
9	70/M	Multiple myeloma	Pulmonary	Community†	Unifocal	Yes	No
10	50/F	Multiple myeloma	None	Community	Multifocal	Yes	Yes
11	72/M	Multiple myeloma	Type 2 diabetes	Community	None	No	No
12	42/F	Multiple myeloma	Type 2 diabetes	Community	Multifocal	Yes	No
13	57/M	Multiple myeloma	None	Community†	Multifocal	No	No
14	30/F	Hodgkins lymphoma	None	Community	Multifocal	No	No
15	52/F	Non-Hodgkins lymphoma	None	Community	None	No	No
16	68/M	Multiple myeloma	Renal	Community	Multifocal	Yes	Yes
Patients with no prior stem cell transplant							
17	24/F	Hodgkins lymphoma	None	Community¶	None	No	No
18	72/M	AML	Type 2 diabetes	Community	Multifocal	No	No
19	63/F	Multiple myeloma	None	Community	Multifocal	No	No
20	80/F	Aplastic anemia	Cardiac	Community	None	No	No
21	70/F	Hodgkins lymphoma	None	Community	None	No	No
22	61/F	CLL	None	Community†	Multifocal	No	No
23	63/F	Non-Hodgkin lymphoma	Pulmonary	Community†	None	No	No
24	68/M	CLL	Pulmonary	Community	None	Yes	Yes
25	76/M	CLL	Pulmonary	Community	Multifocal	No	No
26	59/F	AML	None	Community	None	No	No
27	47/F	CLL	None	Community	No imaging	No	No
28	57/M	Multiple myeloma	None	Community	Unifocal	No	No
29	56/M	Hodgkin lymphoma	Type 2 diabetes	Community	Multifocal	No	No
30	26/M	ALL	None	Nosocomial	No imaging	No	No
31	55/F	Cervical cancer	None	Community	None	No	No
32	50/F	Breast cancer	None	Community	Multifocal	No	No

*HSCT, hematopoietic stem cell transplant; ICU, intensive care unit; GVHD, graft-versus-host disease; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia.
†Household contacts with influenza-like illness identified.
‡Severe GVHD.
§Chronic GVHD.
¶Patient 17 had onset of influenza-like symptoms on returning to Victoria from interstate travel.

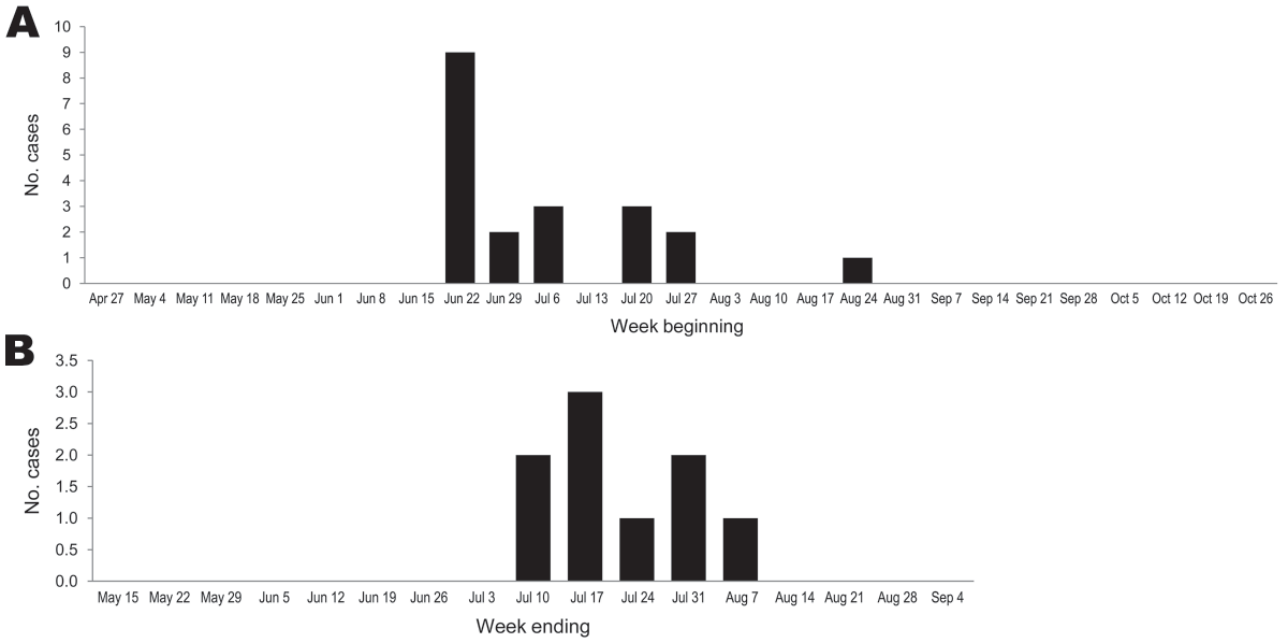

Figure. Date of admission to hospitals in Victoria (A) and New South Wales (B), Australia, for patients with underlying malignancy who were infected with pandemic (H1N1) 2009, April–October 2009. Twelve Victorian and 4 New South Wales patients were recipients of a hematopoietic stem cell transplant. Rates of laboratory detection of all influenza viruses, obtained from population-based epidemic surveillance for Victoria and New South Wales, are given in the online Technical Appendix (www.cdc.gov/EID/content/16/7/1068-Techapp.pdf).

other patients had the diagnosis confirmed on NAT of nasopharyngeal swab samples. Six patients (19%) had a baseline peripheral blood lymphocyte count ≤200 cells/mL, and 18 (56%) patients had pneumonia shown on baseline chest radiograph.

Antiviral Therapy

Thirty patients (94%) were administered oseltamivir therapy. Of these patients, 13 (41%) were administered therapy within 48 hours, and 16 began therapy >48 hours after symptom onset. Duration of illness before receiving oseltamivir was unknown for patient 2. Nine patients received NA inhibitors for >5 days. Three of these patients (1, 12, 18) had a positive NAT result >5 days after oseltamivir treatment began. Antimicrobial drug treatment was changed to zanamir for patients 1 and 8 after they received initial therapy with oseltamivir (see below). The longest duration of antiviral therapy was 43 d (patient 1). Patients 15 and 31 had uncomplicated infections and were not administered oseltamivir therapy.

Co-pathogens

Four patients had notable co-pathogens identified. Patient 2 had 4 concurrent herpesviridae (cytomegalovirus, Epstein-Barr virus, herpes simplex virus 2, and human herpesvirus 6) detected by NAT in respiratory specimens and blood. Treatment for patient 2 included liposomal amphotericin B and foscarnet. Patient 7 had *Staphylococcus aureus* bacteremia from an unrelated source. Patient 17, who came to transplant with an ILI, had bronchial washings that were positive for pandemic (H1N1) 2009 by NAT, galactomannan antigen, and *Aspergillus* spp. by NAT 5 days after transplant. Pulmonary nodules consistent with invasive fungal infection were seen on a high-resolution computed tomography scan. Patient 13 was infected with respiratory syncytial virus, which was detected by multiplex NAT on a nasopharyngeal swab.

Patients Admitted to Intensive Care Unit

Ten (31.3%) patients were admitted to intensive care (Table 1). In each case, the primary reason for admission to intensive care was respiratory failure. Seven (70%) of these patients died, 6 in intensive care and 1 (patient 5) of recurrent pneumonia after being discharged from intensive care. Preterminal events included progressive respiratory failure (n = 5) and multiorgan failure (n = 2).

All allogeneic HSCT recipients with the following features required admission to intensive care for mechanical ventilation: transplantation within 100 d, severe GVHD, and nosocomial acquisition of pandemic (H1N1) 2009. However, onset of symptoms for patient 1 was day 119 after allogeneic transplantation.

Eight of 10 patients admitted to intensive care had evidence of pneumonia on baseline chest radiograph. Patient

1 initially had normal chest radiograph results, despite the acute onset of hypoxia. Patient 24 was transferred to intensive care after 3 days in the hospital, at which point bilateral infiltrates were seen on chest radiograph, and oseltamivir therapy was begun.

Oseltamivir Resistance

Ten patients had repeat NAT testing to determine clearance of viral shedding. Eight had ≥1 further positive NAT (2 on sputum, 5 on nasopharyngeal swab specimen, and 1 on bronchoalveolar lavage sample) after receiving oseltamivir. Five of these patients had a positive NAT after >5 d of oseltamivir therapy. The longest recorded duration of viral shedding during oseltamivir therapy was 28 d (patient 1).

The H275Y NA mutation, a substitution known to confer a high level of oseltamivir resistance, was detected in 4 (57%) of 7 patients who had detectable nucleic acid after ≥4 d of oseltamivir therapy. These 4 patients comprised 13.3% of the 30 patients who received oseltamivir. The findings for the 4 patients who were infected with oseltamivir-resistant influenza virus are summarized in Table 2. The H275Y mutation was undetectable in initial diagnostic samples from these patients. Additionally, the H275Y mutation was detected in all available samples collected from these patients after they received oseltamivir therapy. Three of the 4 patients who had oseltamivir-resistant pandemic (H1N1) 2009 virus infection were HSCT recipients who had been admitted to intensive care. Virus isolation in MDCK cells was attempted for the samples that contained the H275Y mutation but was unsuccessful after 2 passages. This precluded the use of the phenotypic NA inhibition assay to further analyze the samples.

Patient 12, who survived despite shedding oseltamivir-resistant pandemic (H1N1) 2009 virus, had a biphasic clinical course. She initially stabilized while she was treated with oseltamivir; progressive respiratory failure then developed, which coincided with her recovery from neutropenia before later improvement. The other patient who survived despite shedding oseltamivir-resistant 2009 pandemic (H1N1) virus (patient 20) had an ILI without pneumonia. Of the 2 patients with resistant isolates who died, patient 5 first experienced the apparent resolution of pneumonia but later succumbed to recurrent pneumonia, and patient 1 had persisting pneumonitis despite 36 days of oseltamivir therapy.

Patient 24 had multiple positive NATs, none with the H275Y mutant detected. His NAT was positive 4 d after he began oseltamivir therapy, and he died 1 d after his last specimen, a nasopharyngeal swab, was collected.

Conclusions

We report a case series of hospitalized cancer patients with influenza A pandemic (H1N1) 2009 virus infection and their outcomes. Patients with hematologic malignancies accounted for 50% of deaths of persons with pandemic influenza in Victoria during the first 3 months of the pandemic (*15*). The strongest effects of illness from pandemic influenza among hospitalized cancer patients in the present series occurred in HSCT recipients. Nine of the 10 cancer patients admitted to intensive care were HSCT recipients. Furthermore, of 7 deaths from pandemic (H1N1) 2009 in this series of hospitalized cancer patients, 6 occurred in HSCT recipients (comprising 37.5% of these patients). Our observations are similar to those seen with seasonal influenza. In a series of hematology patients with respiratory virus infection, including seasonal influenza, from 1 large cancer center, the largest number of infections and deaths occurred in recipients of allogeneic HSCTs (*21*). Our observations support the importance of existing recommendations for control of transmission of influenza infection in HSCT recipients during an influenza pandemic (*22*).

Table 2. Characteristics of 4 patients infected with oseltamivir-resistant pandemic influenza A (H1N1) virus isolates, Australia*†

Characteristic	Patient no. 1	5	12	20
Within 100 days of HSCT‡	No	Yes	No	–
Time to development of resistance, d	22	11§	8	4
Time of last positive NAT result, d	28	16	8	4
Change to zanamivir	Yes	No	No	No
Time to zanamivir, d	36	–	–	–
Died	Yes	Yes	No	No
LOS, d	39	66	21	9

*HSCT, hematopoietic stem cell transplant; NAT, nucleic acid test; LOS, length of stay.
†Oseltamivir resistance was influenza virus with H275Y mutation.
‡Time from commencement of oseltamivir.
§Detected in bronchoalveolar lavage specimen with negative NAT on nasopharyngeal swab 3 d before first and 10 d after last bronchoscopy. This patient received oseltamivir for 5 d.

Half of the 24 cancer patients who had not received an allogeneic HSCT had underlying multiple myeloma (n = 8) and chronic lymphocytic leukemia (n = 4). There was also a paucity of patients with solid tumors (n = 2). The severity of illness from influenza in patients who have underlying multiple myeloma and chronic lymphocytic leukemia has not previously been widely appreciated, although a high rate of pneumonic illness from influenza has previously been demonstrated at 1 center (*21*). Patients with multiple myeloma have impaired cell-mediated immunity, in addition to humoral immune deficits (*6*,*23*,*24*). All 4 patients with multiple myeloma admitted to intensive care had previously received an autologous HSCT, which likely further compromised immunologic function. They also had been diagnosed with myeloma for several years.

Bacterial co-pathogens that cause pneumonia were not identified in our study cohort. However, patients received broad-spectrum antibacterial agents on admission. In con-

trast, a small number of investigators have identified bacterial superinfections in up to 30% of fatal cases of pandemic influenza (*3*,*25*). For patient 17 in this series, a diagnosis of pulmonary aspergillosis was made after autologous HSCT, an association previously reported with seasonal influenza in allogeneic HSCT recipients (*26*).

When there is a circulating strain of influenza not contained in the recent influenza vaccine, postexposure oseltamivir prophylaxis is an attractive strategy that can prevent the development of influenza infection (*27*–*29*). Postexposure prophylaxis of healthcare workers and family members may reduce the likelihood of exposure through prevention of influenza infection in close contacts of HSCT recipients. Our observations that would support post-exposure oseltamivir prophylaxis are 1) 4 of 5 allogeneic HSCT recipients who died or were admitted to intensive care had either nosocomial or household acquisition; 2) 5 of 11 HSCT recipients who had pneumonia before beginning oseltamivir therapy died; 3) none of the patients in this study were known to have received postexposure prophylaxis at the time of symptom onset, despite use of this strategy during the study period (*29*); and 4) 3 patients acquired the infection in the hospital from unidentified sources. This nosocomial transmission occurred despite heightened awareness during this pandemic, which demonstrates the difficulties of effective containment in the hospital setting (*28*,*29*).

The finding of oseltamivir-resistant influenza virus in 4 of 7 patients with a positive pandemic (H1N1) 2009–specific RT-PCR result after ≥4 d of oseltamivir therapy is a cause for concern. In Australia, oseltamivir-resistant pandemic (H1N1) 2009 has been described in another immunocompromised patient, a renal transplant recipient (*30*). No oseltamivir-resistant pandemic (H1N1) 2009 strains have been detected in nonhospitalized patients in Australia (B. Wang and A. Hurt, pers. comm.). The risk of pandemic influenza developing the H275Y mutation that confers oseltamivir resistance is considered to be higher in immunocompromised patients (*10*,*12*). Immunocompromised patients have also been overrepresented in deaths associated with oseltamivir-resistant seasonal influenza (*31*,*32*). If repeat NAT on respiratory specimens had been routinely performed for all treated patients in the present series, rates of oseltamivir resistance may have been even higher than we observed. Our findings indicate an urgent need to optimize oseltamivir dosing in immunocompromised patients. Further research into the role of combination antiviral therapy should be considered (*33*,*34*). Immunocompromised patients should have serial screening for ongoing viral shedding and oseltamivir resistance. The oseltamivir-resistant H275Y mutants remain susceptible to the alternative NA inhibitor zanamivir, and therefore the use of this drug should be considered in patients who are shedding these viruses (*10*).

Only 1 of the patients who was in intensive care and had oseltamivir-resistant pandemic (H1N1) 2009 virus survived. The effects of oseltamivir-resistant influenza virus in this patient are uncertain. The deterioration of her condition after initial stabilization may have been related to development of oseltamivir-resistance and persistent influenza viral replication similar to that seen in a recent case report (*30*). Others have shown development of resistance to oseltamivir within 3 d of therapy (*30*), and we observed resistance develop within 4 d for 1 patient. Undoubtedly, other explanations for her biphasic illness are possible. Immune recovery with resolution of neutropenia may have enabled viral clearance and recovery.

Universal chemoprophylaxis for HSCT recipients during an outbreak with an influenza strain that is not contained in the available influenza vaccine has been recommended by international guidelines supported by North American and European Blood and Marrow Transplant groups, Infectious Diseases Society of America, and the US Centers for Disease Control and Prevention (*22*). One of the study centers instituted universal chemoprophylaxis for patients admitted to receive an allogeneic HSCT after 2 cases of nosocomial-acquired infection before engraftment (P. Ferguson, pers. comm.). Although the strategy led to apparent success, with no further nosocomial infections observed, we have concern about this approach based on 1) the high rate of oseltamivir resistance (4/30 [13.3%] administered oseltamivir) observed in this study; 2) overrepresentation of postexposure prophylaxis in cases of oseltamivir-resistant pandemic (H1N1) 2009 influenza (*12*); 3) the potential that pandemic (H1N1) 2009 H275Y mutant viruses may transmit and spread throughout the community, similar to outcomes recently observed with seasonal influenza A (H1N1) viruses with the same H275Y mutation (*8*,*11*,*35*); and 4) the paucity of hospital-acquired cases seen in this series during heightened surveillance and control measures of a pandemic. A more judicious approach may be surveillance, infection control measures, and early treatment until vaccination becomes available (*12*,*22*,*28*). Additionally, education of patients and their close contacts to facilitate early treatment and avoidance of exposure is essential. When a vaccine becomes available, close contacts of those infected, their family members, and healthcare workers should be vaccinated.

One of the strengths of this study is the relatively high coverage of the major hematology centers in a single state, which reduces the effect of bias on patient ascertainment. However, patients whose treatment was managed in the community were excluded, and this information will be essential to our understanding as to which co-factors predict clinical progression and outcomes for pandemic (H1N1) 2009 infection in immunocompromised patients.

A high rate of oseltamivir resistance developed in critically ill HSCT recipients, particularly in those who continued to shed pandemic (H1N1) 2009 virus after 4 d of oseltamivir treatment. Consequently, surveillance, infection control measures, and early treatment of those at greatest risk of pandemic (H1N1) 2009 infection may prove more useful than universal chemoprophylaxis during an outbreak with an influenza strain that is not contained in the available influenza vaccine. Continued surveillance for oseltamivir-resistant influenza virus strains is needed, particularly in the immunocompromised.

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Dr Tramontana is an infectious diseases and internal medicine physician at Peter MacCallum Cancer Centre and Western Hospital in Melbourne, Victoria, Australia. His interests include mycobacterial infections and infections in immunocompromised hosts.

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Address for correspondence: Adrian R. Tramontana, Peter MacCallum Cancer Centre and Western Hospital, 1 St Andrews Pl, East Melbourne, Victoria 3002, Australia; email: adrian.tramontana@petermac.org

Population Structure of East African Relapsing Fever *Borrelia* spp.

Sally J. Cutler, E. Margarita Bonilla, and Rajbir J. Singh

Differentiation of endemic East African tick-borne relapsing fever *Borrelia duttonii* spirochetes from epidemic louse-borne relapsing fever (LBRF) *B. recurrentis* spirochetes into different species has been questioned. We assessed a noncoding intragenic spacer (IGS) region to compare genotypes found in clinical samples from relapsing fever patients. Although IGS typing was highly discriminatory and resolved 4 East African tick-borne relapsing fever groups from a disease-endemic region in Tanzania, 2 IGS clades were found among LBRF patients in Ethiopia. The 2 IGS sequence types for *B. recurrentis* overlapped with 2 of the 4 groups found among *B. duttonii*. All cultivable isolates of *B. duttonii* fell into a single IGS cluster, which suggests their analysis might introduce selective bias. We provide further support that *B. recurrentis* is a subset of *B. duttonii* and represents an ecotype rather than a species. These observations have disease control implications and suggest LBRF *Borrelia* spp. could reemerge from its tick-borne reservoirs in which vectors coexist.

Relapsing fever is a recurrent febrile infection caused by various *Borrelia* spirochetes transmitted either by lice (epidemic relapsing fever) or by ticks (endemic relapsing fever). Relapsing fever was once a disease of global epidemic importance. However, largely as a result of the demise of the clothing louse *Pediculus humanus*, it is now restricted to areas where clothing lice are still commonplace, such as Ethiopia (*1*). In these regions it still has a major impact and was documented in a recent Ethiopian Department of Health report as being the seventh most common cause of hospital admission (2.5% of total; 3,777 cases) and fifth most common cause of death (0.9%, 42 cases) (2004) (*1*). The epidemic tendency of this infection is likely to reside in its vector transmission, with waves of clothing lice fleeing febrile patients and thus facilitating epidemic transmission. The endemic tick-borne relapsing fever spirochetes are transmitted by *Ornithodoros* ticks; *O. sonrai* serve as the principle vector for *Borrelia crocidurae* in West Africa, and *O. moubata* complex ticks effectively maintain these spirochetes in East Africa (*1*). Because soft ticks are only associated with hosts for their typically rapid nocturnal feeding, this limits the spread of infection beyond the confines of the areas where tick vectors reside.

Although these differing life styles of the vectors can account for the epidemiologic differences between these infections, recent studies have highlighted the similarity between *B. recurrentis*, the cause of louse-borne relapsing fever, and *B. duttonii*, the agent of East African of tick-borne relapsing fever (*2*–*4*). It has been postulated that *B. recurrentis* is a louse-adapted variant of *B. duttonii* (*5*). In these disease-endemic regions, diagnosis is typically achieved through demonstration of spirochetes in stained blood films from patients. However, this technique is unable to discriminate between the different *Borrelia* spp. that cause relapsing fever.

To provide a method able to reliably identify these spirochetes, we validated the use of sequence analysis of an intragenic spacer (IGS) region for typing these spirochetes (*3*). This method was used to analyze a noncoding spacer region and proved to be highly discriminatory; it resolved 4 groups among *B. duttonii* found among isolates and directly in tick vectors. Two groups were found among *B. recurrentis* isolates and louse vectors (*3*). Furthermore, a novel *Borrelia* species detected previously was found, and some sequence types resembled *B. crocidurae*, previously believed to be only in West Africa (*3*,*6*). The surprising finding was that 1 phylogenetic group of *B. duttonii* overlapped with a group of *B. recurrentis* (*3*).

Author affiliation: University of East London, London, UK

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This colinearity was further supported by complete genomic sequencing of 1 isolate of *B. recurrentis* and 1 of *B. duttonii*, which suggests that *B. recurrentis* is a decaying genome that evolved from either *B. duttonii* or a common ancestral strain (*5*).

To further explore this apparent overlap between *B. duttonii* and *B. recurrentis*, we sequenced and compared additional gene targets. However, this study focused upon coding genes that were under different selective pressure and thus were not necessarily comparable to the noncoding IGS previously used. Furthermore, these investigations used only cultivable strains, and thus could represent bias toward those able to grow under axenic conditions. These investigations disclosed a clear demarcation between *B. duttonii* and *B. recurrentis* (*4*).

To resolve this apparent dichotomy, we undertook additional IGS typing directly on serum samples collected from patients with clinical cases, thus removing the selective pressure of cultivation. We report the results of those investigations.

Materials and Methods

Clinical Samples

Eighty-eight serum samples from patients in Ethiopia with relapsing fever that were blood film–positive for spirochetes were collected and stored frozen at –20°C before testing. Similarly, 23 samples collected from patients in Tanzania were collected and stored frozen. An additional series of 45 samples from family members accompanying patients to hospital as potential blood donors were available for analysis. Ethical approval for their collection and testing for relapsing fever had been granted for earlier studies (Ethiopia [*7*] and Commission for Science and Technology [COSTECH] 2001–330-NA-2001–25 for Tanzania).

DNA Extraction

Serum samples were centrifuged at 13,000 rpm in a microfuge for 30 min and 100 μL of the pellet was used for DNA extraction. After an initial proteinase K digestion in a waterbath at 56°C for 1 h, DNA extraction was conducted by using DNeasy reagents (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol for the QIAcube robotic platform with a final elution volume of 200 μL.

Preliminary Screening for *Borrelia* spp.

A real-time TaqMan flagellin assay was used to screen samples for the presence of borrelial DNA before investigation. The primers are shown in Table 1. Primers were used at a final concentration of 1,000 mmol/L, and the probe was used at a final concentration of 50 mmol/L. A mastermix composed of PCR working concentration buffer without $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.2 mmol/L of each dNTP, 5 mmol/L $MgCl_2$, 0.075 nmol/L Rox, and 0.06 U Taq polymerase (Invitrogen). Mastermix was divided into aliquots of 23 μL to which 2 μL of extracted DNA was added. A MX3000 thermocycler (Stratagene, La Jolla, CA, USA) was used with an initial denaturation of 95°C for 10 min, followed by 50 cycles each at 94°C for 15 s and 60°C for 60 s. Results were read at a Fam wavelength (emission 516) by using Rox as a reference wavelength (emission 610).

IGS Typing

Samples positive for *Borrelia* DNA by the flagellin real-time assay were further subjected to IGS typing. A nested PCR was used to amplify the IGS, with outer primers anchored within the 3′ end of the *rrs* ribosomal gene and the 5′ end within the *ileT* gene as used previously (*3*). Primer sequences are shown in Table 1. Conventional PCR was performed by using 25-μL reaction volumes in My Cycler thermocylers (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 74°C for 60 s, and a final extension at 72°C for 7 min. A 2-μL volume from the first-round reaction was added to 23 μL of fresh second-round mastermix. This mixture was heated at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 74°C for 60 s, and a final extension at 72°C for 7 min. Resulting amplicons were resolved by electrophoresis on 1% agarose gels stained with ethidium bromide (0.5 μg/mL). Different manipulations of the PCR were conducted in different laboratory areas to avoid contamination, and nontemplate controls were included at a ratio of 1 for every 15 samples tested.

Table 1. Primer and probe sequences used in study of East African relapsing fever *Borrelia* spp.*

Primer specificity	Primer/probe sequence, 5′ → 3′
Flagellin forward	CTAGTGGGCATAGAATTAATCGTGC
Flagellin reverse	GCTTGGGATAACCCTCTAATTTGA
Flagellin probe	fam-TGGTATGGGTGTTGCTGGGAAAATTACG-bhq1
First-round IGS forward	GTATGTTTAGTGAGGGGGGTG
First-round IGS reverse	GGATCATAGCTCAGGTGGTTAG
Second-round IGS forward	AGGGGGGTGAAGTCGTAACAAG
Second-round IGS reverse	GTCTGATAAACCTGAGGTCGGA

*IGS, intragenic spacer.

Sequencing and Phylogenetic Analysis

Resulting amplicons were subjected to sequence analysis on an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the Genome Centre Queen Mary's University, London. The inner nested primers were additionally used for sequencing primers at a concentration of 10 pmol/μL. Sequence results were aligned by using ClustalW (www.ebi.ac.uk/clustalw) trimmed and a neighbor-joining phylogenetic tree was constructed by using MEGA4 (*8*).

Results

Most samples collected from patients with either louse-borne (all 88 serum samples) or tick-borne relapsing fever (21/23 samples), yielded a positive PCR result upon initial screening and subsequently when tested for IGS. The 2 samples positive by initial microscopy, but negative by PCR, may represent DNA degradation over the prolonged period of frozen storage before testing. Additionally, 15 of 45 blood donors who accompanied patients in Tanzania had positive test results upon screening; samples from 9 of these also produced IGS amplicons. Nontemplate controls showed negative results.

IGS sequence data were obtained for 90% of the 124 PCR screen–positive samples (18 from patients with tick-borne relapsing fever, 85 from patients with louse-borne relapsing fever, and 9 from blood donors in Tanzania) and used for phylogenetic analysis. Only 5 of the 34 identical *B. recurrentis* type I sequences (represented by *B. recurrentis* A1 DQ000277) and 7 of the 43 *B. recurrentis* type II sequences (represented by *B. recurrentis* A11 DQ000278) are shown in Figure.

Similar to results found when looking at cultivable strains and IGS data in ticks or lice (*3*), we were able to show 4 clades among *B. duttonii* and 2 for *B. recurrentis* (Figure). Two of the *B. duttonii* clades contained only sequence types from patients in Tanzania or blood donors. Notably, all of the cultivable strains of *B. duttonii* resembled sequence type I. The other previously designated type IV (*3*), represented by *B. duttonii* WM (DQ000282) that had been represented by a single patient sequence, was found in 2 additional patient samples and in 1 serum sample from a blood donor. The remaining 2 *B. duttonii* clades overlapped with the 2 *B. recurrentis* clades. The accession numbers for the sequences types shown in Figure are deposited under GenBank nos. GQ401243–GQ401278 (Table 2).

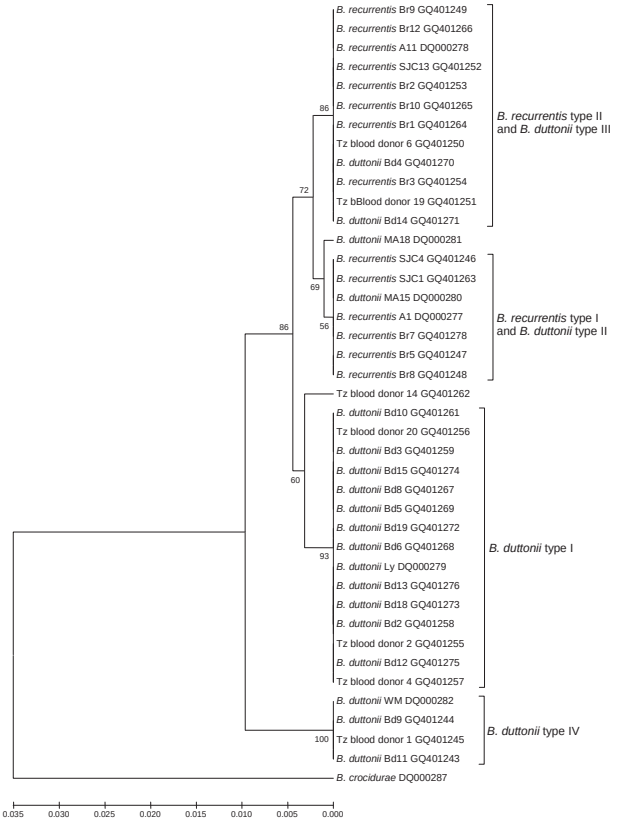

Figure. Phylogenetic tree of intragenic space sequences showing 2 groups among *Borrelia recurrentis* and 4 groups among *B. duttonii*. Tree created by using the unweighted pair group method with arithmetic mean. The *B. recurrentis* groups overlap with *B. duttonii* groups. Scale bar indicates nucleotide substitutions per site. Tz, Tanzania.

Discussion

The findings of this study build upon those previously reported (*3*). As before, on the basis of IGS sequence types, we identified 4 distinct clusters of *B. duttonii*. Thus, the findings of this study, which analyzed sequence types in clinical samples, generally mirror results found when investigating sequence types of bacteria in arthropod vectors (*3*). The notable exception to this was the absence of the novel *Borrelia* sp. previously detected by Scott et al. in ticks (*3*) and by others in ticks and human samples (*6*,*9*). Whether detection of this species in humans with fever was directly related to the fever or incidental remains to be assessed through properly structured epidemiologic investigations. We did not detect this species among samples collected from patients with clinical relapsing fever in our study population. In consequence, our findings do not support the conclusion that this species has the potential to cause human infection.

Our samples from Tanzania were collected from villagers living in traditional mud-built dwellings; these dwellings usually have >80% infestation rates with *O. moubata* ticks. Regular nocturnal feeding by ticks could account for efficient transmission of these spirochetes to humans. Consequently, it is not surprising that samples from these persons, or from the asymptomatic blood donors who lived

in similar conditions, were positive for *B. duttonii*. The absence of evidence of cross-contamination of the assays further substantiates these findings. Others have reported microscopy positive samples and PCR positive results from asymptomatic persons in Tanzania (*6*), which lends further support to this finding. Whether these observations represent persistent infection, as seen in the related spirochete that causes Lyme borreliosis, or reflects continued exposure by feeding ticks, remains to be elucidated.

Conversely, in Ethiopia, data are not available concerning the prevalence of soft tick species; however, infestation with clothing lice is commonplace (*10*). Based on the vector prevalence, in Ethiopia, relapsing fever has always been presumed to result from *B. recurrentis* infection. A subset of serum samples included in the current study were found to harbor a premature stop codon in their *recA* gene (S.J. Cutler et al., unpub. data), a feature disclosed in *B. recurrentis*, but not *B. duttonii,* in the recently completed whole genome sequencing of these spirochetes (*5*). This finding provides further evidence for the identification of infecting spirochetes among these samples.

The distribution of IGS profiles found among Tanzanian blood donors did not appear to differ from that found among persons with clinical cases in this region. The apparent absence of associated clinical signs is likely to correlate with a degree of local immunity. This finding is the basis of much local folk lore on the need to have regular tick exposure to boost immunity (*11*). These spirochetes undergo multiphasic antigenic variation (*12*), and whether they would continue to cause asymptomatic infection if the spirochetes underwent antigenic variation remains speculative. Furthermore, these organisms are able to evade key components of the innate immune response (*13*). This evasion might help account for their presence in asymptomatic persons.

Strikingly, the IGS sequence type for the cultivable isolates obtained for *B. duttonii* all fell into a single clade in which only *B. duttonii* sequence types were represented. This finding would explain the distinct clustering previously reported that separated *B. duttonii* from *B. recurrentis* when a multigene sequence–based approach was used (*4*). It may be that this particular cluster of *B. duttonii* is more readily cultivable that those belonging to other clades, thus highlighting the potential for misinterpretation of population structure analysis that is based only on cultivable isolates.

We had reported that *B. recurrentis* fell into 2 clades, which we can reconfirm with our current in situ findings (*3*). Of the samples tested from patients in Ethiopia with louse-borne relapsing fever, 44% fell into the type I clade and 56% were type II. Interestingly, our earlier work showed that *B. duttonii* type II represented by tick sample MA/15 (DQ000280) had a IGS sequence identical to those found for *B. recurrentis* type I from Ethiopia. Our current findings have provided further support for this observation by showing that another *B. duttonii* type III represented by tick sample MA/18 (DQ000281) aligns more closely with *B. recurrentis* IGS types. Further examples of IGS sequence homology were found between spirochetes that cause louse-borne relapsing fever and those that cause tick-borne relapsing fever, in samples from 2 patients from Tanzania with tick-borne relapsing fever (Bd4 and Bd14) and also from 2 blood donors from Tanzania (Tz blood donor 6 and Tz blood donor 19); the isolates clustered among *B. recurrentis* type II.

Table 2. Sample designation and accession numbers for *Borrelia recurrentis* and *B. duttonii,* East Africa

Strain designation	Species	GenBank accession no.	Reference
Bd11	*B. duttonii*	GQ401243	This study
Bd9	*B. duttonii*	GQ401244	This study
Tz blood donor 1	*B. duttonii*	GQ401245	This study
SJC4	*B. recurrentis*	GQ401246	This study
Br5	*B. recurrentis*	GQ401247	This study
Br8	*B, recurrentis*	GQ401248	This study
Br9	*B. recurrentis*	GQ401249	This study
Tz blood donor 6	*B. duttonii*	GQ401250	This study
Tz blood donor 19	*B. duttonii*	GQ401251	This study
SJC13	*B. recurrentis*	GQ401252	This study
Br2	*B. recurrentis*	GQ401253	This study
Br3	*B. recurrentis*	GQ401254	This study
Tz blood donor 2	*B. duttonii*	GQ401255	This study
Tz blood donor 20	*B. duttonii*	GQ401256	This study
Tz blood donor 4	*B. duttonii*	GQ401257	This study
Bd2	*B. duttoii*	GQ401258	This study
Bd3	*B. duttonii*	GQ401259	This study
Bd7	*B. duttonii*	GQ401260	This study
Bd10	*B. duttonii*	GQ401261	This study
Bd14	*B. duttonii*	GQ401262	This study
SJC1	*B. recurrentis*	GQ401263	This study
Br1	*B. recurrentis*	GQ401264	This study
Br10	*B. recurrentis*	GQ401265	This study
Br12	*B. recurrentis*	GQ401266	This study
Bd8	*B. duttonii*	GQ401267	This study
Bd6	*B. duttonii*	GQ401268	This study
Bd5	*B. duttonii*	GQ401269	This study
Bd4	*B. duttonii*	GQ401270	This study
Bd14	*B. duttonii*	GQ401271	This study
Bd19	*B. duttonii*	GQ401272	This study
Bd18	*B. duttonii*	GQ401273	This study
Bd15	*B. duttonii*	GQ401274	This study
Bd12	*B. duttonii*	GQ401275	This study
Bd13	*B. duttonii*	GQ401276	This study
Bd1	*B. duttonii*	GQ401277	This study
Br7	*B. recurrentis*	GQ401278	This study
A1	*B. recurrentis*	DQ000277	(*3*)
A11	*B. recurrentis*	DQ000278	(*3*)
Ly	*B. duttonii*	DQ000279	(*3*)
MA15	*B. duttonii*	DQ000280	(*3*)
MA18	*B. duttonii*	DQ000281	(*3*)
WM	*B. duttonii*	DQ000282	(*3*)

The vectorial specificity of these spirochetes has been a source of curiosity for many years. Early investigations questioned host and vector specificities of relapsing fever spirochetes; studies were carried out that enabled ticks to feed on patients with the louse-borne form of the disease and subsequently letting these ticks feed upon nonfebrile persons. These studies were unable to conclusively establish whether alternative arthropod vectors could transmit these spirochetes. Haberkorn demonstrated that *B. crocidurae* isolated from *O. erraticus* successfully multiplied in lice (*14*). The investigations by Heisch and Garnham initially claimed successful transmission of *B. duttonii* by body lice (*15*). However, in a later study Heisch concluded that *B. duttonii* from humans is unlikely to be naturally transmitted by lice (*16*). Many of these early investigations were hampered by the inability to reliably identify the organisms used, lack of cultivable strains, and having no means of assessing the immune status of recipient hosts. Speciation of the causative relapsing fever spirochete was based on the 1 vector–1 species criteria that relied on geographic region where infection occurred and identification of the vector responsible for transmission.

Collectively, these findings support the increasing evidence that *B. recurrentis* has evolved either directly from *B. duttonii* or from a common ancestral strain. This raises the question of whether these spirochetes should be considered as separate species or as ecotypes of the same species? With the advent of molecular typing tools, should the traditional classification of relapsing fever spirochetes be readdressed? A precedent for this approach already exists with other highly related organisms such as *Bacillus anthracis, B. cereus*, and *B. thuringiensis* (*17*). Although IGS typing provides a highly discriminatory tool to type these spirochetes, these observations should be underpinned by sequence analysis of multiple gene targets to provide a robust phylogenetic analysis.

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Dr Cutler is a reader in medical microbiology and infectious diseases at the University of East London. She has a long-standing research interest in African relapsing fever, following the first successful in vitro cultivation of East African relapsing fever spirochetes through to their preliminary characterization.

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Address for correspondence: Sally J. Cutler, School of Health and Bioscience, University of East London, Water Lane, Stratford, London E15 4LZ, UK; email: s.cutler@uel.ac.uk

Human Infection with *Rickettsia felis*, Kenya

Allen L. Richards, Ju Jiang, Sylvia Omulo, Ryan Dare, Khalif Abdirahman, Abdile Ali, Shanaaz K. Sharif, Daniel R. Feikin, Robert F. Breiman, and M. Kariuki Njenga

To determine the cause of acute febrile illnesses other than malaria in the North Eastern Province, Kenya, we investigated rickettsial infection among patients from Garissa Provincial Hospital for 23 months during 2006–2008. Nucleic acid preparations of serum from 6 (3.7%) of 163 patients were positive for rickettsial DNA as determined by a genus-specific quantitative real-time PCR and were subsequently confirmed by molecular sequencing to be positive for *Rickettsia felis*. The 6 febrile patients' symptoms included headache; nausea; and muscle, back, and joint pain. None of the patients had a skin rash.

Rickettsial diseases are found worldwide and are caused by infection with obligate intracellular rickettsiae, which are transmitted to humans by arthropod vectors (e.g., lice, fleas, ticks, and mites). Rickettsiae are associated with arthropods for a least a part of their life cycle and are passed to other arthropods by transovarial transmission or horizontal transmission involving vertebrate hosts (*1*). Rickettsiae are small, gram-negative, fastidious bacteria of the α subdivision of Proteobacteria, which are frequently divided into 2 groups based on antigenicity, G+C content, culture conditions, and actin polymerization: 1) the typhus group including *Rickettsia prowazekii* and *R. typhi,* the causative agents of louse-borne epidemic and flea-borne murine typhus, and 2) the spotted fever group including >20 species that may cause tick-, flea-, and mite-borne rickettsioses (*2*). Infection of humans by rickettsiae often begins at the site of the bite of the arthropod vector and subsequently spreads through the draining lymph nodes throughout the body, resulting in infection of endothelial cells, which can lead to multiorgan pathologic changes associated with potentially life-threatening diseases (*1*).

Little is known about the full spectrum of rickettsial diseases that occur in Kenya. One frequently reported rickettsial disease has been described in many parts of Kenya as Kenya fever, Kenya typhus, Kenya tick fever, or Kenyan tick typhus (*3*,*4*). This disease was initially thought to be caused by a single pathogen, *R. conorii*, the causative agent of Mediterranean spotted fever (*5*). However, recently, a second agent has been suggested as a cause of at least a portion of these rickettsioses (*6*,*7*), *R. africae*, the causative agent of African tick-bite fever. In addition to the tick-borne spotted fever disease, flea-borne murine typhus is known to occur in Kenya, especially in urban areas where the house rat (*Rattus rattus*) is prevalent (*4*,*8*). However, louse-borne epidemic typhus does not appear to be endemic to Kenya (*4*).

The objective of the hospital-based surveillance we report here was to determine the etiologic agents of non-malaria acute febrile illnesses in the North Eastern Province, Kenya, during a period when there is no Rift Valley fever (RVF) epidemic in the country. The North Eastern Province was severely affected by the 2 most recent RVF epidemics in the Eastern Africa region; >89,000 human RVF infections occurred during the 1997–98 epidemic and >300 acute cases occurred during the 2006–2007 epidemic (*9*,*10*). Therefore, the surveillance was established to investigate whether acute RVF cases occurred during interepidemic periods. As part of this investigation, we also investigated the occurrence of other causes of fever that are not routinely tested for at the local hospitals, including

Author affiliations: Naval Medical Research Center, Silver Spring, Maryland, USA (A.L. Richards, J. Jiang); Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA (A.L. Richards); US Centers for Disease Control and Prevention, Nairobi, Kenya (S. Omulo, R. Dare, D.R. Feikin, R.F. Breiman, M.K. Njenga); and Kenya Ministry of Health, Nairobi (K.A. Abdirahman, A. Ali, S.K. Sharif)

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rickettsiosis, brucellosis, leptospirosis, and other viral infections. The study protocol was approved by the institutional review boards of the US Centers for Disease Control and Prevention, US Naval Medical Research Center, and Kenya Medical Research Institute in compliance with all applicable regulations governing the protection of human subjects.

Materials and Methods

Study Site

This hospital-based study was conducted at the Garissa Provincial Hospital in Garissa town, located in North Eastern Province, Kenya (Figure). The semiarid province has an average annual rainfall <40 mm; is inhabited by a predominantly seminomadic pastoralist population of Somali origin who keep cattle, sheep, goats, and camels; and carries a wide range of wildlife, including zebras, antelopes, waterbucks, giraffes, warthogs, monkeys, gerenuks, dik diks, lions, hyenas, and cheetahs. Livestock interacts freely with wildlife, particularly with the large population of zebras, waterbucks, and warthogs with which they share pastures and watering holes. Local residents keep dogs primarily for security and for herding livestock.

Figure. North Eastern Province (shaded area), Kenya. The city of Garissa is marked with a black circle.

Study Participants

Rickettsial infections were evaluated as potential causes of non-malaria fever among 163 patients with fever, muscle pain, back pain, headache, and joint pain treated at the Garissa Provincial Hospital. A consenting patient was enrolled in the study if he or she was ≥5 years of age (inpatients or outpatients), had an axillary temperature ≥38°C, had a negative test for malaria by blood smear, and had a history of animal contact. Animal contact was described as herding, milking, or slaughtering. Blood samples were obtained from all consenting patients who met the criteria listed. A standardized questionnaire was administered by a clinical officer to record the patient's clinical signs, symptoms, and potential risk factors, such as contact with animals, consumption of unpasteurized dairy products, and unsanitary sources of drinking water.

Specimen Collection and Transportation

Approximately 3 mL of blood was collected from each patient by venipuncture using sterile technique. Serum samples were separated and stored at –20°C before being transported to the Kenya Medical Research Institute/US Centers for Disease Control and Prevention laboratory in Nairobi for acute-phase PCR and serologic testing.

DNA Extraction

DNA was extracted from 300 μL of serum by using the QiaAmp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). The sample DNA was first treated with 30 μL of proteinase K and then lysed with 300 μL of the kit buffer. An equal volume of absolute ethanol was added to the lysed sample and the mixture added to the DNA trapping columns provided. The columns were then freed of cell debris and other contaminants by a series of washes before releasing the matrix-trapped DNA in 50 μL of the kit elution buffer.

Quantitative Real-time PCRs

Two quantitative real-time PCR (qPCR) assays were used to screen for rickettisal nucleic acid in the patients' serum samples: 1) the *Rickettsia* genus–specific Rick17 qPCR that amplifies and detects a 115-bp segment of the 17-kDa antigen gene (*11*) and 2) the tick-borne rickettsiae-specific qPCR that amplifies and detects a 128-bp segment of the outer membrane protein (*ompB*) gene (*12*). The *R. felis*–specific qPCR that amplifies and detects a 129 bp segment of the *ompB* gene was also used, as previously described (*13*,*14*). The primer and probe oligonucleotides were synthesized by Sigma Genosys (The Woodlands, TX, USA). Fluorescence was monitored each cycle during the annealing step, and data were analyzed with SmartCycler software version 2.0d (Cepheid, Sunnyvale, CA, USA).

A plasmid DNA constructed with a PCR product amplified from the *R. rickettsii* 17-kDa antigen gene was used

as the positive control at 10^3 copies/reaction for the Rick17 qPCR . Plasmid DNAs constructed with the target sequences amplified from *R. rickettsii* and *R. felis ompB* were used as positive controls at 10^3 copies/reaction for tick-borne rickettsiae-specific and *R. felis* qPCRs, respectively. Three negative controls included in each run for all qPCRs were consistently negative.

PCR, Nested PCR, and Sequencing

Oligonucleotide primers and procedures used for PCR and nested PCR amplification of 17-kDa antigen gene, *ompB*, and *R. felis* plasmids have been described elsewhere (*15*,*16*). Briefly, the PCR products from the 17-kDa gene, *ompB*, and pRF plasmid were purified by using a QIAquick PCR purification kit (QIAGEN). No positive controls were used in these PCR and nested PCR procedures to decrease the chance of contamination; however, a single negative control was run with the samples and did not produce any products. The BigDye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used in the subsequent sequencing reactions according to manufacturer's instructions. Sequencing products were purified by using Performa Gel Filtration Cartridges (EdgeBioSystems, Gaithersburg, MD, USA), and sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The primers used for PCR amplification were the same primers as those used for the sequencing reactions. At least 2 sequencing reactions were performed for each strand of DNA. Sequences were assembled by using Sequencher 4.0 (Gene Codes Corporation Inc., Ann Arbor, MI, USA), and BLAST searches were managed on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov).

Results

A total of 163 patients whose illnesses met the case definition (fever, but not malaria) were seen at Garissa Provincial Hospital from August 1, 2006, through June 30, 2008. No patients had evidence of acute RVF disease either by PCR or immunoglobulin (Ig) M testing, but samples obtained from 35 (21.5%) patients were positive for RVF-specific IgG, indicating previous infection with the RVF virus.

To ascertain whether the patients' serum samples contained molecular evidence of rickettsiae, DNA was extracted and the preparations were assessed by qPCRfor genus-specific 17-kDa gene (Rick17 assay) and a segment of the *ompB*, which is specific for tick-borne rickettsiae. Six samples of 163 (3.7%) tested were positive by Rick17, but none were positive by tick-borne rickettsiae–specific assay, suggesting nontick-associated rickettsiae were responsible for the patients' fevers.

To identify which rickettsiae were responsible for the patients' illnesses, a 434-bp fragment from the 17-kDa antigen gene was amplified from 3 of 6 Rick17-positive samples by nested PCR. Amplicons (434 bp) were produced and represented most of the open reading frame (ORF 480 bp). They were subsequently sequenced, and a BLAST search was conducted. For 3 of 6 Rick17-positive samples, amplicons were produced and assessed, and sequences of 389, 412, and 412 bp were obtained and determined to be 100% identical to that provided in GenBank for *R. felis* Cal2 (GenBank accession no. CP000054). To confirm the identity of the rickettsial agents, the 6 DNA sample preparations were assessed by an *R. felis* qPCR selective for a unique portion of *ompB*. All 6 samples were positive (Table 1). In addition, after nested PCR for a fragment of *ompB*, two 599-bp amplicons were successfully produced; the sequences of these products were 100% identical with that of *R. felis* Cal2 (GenBank accession no. CP000054). Of the 6 samples tested, the *R. felis* plasmid RF was detected only in sample 1 (GenBank accession no. CP000054), and none of the 6 samples produced an RFδ product (Table 1). Collectively, these results show that *R. felis* DNA was detected in samples from 6 patients even though the rickettsial nucleic acid concentrations were low in the serum samples assessed (cycle threshold values 36.4–44.7, data not shown).

The 6 patients with positive test results for *R. felis* were 7–47 years of age. Three patients were men, 2 herdsmen and 1 farmer; 2 of the 3 female patients were housewives and 1 was a student 7 years of age. All patients reported contact with livestock animals such as cattle, sheep, goats, or camels. Although this was not captured in the questionnaire, almost all livestock owners in the region had dogs

Table 1. Molecular detection results and characteristics of rickettsial DNA preparations in acute-phase serum samples from 6 patients who had fever, Garissa Provincial Hospital, North Eastern Province, Kenya, 2006–2008*

Patient no.	17-kDa gene sequence	*ompB* sequence	RF plasmid
1	100% identification with *Rickettsia felis*	100% identification with *R. felis*	100% identification with *R. felis*
2	100% identification with *R. felis*	–	–
3	–	–	–
4	–	100% identification with *R. felis*	–
5	–	–	–
6	100% identification with *R. felis*	–	–

**ompB*, outer membrane protein B gene. All samples were positive by Rick17 PCR and Rfelis qPCR and negative by Trick quantitative PCR (qPCR), RF plasmid, and RFδ plasmid. Rick17 qPCR is specific for the 17-kDa antigen gene of *Rickettsia* species; Trick qPCR is specific for a portion of *ompB* that is common to tick-borne rickettsiae; Rfelis qPCR is specific for a portion of *ompB* that is common to *R. felis*.

that assisted with livestock herding and security. All of the *Rickettsia*-positive patients reported having had fever for 3–8 days, as well as nausea, muscle ache, back pain, headache, and joint pain (Table 2). No patients reported skin rash, and no information about possible eschars was collected. Of the 6 patients, 5 sought care from September through February, which is the rainy season, and the other was seen in May. None of the *Rickettsia*-positive patients had evidence of jaundice, neurologic signs, bleeding, or vision impairment. Of 6 *R. felis* positive patients, 1 (16%) had IgG to RVF virus—a prevalence similar to that seen in the 163 cases (21.5%). Moreover, all 6 *R. felis* patients had negative tests for leptospirosis and brucellosis (data not shown). Though no cases of leptospirosis were identified among the patients, 14.5% of the fever patients had positive tests for brucellosis (data not shown).

Discussion

R. felis is the causative agent of flea-borne spotted fever, an emerging zoonotic disease with wide cosmopolitan distribution in >20 countries and 5 continents (*17*). The large dissemination of *R. felis* and thus the risk for flea-borne spotted fever can be attributed to the pervasiveness of infected arthropods worldwide, including 10 flea species (*Ctenocephalides felis, C. canis, Xenopsylla cheopis, X. brasilliensis, Pulex irritans, Archeopsylla erinacei, Tunga penetrans, Ceratophyllus gallinae, Spilospsyllus cuniculi,* and *Echidnophaga gallinacean*) (*13*,*18*–*21*), as well as mites and ticks (*22*,*23*; J. Jiang and A. Richards, unpub. data). The cat flea, *C. felis,* is considered the primary vector for *R. felis* infections because this arthropod can maintain stable infected progeny through transovarial and transtadial transmission (*24*–*26*), and antibodies against *R. felis* have been detected in animals after they have been exposed to infected cat fleas (*27*,*28*). Hosts for the infected arthropod vectors include cats, dogs, opossums, and rodents; however, no viable *R. felis* has been isolated from a vertebrate host (*17*), although *R. felis* DNA has been detected in the blood of cats (*28*), opossums (*29*), dog (*30*), rodents (*21*), and humans (*14*,*30*,*31*). However, Hawley et al. (*32*) did not detect *R. felis* DNA in cats naturally infested with *R. felis*–infected cat fleas, nor did the authors of a recent study find an association between rickettsial DNA or antibodies and the presence of fever in clinically ill cats (*33*).

The clinical signs and symptoms of flea-borne spotted fever in humans are similar to those of flea-borne murine typhus and other rickettsioses and include high fever, headache, myalgia, and rash; other manifestations occur, such as abdominal pain, nausea, vomiting, cough, eschar, photophobia, and hearing loss, occur less frequently (*17*,*18*,*31*,*34*,*35*). All of the 6 patients we described with flea-borne spotted fever reported fever, headache, and back and joint pain; 4 reported muscle aches; and 3 reported nausea and appetite loss. However, none reported rash.

Because of the nonspecific signs and symptoms of flea-borne spotted fever, the disease is difficult to diagnose clinically. Molecular diagnostics, such as qPCRs, are the methods of choice for detecting *R. felis* and diagnosing flea-borne spotted fever because *R. felis* has yet to be cultured from clinical samples and because serologic assays to detect rickettsia-specific antibodies take 1–2 weeks after onset of disease to reach detectable levels (*17*,*26*,*35*). qPCRs have detected rickettsial nucleic acid in various clinical specimens (*14*,*15*,*31*). Because of the obligate intracellular nature of rickettsiae, clinical samples that include more infected cells, such as tissue biopsy samples, peripheral blood mononuclear cells, and buffy coats, will increase the likelihood of obtaining a positive result even after patients have received proper treatment with antimicrobial drugs (*15*,*36*). For this investigation, we used qPCR screening assays, PCR and multilocus sequence typing, and a species-specific qPCR algorithm to assay acute-phase se-

Table 2. Clinical and demographic information of patients with flea-borne spotted fever who were treated at Garissa Provincial Hospital, North Eastern Province, Kenya, 2006–2008

Patient no.	Date	Age, y/sex	Occupation	Clinical signs and symptoms	Fever duration, d	Animals with which patient had contact
1	2006 Sep 6	47/M	Herdsman	Back pain, headache, joint pain, appetite loss, nausea	8	Cattle, sheep, goats, dogs
2	2007 Oct 19	7/F	Student	Muscle aches, back pain, headache, joint pain	7	Unknown*
3	2007 Oct 30	38/F	Housewife	Muscle aches, back pain, headache, joint pain, chills, malaise, nausea	3	Cattle, sheep, goats, dogs
4	2007 Dec 10	20/M	Herdsman	Cough, back pain, headache, joint pain	3	Cattle, sheep, goats, dogs
5	2008 Feb 28	35/M	Farmer	Cough, muscle aches, back pain, headache, joint pain, malaise, fatigue, appetite loss, dizziness	7	Unknown*
6	2008 May 2	25/F	Housewife	Muscle aches, back pain, headache, joint pain, chills, malaise, fatigue, appetite loss, vomiting, nausea	3	Cattle, sheep, goats, dogs

*Responses to the questionnaires indicated these patients came in contact with livestock; however, the responses did not list specific animals.

rum samples to determine the rickettsial agent causing fever if 6 of 163 patients as *R. felis*. This algorithm, derived initially from the methods of Fournier et al. (*37*), has been used successfully to identify the rickettsial agents in human clinical samples (*14*,*15*).

We report the detection and clinical signs and symptoms of *R. felis* infection of humans in Kenya. Notably, the agents for the rickettsiosis were not *R. conorii, R. africae*, or *R. typhi*, agents previously known to cause rickettsial disease in Kenya. Identification of flea-borne spotted fever and its agent *R. felis* has been reported sporadically in other locations in Africa, e.g., Democratic Republic of Congo (*20*), Tunisia (*38*), Algeria (*39*), Gabon (*40*), and Egypt (*14*). Therefore, flea-borne spotted fever needs to be included in the differential diagnosis of febrile diseases in Kenya.

We identified illness caused by *R. felis* in 6 (3.7%) of 163 fever patients in a 23-month period. This number of patients in 1 portion of the country during this short period contrasts with a total of only 68 patients known worldwide (*17*). This finding suggests that risk factors associated with flea-borne spotted fever in North Eastern Province, Kenya, exist and that civilians, military personnel, displaced populations, and foreign travelers are threatened. Therefore, healthcare providers in Kenya and in countries in which travelers return from Kenya should be aware of the possibility that febrile patients might be infected with *R. felis*.

It is also of interest that 1 of 93 patients was found in the first year of the study (August 2006–July 2007), whereas 5 of 70 patients were found in the subsequent 11 months (August 2007–June 2008). Why the number of patients increased in the two ≈1-year periods is unknown. Ongoing surveillance should determine whether this increase suggests *R. felis* infection is an emerging disease or is an endemic disease just now being recognized. In addition, future studies of rickettsioses in Kenya should include an in-depth assessment of the pathogenesis, prevalence, incidence, and risks associated with flea-borne spotted fever.

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Dr Richards is an associate professor in the Department of Preventive Medicine and Biometrics at the Uniformed Services University of the Health Sciences. His research interests include rickettsial epidemiology, study of the host immune response to rickettsial infection, and development of a rapid diagnostic assay and vaccine.

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Address for correspondence: M. Kariuki Njenga, Centers for Disease Control and Prevention Global Disease Detection Program–Kenya, Nairobi, Kenya, Unit 8900, Box 6610, DPO, AE 09831-6610, USA; email: knjenga@ke.cdc.gov

Ebola Hemorrhagic Fever Associated with Novel Virus Strain, Uganda, 2007–2008

Joseph F. Wamala, Luswa Lukwago, Mugagga Malimbo, Patrick Nguku, Zabulon Yoti, Monica Musenero, Jackson Amone, William Mbabazi, Miriam Nanyunja, Sam Zaramba, Alex Opio, Julius J. Lutwama, Ambrose O. Talisuna, and Sam I. Okware

During August 2007–February 2008, the novel *Bundibugyo ebolavirus* species was identified during an outbreak of Ebola viral hemorrhagic fever in Bundibugyo district, western Uganda. To characterize the outbreak as a requisite for determining response, we instituted a case-series investigation. We identified 192 suspected cases, of which 42 (22%) were laboratory positive for the novel species; 74 (38%) were probable, and 77 (40%) were negative. Laboratory confirmation lagged behind outbreak verification by 3 months. *Bundibugyo ebolavirus* was less fatal (case-fatality rate 34%) than Ebola viruses that had caused previous outbreaks in the region, and most transmission was associated with handling of dead persons without appropriate protection (adjusted odds ratio 3.83, 95% confidence interval 1.78–8.23). Our study highlights the need for maintaining a high index of suspicion for viral hemorrhagic fevers among healthcare workers, building local capacity for laboratory confirmation of viral hemorrhagic fevers, and institutionalizing standard precautions.

Ebola hemorrhagic fever (EHF) is a severe, often fatal disease of humans and nonhuman primates caused by a single-stranded RNA virus belonging to the *Filoviridae* family. The virus was first isolated in 1976 after hemorrhagic fever outbreaks in Zaire and Sudan that resulted in >250 deaths (*1*,*2*). Before the outbreak described here, 4 distinct species of Ebola virus were known: *Zaire ebolavirus*, *Sudan ebolavirus*, *Côte d'Ivoire ebolavirus*, and *Reston ebolavirus*.

Although the reservoirs for the virus and mechanisms of transmission have not been fully elucidated, a recent study reported that 4% of bats from Gabon were positive for Zaire Ebola virus immunoglobulin (Ig) G (*3*). Initial human infection presumably occurs when humans are exposed to infected body fluids of the animal reservoir or intermediate host. Thereafter, person-to-person transmission occurs through direct contact with body fluids of an infected person (*1*,*2*,*4*). After an incubation period of 1–21 days (*1*,*2*,*5*), an acute febrile illness develops in infected persons; the disease is characterized by the sudden onset of fever, chills, headache, and myalgia, followed later by rash, sore throat, nausea, vomiting, diarrhea, and abdominal pain (*1*,*2*,*5*). Approximately half of infected persons manifest hemorrhagic signs, e.g., bleeding from the nasal cavity, passing of blood in the urine, and/or gastrointestinal and vaginal bleeding (*5*,*6*). The case-fatality rate (CFR) for Zaire Ebola virus and Sudan Ebola virus (SEBOV) infections varies from 53% to 90% (*7*). The first EHF outbreak in Uganda occurred in 2000 and affected Gulu, Masindi, and Mbarara districts, with a total of 425 case-patients and 224 deaths (CFR 53%). The outbreak was caused by the *Sudan ebolavirus* species (*8*).

On November 5, 2007, the Uganda Ministry of Health (UMOH) received a report of the deaths of 20 persons in Bundibugyo district, western Uganda. However, UMOH had received initial reports of 2 suspected cases of a febrile diarrheal illness on August 2, 2007. A UMOH team investigated these 2 cases, but the findings were not conclusive because of inadequate in-country laboratory capacity. In the

Author affiliations: Ministry of Heath, Kampala, Uganda (J.F. Wamala, L. Lukwago, M. Malimbo, M. Musenero, J. Amone, S. Zaramba, A. Opio, J.J. Lutwama, A.O. Talisuna, S.I. Okware); World Health Organization Country Office, Kampala (Z. Yoti, W. Mbabazi, M. Nanyunja); and African Field Epidemiology Network Secretariat, Kampala (P. Nguku)

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second report, ill persons had an acute febrile hemorrhagic illness, and tests conducted by the US Centers for Disease Control and Prevention (Atlanta, GA, USA) confirmed the illness as EHF on November 29, 2007.

Methods

Outbreak Site

Bundibugyo district is located in western Uganda (Figure 1). Approximately 60% of the district is covered by the Rwenzori Mountains and Semliki National Park and Game Reserve, which have a broad range of wildlife, including primates. The district has a population of 253,493 and a population density of 108 persons/km^2 (*9*), 1 hospital, and 26 health centers. The main economic activities are cocoa farming, fishing, and tourism. Hunting of wild animals is common among settlements near the national park.

Epidemiologic Activities

During November 29, 2007–February 20, 2008, village health teams conducted active searches in the communities through daily door-to-door visits in their respective villages while investigation teams of local and international experts reviewed case notes at health facilities and verified suspected cases reported by the village health teams. Cases were categorized as suspected EHF, probable EHF, and confirmed EHF (Table 1).The UMOH developed working case definitions and established an enhanced surveillance system for identifying suspected case-patients in the affected district and in contiguous districts.

Investigative Activities

Investigation teams actively searched for case-patients in health facilities and communities and retrospectively reviewed hospital records. Increased awareness of the disease through public education campaigns and the media facilitated reporting of suspected cases to health authorities. Village health teams assisted in case identification and reports and contact follow-up at the community level. Clinical and epidemiologic data were systematically collected from persons with suspected, probable, and confirmed cases. Proxies (usually parents, spouses, or adult siblings) were interviewed for information about case-patients who had died before an interview could be conducted. Persons with suspected cases identified in the community were transported by a mobile ambulatory team to designated isolation facilities. A triage desk was established in the outpatient departments of each health facility in the district to screen for suspected cases and notify the surveillance system. Clinical specimens, including blood, were collected for laboratory testing from all persons with suspected EHF. All contacts were entered into, and follow-up schedules were drawn by using, the Field Information Management System database (*10*).

Figure 1. Major towns and districts in Uganda; districts and major towns share the same names. Green shading, national parks; red lines, main roads; blue shading, perennial lakes.

Laboratory Methods

Five milliliters of blood was collected from all persons with suspected EHF at least 4 days after symptom onset when possible. Specimens initially were transported to the Uganda Virus Research Institute (UVRI) and then transferred to US Centers for Disease Control and Prevention for laboratory analysis. However, on December 4, a laboratory was set up at UVRI, and subsequent specimens were tested there, as described by Towner et al. (*11*). Virus isolation was not attempted at UVRI.

Data Analysis

We used Epi Info software version 3.4 (*12*) to create a database into which information from individual case investigation forms was entered and updated daily. The age and sex population structure and projections for Bundibugyo district were obtained from the population and housing census 2002 data (*9*), and attack rates were computed by using the district population projections. Current geographic maps were obtained from the World Health Organization Health Mapper Mapping Software, version 4.2 (*13*).

We tabulated risk factors by case status and calculated odds ratios (ORs) using as the reference group persons with suspected EHF who had negative test results. To control for confounding and to test for effect modification, we entered variables with p values <0.1 into a multivariable logistic regression model. By using backward elimination, we eliminated all variables that were not statistically significant at p<0.05. The model had the following co-variables: admission to hospital or visit with a sick person, consulta-

tion with traditional healer, participation in funeral rituals, travel to area with cases, contact with a known suspected/confirmed case-patient, contact with wildlife, subcounty of residence, age, and sex. The Uganda National Council of Science and Technology expedited ethical review and clearance because the data were being collected to guide outbreak control.

Results

Outbreak Response

UMOH declared an EHF outbreak on November 29. Consequently, the National Task Force, composed of both local and international partners, was activated to determine the magnitude of the outbreak and coordinate immediate outbreak response.

All case-patients were treated in isolation wards set up in Kampala at the National Referral Hospital, Mulago, and in Bundibugyo at Kikyo and Bundibugyo health facilities. Health workers on the case-management teams had participated in patient management during the SEBOV outbreak in Gulu in 2000, thus, their experience provided a valuable resource during the response. Countrywide social mobilization and health education during the outbreak involved developing and disseminating health education materials and messages through mobile video shows, community meetings with opinion leaders, radio talk shows, and newspaper inserts.

Index Case Investigation

The putative index patient was a 26-year-old woman from Kabango village, Kasitu subcounty, in Bundibugyo district. Hunting spears were found at her home, but hunting as a practice was denied. Fever and general weakness developed in this woman, for which she was hospitalized on August 1. She delivered a preterm infant the following day. Diarrhea and difficulty breathing developed, but hemorrhagic manifestations did not appear. She died on August 4.

In this cluster, 9 case-patients and 6 deaths (the neonate, sister, mother, and 2 nieces of the index patient) (CFR 67%) were reported. The mother and sister were involved in nursing and handling the remains of the index patient because, at the time, barrier nursing and supervised burials had not been initiated. Because these persons were affected before the outbreak was confirmed, the high CFR was attributable to inadequate supportive care. Three survivors were tested; 2 were positive for *Bundibugyo ebolavirus*–specific IgG.

Description of Cases

Illnesses in 192 persons met the definition for suspected EHF. Of these, 42 (22%) were laboratory confirmed as positive for a novel Ebola virus species *(Bundibugyo ebolavirus*); 74 (38%) remained probable, and 76 (40%) were laboratory negative and classified as noncases.

Table 1. Case definitions for epidemiologic investigation of EHF outbreak, Bundibugyo district, Uganda, 2007–2008*

Classification	Definition
Suspected case	Sudden onset of fever and at least 4 of the following symptoms in a resident of or visitor to the affected subcounties in Bundibugyo district: vomiting, diarrhea, abdominal pain, conjunctivitis, skin rash, unexplained bleeding from any body part, muscle pain, intense fatigue, difficulty swallowing, difficulty breathing, hiccups, or headache since August 1, 2007, OR sudden onset of fever in any person who had had contact with a person with suspected, probable, or confirmed EHF, OR sudden death in a person in the community without any other explanation.
Probable case	Suspected EHF in any person (dead or alive) with at least 3 of the following symptoms; vomiting, diarrhea, or unexplained bleeding from any site, conjunctivitis, or skin rash; AND with an epidemiologic link to a person with probable or confirmed EHF, OR either no specimen collected for laboratory testing or a negative laboratory result in a specimen collected 0–3 days after onset of symptoms in a person with suspected EHF.
Confirmed case	Laboratory confirmation of infection by isolation of virus from any body fluid or tissue, OR detection of viral antigen in any body fluid or tissue by antigen-detection ELISA, reverse-transcription–PCR, or immunohistochemistry, OR demonstration of serum Ebola virus–specific IgG antibodies by ELISA, with or without IgM, in any person with suspected or probable EHF.
Contact	A person who had slept in the same household and/or had direct physical contact with a person (dead or alive) with suspected, probable, or confirmed EHF and/or had been exposed to an infected person or to an infected person's secretions, excretions, tissues, or linens within 3 weeks after that person's onset of illness.

*EHF, Ebola hemorrhagic fever; Ig, immunoglobulin.

Overall, 39 of the 116 persons with confirmed and probable EHF died (CFR 34%). For male patients, the CFR was 35% (23/65) and for females, 31% (16/51). The CFR for confirmed EHF was 33% (14/42) and for suspected EHF, 34% (25/74). Case-patients' ages ranged from 3 weeks to 70 years (mean 34 years, median 35 years). Most 40 (36%) case-patients were crop farmers; 14 (12%) were healthcare workers.

Symptoms

The median incubation period from contact with an infected person to symptom onset was 7 days (range 2–20 days). The most frequent symptoms were fever (100%), intense fatigue (92%), headache (87%), abdominal pain

(87%), vomiting (83%), and diarrhea (83%). Bleeding manifestations (including hematuria; hematemesis; bleeding from the eyes, nose, and vagina; and/or bloody stool) occurred in 52% of case-patients, and most (59%) case-patients who died had some form of bleeding. Other symptoms reported were dysphagia (54% of case-patients), dyspnea (52%), and rash (50%). Median duration from symptom onset to recovery was 10 days (range 2–26 days). Median duration from symptom onset to death was 10 days (range 3–21 days).

The outbreak showed 3 successive cycles of transmission (Figure 2). Each transmission cycle lasted 6 weeks, with an interval of 3–11 days, and was characterized by a typical epidemic peak followed by gradual decline. The highest peak occurred on November 25. Despite the timely detection and initiation of investigations during the 30th epidemiologic week, the successive transmission cycles indicated delayed laboratory confirmation and declaration of the outbreak. Outbreak confirmation was delayed until the 48th epidemiologic week, resulting in inadequate control measures before the outbreak was confirmed.

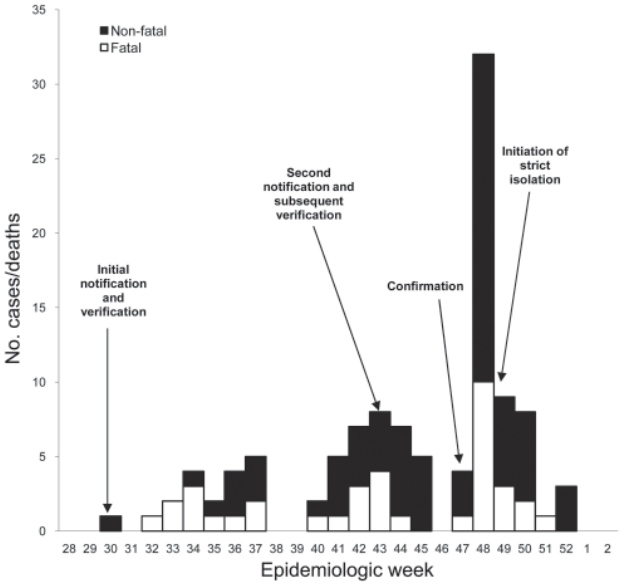

Figure 2. Ebola outbreak, by week of onset for probable and confirmed cases (n = 116), Bundibugyo district, Uganda, August–December 2007.

Geographic Characteristics and Risk Factors

The outbreak was confined to Bundibugyo district. More than 97% of cases were detected in 4 of the 10 subcounties in the district (Kasitu, Bundibugyo town council, Bubukwanga, and Busaru), with >54% of cases occurring in Kasitu subcounty (Table 2).

The overall attack rate in the district was 43 cases/100,000 population. The highest rate occurred in Kasitu subcounty, followed by the rate in Bundibugyo town council (within Bundibugyo district (Table 2). By sex, the attack rate for men was higher than that for women (64 vs. 47/100,000 population). By age group, the attack rate was higher for persons 41–50 years of age than for persons 51–60 years of age (146 vs. 122/100,000 population).

Analysis of Possible Risk Factors

All case-patients were investigated for exposures within 3 weeks before development of symptoms. To better determine risk factors for the disease, we conducted a bivariate analysis using the 76 non–case-patients as the reference group to separately assess persons with probable and confirmed EHF (Table 3). Before the institution of strict isolation policies, visitors had direct contact with patients through shaking of hands, hugging, or contact with potentially infected surfaces. Patients with confirmed EHF (OR 8.71, 95% confidence interval [CI] 3.03–26.30) and patients with probable and confirmed cases combined (OR 2.56, 95% CI 1.35–4.85) were significantly more likely to have visited sick persons or to have visited the hospital 3 weeks before becoming sick. Consultation with a traditional healer within 3 weeks before illness onset was not significantly associated with having EHF (OR 0.16, 95% CI 0.01–1.15) (Table 3).

The other risk factor identified was participation in funeral rituals before onset of illness. The funeral rituals, performed by close relatives, involved washing and dressing the body of the decedent. Patients with probable EHF (OR

Table 2. Geographic distribution of persons with Ebola hemorrhagic fever, Bundibugyo district, Uganda, 2007–2008

Subcounty	Population	No. cases	No. deaths	Case-fatality rate, %	Attack rate*
Kasitu	33,968	63	18	29	185
Bundibungyo town council	17,590	25	8	32	142
Bubukwanga	23,398	17	7	41	73
Busaru	40,547	8	3	38	20
Harugali	29,162	1	1	100	3
Karugutu	19,384	1	1	100	5
Bubandi	22,063	1	1	100	5
Other subcounties	81,879	0	0	0	0
Total	267,991	116	39	34	43

*Per 100,000 population.

Table 3. Bivariate analysis of risk factors for Ebola viral hemorrhagic fever, Bundibugyo district, Uganda, 2007–2008*

Potential risk factor	Probable case, n = 74	Confirmed case, n = 42	Probable/confirmed case, n = 116	Noncase, n = 76 (ref.)
Hospitalized/visited hospital, no. (%)	38 (51.40)	36 (85.70)	74 (63.79)	31 (40.80)
OR (95% CI)	1.5 (0.8–3.1)	**8.7 (3.0–26.3)**	**2.6 (1.4–4.9)**	1
p value	0.2	**<0.001**	**<0.05**	
Consulted traditional healer, no. (%)	1 (1.4)	0	1 (0.9)	4 (5.3)
OR (95% CI)	0.25 (0.01–2.4)	Undefined	**0.2 (0.01–1.5)**	1
p value	0.2		**0.06**	
Participated in funeral rituals, no. (%)	43 (58.1)	32 (76.2)	75 (64.7)	23 (30.2)
OR (95% CI)	**3.2 (1.6–6.6)**	**7.4 (2.9–19.3)**	**4.22 (2.2–8.2)**	1
p value	**<0.001**	**<0.001**	**<0.001**	
Traveled before illness, no. (%)	**29 (39.20)**	11 (26.20)	**40 (34.50)**	15 (19.74)
OR (95% CI)	**2.62 (1.2–5.8)**	1.4 (0.5–3.8)	**2.1 (1.0–4.5)**	1
p value	**<0.05**		**0.03**	
Had contact with person with known suspected case, no. (%)	48 (64.90)	42 (100.00)	90 (77.60)	43 (56.58)
OR (95% CI)	1.4 (0.7–2.9)	Undefined	**2.7 (1.35–5.24)**	1
p value	0.3		<0.05	
Had contact with wildlife, no. (%)	1 (1.4)	0	1 (0.9)	1 (1.3)
OR (95% CI)	1.0 (0.0–38.4)	Undefined	0.7 (0.02–24.30)	1
p value	1.0		0.8	
Male sex, no. (%)	40 (54.00)	25 (59.52)	65 (56.00)	37 (29.31)
OR (95% CI)	1.2 (0.6–2.5)	1.6 (0.7–3.6)	1.3 (0.7–2.5)	1
p value	0.5	0.3	0.3	
Age 41–60 y, no. (%)	18 (24.30)	16 (38.10)	34 (0.90)	18 (1.32)
OR (95% CI)	1.0 (0.5–2.3)	2.0 (0.8–4.9)	1.3 (0.7–2.7)	1
p value	0.9	0.1	0.4	

*Ref., reference; OR, odds ratio; CI, confidence interval. **Boldface** indicates significant associations.

3.20, 95% CI 1.55–6.64) and confirmed EHF (OR 7.37, 95% CI 2.89–19.27) were significantly more likely than persons in the reference group to have participated in funeral rituals before they became sick (Table 3). Similarly, when both groups of patients with probable and confirmed EHF were combined (OR 4.22, 95% CI 2.17–8.24), the members of the new group were significantly more likely to have participated in funeral rituals before becoming sick (Table 3).

Ebola case-patients were asked whether they had had any form of contact with a person known to have suspected or confirmed EHF. Patients with probable and confirmed EHF combined were significantly more likely (OR 2.66, 95% CI 1.35–5.24) to have had contact with a person known to have suspected or confirmed EHF before becoming sick (Table 3).

We performed multivariate analysis using binary logistic regression to control for confounding and to test for effect modification. After backward elimination, participating in funeral rituals remained as the sole significant risk factor associated with being a probable/confirmed case-patient (adjusted OR 3.83, 95% CI 1.78–8.23).

Discussion

The EHF epidemic in Bundibugyo district in western Uganda during August 1, 2007–February 20, 2008, was caused by a new species of the virus, *Bundibugyo ebolavirus*. It most likely was transmitted from wildlife located within Semliki National Park. The median incubation period of 1 week was less than that reported previously (*5*) but within the range reported in other Ebola outbreaks (*1*,*2*). In addition, patients' symptoms were similar to those reported previously, except for the manifestation of bleeding, which occurred >2× more often than during the SEBOV outbreak in Gulu in 2000 (*5*).

The *Bundibugyo ebolavirus* outbreak (CFR 34%) caused a lower proportion of deaths than did the SEBOV outbreak in Gulu (CFR 53%) (*5*). Similarly, the CFR for the Bundibugyo outbreak was lower than that reported from other outbreaks outside Uganda (*1*,*2*), which indicates either that the new virus strain may be less virulent or that improved interventions led to more timely case identification and better case management.

During the Bundibugyo outbreak, vital functions were sustained by supportive treatment, including administering antipyretics, monitoring fluid balance, and giving antibacterial or antimalarial drugs for concurrent bacterial or protozoal infections. Oral rehydration and oral administration of antibacterial drugs were encouraged for all patients, provided they were conscious and not vomiting; otherwise, fluids and antibiotics were administered intravenously. To streamline case detection, village health teams and ambulance teams were trained early to conduct active case search and referral. Strict isolation measures included the establishment of triage in all health facilities, designation

of isolation wards, training of healthcare workers in adherence to standard precautions, barrier nursing, supervised burial, and ambulance services.

During the Bundibugyo outbreak, case-patients were more likely than non–case-patients to have participated in funeral rituals. The practice exposes contacts to infectious body fluids that have been associated with acquiring EHF (*4*).

Fourteen health workers were infected during the Bundibugyo outbreak before strict isolation procedures were initiated. During the SEBOV outbreak in Gulu, 64% of health workers were infected after isolation wards were established (*14*).

The outbreak response had 2 challenges and at least 1 limitation. Investigations at the local and national levels were conducted in a timely manner, but the lack of capacity for laboratory confirmation delayed initial outbreak confirmation and therefore the initiation of an appropriate response. Because hunting in the national parks is illegal, attempts to link the Bundibugyo outbreak to wildlife were futile because none of the families investigated admitted to participating in hunting. The use of hospitalized patients as a comparison group was economical, but their illnesses could have been related to risk factors for *Bundibugyo ebolavirus* infection, hence rendering those risk factors undetectable.

We recommend that an index of suspicion for Ebola viruses (and Marburg virus) be maintained for clusters of cases with fever of sudden onset, intense fatigue, abdominal upsets, and evidence of person-to-person transmission. In concert with timely initiation of active case searching, use of ambulance and burial teams, and strict adherence to patient isolation practices, an index of suspicion should ensure mitigation of the identified risk factors.

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Dr Wamala is a medical epidemiologist at the Ministry of Health, Uganda. His research interests include disease surveillance for the control of epidemic prone diseases.

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Address for correspondence: Joseph F. Wamala, Epidemiological Surveillance Division, Ministry of Health Uganda, PO Box, 7272, Kampala, Uganda; email: j_wamala@yahoo.com

High Diversity and Ancient Common Ancestry of Lymphocytic Choriomeningitis Virus

Cesar G. Albariño,[1] Gustavo Palacios,[1] Marina L. Khristova, Bobbie R. Erickson, Serena A. Carroll, James A. Comer, Jeffrey Hui, Thomas Briese, Kirsten St. George, Thomas G. Ksiazek,[2] W. Ian Lipkin, and Stuart T. Nichol

Lymphocytic choriomeningitis virus (LCMV) is the prototype of the family *Arenaviridae*. LCMV can be associated with severe disease in humans, and its global distribution reflects the broad dispersion of the primary rodent reservoir, the house mouse (*Mus musculus*). Recent interest in the natural history of the virus has been stimulated by increasing recognition of LCMV infections during pregnancy, and in clusters of LCMV-associated fatal illness among tissue transplant recipients. Despite its public health importance, little is known regarding the genetic diversity or distribution of virus variants. Genomic analysis of 29 LCMV strains collected from a variety of geographic and temporal sources showed these viruses to be highly diverse. Several distinct lineages exist, but there is little correlation with time or place of isolation. Bayesian analysis estimates the most recent common ancestor to be 1,000–5,000 years old, and this long history is consistent with complex phylogeographic relationships of the extant virus isolates.

The rodent-borne arenaviruses (family *Arenaviridae*) are enveloped viruses with bisegmented RNA genomes that include several causative agents of hemorrhagic fevers in the New World and Africa (*1*). The large (L) genome RNA segment encodes the virus polymerase L and the Z protein, whereas the small (S) genome RNA segment encodes the nucleocapsid protein (NP) and glycoprotein precursor (GPC). The prototypic arenavirus, *Lymphocytic choriomeningitis virus* (LCMV), is distributed worldwide (due to its association with rodents of the species *Mus musculus*). This virus is typically associated with mild, self-limited, or asymptomatic infections in immunocompetent persons, but infections can lead to aseptic meningitis (*1*). In immunocompromised persons, LCMV exposure may result in serious systemic infections and death (*2*). Prenatal infection can cause spontaneous abortion or severe birth defects, including hydrocephalus, chorioretinitis, blindness, or psychomotor retardation (*3*,*4*).

Recent clusters of fatal disease in organ transplant recipients have focused new attention on the potential for iatrogenic transmission of LCMV. In December 2003 and April 2005, recipients of solid-organ transplants linked to single donors, died of unexplained infections. LCMV was implicated after the results of viral culture and electron microscopy triggered specific immunohistochemical and molecular tests for arenaviruses (*2*). In the 2005 cluster, a pet hamster that had been introduced into the donor's household was infected with the same virus that was later detected in the recipients (*5*). In early 2007, three patients who received visceral transplants on the same day from a single donor died of a febrile illness 4–6 weeks after transplantation. Unbiased high-throughput sequencing yielded sequences that identified a novel LCMV-related arenavirus (*6*). However, phylogenetic characterization was limited by the paucity of available sequences deposited in public databases. In April 2008, a public health investigation showed evidence of acute LCMV infection in 2 transplant recipients who had received kidneys from a common donor. Both patients died 4 and 10 weeks after transplantation despite intensive supportive care (*7*).

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C.G. Albariño, M.L. Khristova, B.R. Erickson, S.A. Carroll, J.A. Comer, T.G. Ksiazek, S.T. Nichol); Columbia University, New York, New York, USA (G. Palacios, J. Hui, T. Briese, W.I. Lipkin); and New York State Department of Health, Albany, New York, USA (K. St. George)

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[1] These authors contributed equally to this article.

[2] Current affiliation: University of Texas Medical Branch, Galveston, Texas, USA.

In spite of the increasing awareness of the public health importance of LCMV, little is known about the genetic diversity or relationships of LCMVs found in various parts of the world. Previous studies have suggested that nucleotide sequence divergence is high, up to 22% between some LCMVs (*8*–*10*). In the current study, we investigate the genetic diversity of 29 LCMVs, and infer from those sequences a history reaching back >1,000 years, findings consistent with the existing complex virus phylogeographic patterns.

Materials and Methods

Most of the sequences included in the alignments correspond to complete segment sequences. However, some short sequences, such as those from Kodoko virus, were also included in the analysis. This approach was taken to obtain the best reconstruction of the evolutionary history of the taxa (viruses, in our case) by using the maximum number of informative sites available (*11*–*13*). Although the validity of including missing data has been debated in the past, more recent studies have shown that even highly incomplete taxa can be placed accurately within the phylogeny (*11*,*12*,*14*).

The appropriateness of this approach was further examined by running several preliminary analyses. Initially, only full-length segment sequences were analyzed. Once the relationships between taxa and rate estimates were established, partial sequences (e.g., Kodoko virus) were also added to the analyses. No rate shifts were observed nor were any strongly supported phylogenetic relationships obscured. As a result, the tree figures shown in this report were based on the dataset including both whole segment and partial segment sequences.

From virus collections at the Centers for Disease Control and Prevention, the New York State Department of Health, Columbia University, and the World Reference Center of Emerging Viruses and Arboviruses (University of Texas Medical Branch), we selected 12 LCMVs for genetic characterization; origins spanned >70 years with broad geographic distribution (Table 1). Included in the study were representative virus stocks of the classic WE LCMV strain. This strain was originally isolated from a meningoencephalitis patient in New York in 1935 (*15*). In that era, virus isolation and passage were performed by intracranial inoculation into mice, which resulted in isolates that had multiple passages in mice as part of their passage history. Although the WE strain is used extensively in immunobiology experiments, the exact passage history of these viruses has been poorly documented. We located 2 old stocks of WE, 1 lyophilized in 1950 with a record of 7 passages in mouse brain, and 1 lyophilized in 1960 with a record of 7 passages in mouse brain and virus plaque purification (Table 1). In the 1940s, the WE LCMV strain was transferred to the University of British Columbia from the Rockefeller Institute in New York, only to be returned to the New York State Department of Health some years later. This substrain of WE LCMV became known as UBC (*16*). The 2 lyophilized vials were both labeled as UBC WE LCMV. Two other early LCMV isolates were also found. The Douglas-4707 and WHI-5107 strains were isolated by intracranial inoculation of suckling mice from the cerebrospinal fluid of patients in New York who had aseptic meningitis in 1947 and 1949, respectively. These viruses were recovered from lyophilized stocks prepared in 1960 and 1950, respectively (*17*), and represent some of the oldest low passage LCMV stocks still in existence.

The Lyles LCMV strain was isolated in Vero cells from the cerebrospinal fluid (CSF) of a 58-year-old woman from Winder, Georgia, who had nonfatal aseptic meningitis and a history of exposure to mice in her home (*18*). Similarly, the Michigan 2005 LCMV strain was isolated in 2005 from a mouse captured around the home of a 46-year-old woman with a diagnosis of acute meningitis and mild pancreatitis (*19*). The California 2003 LCMV was isolated in 2003 from the CSF of a congenitally infected infant with severe neurologic sequelae, including hydrocephalus, chorioretinitis, blindness, and developmental delay (*20*). The other LCMV isolates were from investigations of clusters of deaths and severe illness in transplant recipients associated with LCMV infection from transplanted organs. Four isolates were obtained during 2003–2008 from infected transplant recipients or rodents suspected of being involved in the exposure of the transplant donor in various locations in the United States (*2*,*5*,*7*). The Dandenong isolate was obtained from the liver of a patient who died after transplantation in Australia; the donor was suspected to have acquired the infection while traveling in the Balkans shortly before death and the harvesting of his organs (*6*). Finally, the isolate from Bulgaria (1956) is strongly suspected of being the first isolate obtained in Bulgaria from a case-patient with confirmed lymphocytic choriomeningitis (*21*).

RNA was extracted either directly from virus stocks or from supernatant harvested from infected cell cultures. A 300-μL aliquot of virus stock or cell culture supernatant was mixed with 900 μL of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and 240 μL of chloroform and extracted according to standard protocols. The nucleic acid obtained was reverse transcribed and amplified by PCR; a total of 12 LCMV S segment sequences and 10 LCMV L segment sequences were amplified and sequenced by dideoxy-sequencing (Applied Biosystems, Foster City, CA, USA). We were unable to amplify by PCR the L segments of LCMV strains WHI-5107 and UBCA337 from the original virus ampoules, and the viruses were found to be no longer viable. The origins of 16 LCMV isolates for which sequences were already available, and that were included in the study, are also shown in Table 1. Multiple

sequence alignments were generated using Multiple Alignment with Fast Fourier Transform (*22*) in SeaView (*23*) and sequence diversity was calculated by using molecular evolutionary genetics analysis (MEGA) 4 (*24*). Bayesian phylogenetic analyses of the sequence differences among the S and L segments of LCMV and Kodoko viruses were conducted using the BEAST, BEAUti and Tracer analysis software packages (*25*). Preliminary analyses were run for 10,000,000 generations with the Hasegawa, Kishino, and Yano + Γ nucleotide substitution model to select the clock and demographic models most appropriate for the S and L data sets. An analysis of the marginal likelihoods indicated that the relaxed lognormal molecular clock and constant population size model was decisively chosen ($\log_{10}$ Bayes

Table 1. Origins of lymphocytic choriomeningitis virus strains analyzed*

Strain (other names)	Collection date (passage history)	Associated case (sample source)	GenBank accession no.
Massachusetts-2008 (811316, 200802972)	Massachusetts, USA, 2008	Transplant case (human blood)	FJ607022,† FJ607031†
Dandenong	Former Yugoslavia, 2006	Transplant case (human liver)	EU136039, EU136038
Rhode Island-2005 (810850, 200501927)	Rhode Island, USA, 2005	Transplant case (hamster kidney)	FJ607021,† FJ607030†
Ohio-2005(810896, 200504261)	Ohio, USA, 2005	Transplant case (hamster kidney)	FJ607026,† FJ607037†
Michigan-2005 (810885, 200504219)	Michigan, USA, 2005	House rodent infestation (mouse spleen)	FJ607023,† FJ607032†
California-2003 (810366, 200312154)	California, USA, 2003	Congenital infection (human CSF)	FJ607019,† FJ607028†
Wisconsin-2003 (810362, 200312181)	Wisconsin, USA, 2003	Transplant case (human CSF)	FJ607027,† FJ607038†
Lyles (810935, Georgia-1984)	Georgia, USA, 1984	House rodent infestation (human CSF)	FJ607020,† FJ607029†
Douglas-4707 (810938, NY-H938)	New York, USA, 1947 (stock lyophilized in 1960; 1 passage in VE6 in 2005)	Human CSF	FJ607024,† FJ607035†
WHI-5107 (810906, NY-H906)	New York, USA,1949 (stock lyophilized in 1950)	Human CSF	FJ607033†
WE-UBC-57135 (810940, NY-H940)	New York, USA,1935 (7 passages in MB, plaque purified; stock lyophilized in 1960; 1 passage in VE6 in 2005)	Human specimen	FJ607025,† FJ607036†
WE-UBC-A337 (810909, NY-H909)	New York, USA, 1935 (7 passages in MB, stock lyophilized in 1950)	Human specimen	FJ607034†
Bulgaria	Bulgaria, 1956? (WRCEVA collection at UTMB)	?	GQ862981,† GQ862982†
M1, M2	Austria, 2005	Infection of mouse colony (mouse spleen)	AB261990, AB261991
LE	France, 2006	Congenital infection (amniotic fluid)	EF164923
Marseille	France, 2004	House rodent infestation (mouse kidney)	DQ286932, DQ286931
CH5692, CH5871	Germany, 1999; Germany, 2000	Infection of monkey colony (monkey spleen and serum)	AY363903, AF325214, AF325215,
CHV1, CHV2, CHV3	Oklahoma, USA,1986	Infection of monkey colony (monkey liver)	U10157, U10158, U10159
MX	Slovakia, 1998	Persistently infected cell line	EU195888, EU195889
Yale (Y)	Connecticut, USA, 1977	Mouse	DQ118959
WE	New York, USA, 1935	First recognized aseptic meningitis by LCMV	M22138, AF004519
Armstrong	Missouri, USA, 1933	St. Louis encephalitis epidemics	AY847351, M20869
CABN, GR01, SN05	Spain, 2004	Wild mice (mouse lungs)	FJ895882, FJ895883, FJ895884

*CSF, cerebrospinal fluid; MB, mouse brains; WRCEVA, World Reference Center of Emerging Viruses and Arboviruses; UTMB, University of Texas Medical Branch; LCMV, lymphocytic choriomeningitis virus. ? represents the uncertainty of this virus origin.
†New sequences.

factors = 3.032 for S segment; 13.472 for L segment). Final data analyses included Markov chain Monte Carlo chain lengths of 20,000,000–480,000,000 generations, with sampling every 1,000 states.

Results

Initial S and L segment sequence comparison and phylogenetic analysis confirmed that all the LCMV and LCMV-like (including Dandenong and Kodoko) virus genome sequences were monophyletic and distinctly related to the other Old World arenaviruses (data not shown and [*26*]). Only fragments of the Kodoko virus genome sequence were available, but results of our analysis were consistent with the previous conclusion that this virus is distinct from LCMV. The S and L segment sequences of all LCMVs (including Dandenong) were distributed in 3 (L segment) or 4 (S segment) different genetic groups or lineages (Figures 1, 2). High levels of virus genetic diversity (Table 2) and protein amino acid differences were found within and between the virus lineages. Up to 18% nucleotide divergence was observed within the S segment lineages, and 22%–25% divergence between 4 characterized lineages (Table 2). Similarly, up to 25% nucleotide divergence was observed within the L segment lineages, with 27%–28% between the 3 currently identified lineages. This nucleotide divergence translates to 18%, 13%, 10%, and 6% divergence in the amino acid sequences of the Z, L, GPC, and NP proteins, respectively. While this level of diversity is considerable, it is comparable to that observed in Lassa virus (LASV), another Old World arenavirus (*27*,*28*).

The S segment tree generated from analysis of 31 virus strains (29 LCMV strains and 2 Kodoko virus strains) is shown in Figure 1. The nodes separating the 4 major lineages are highly supported (posterior probability values >95). Kodoko virus is located on an ancestral branch, sister to the monophyletic clade that contains all of the LCMV strains. Most of the LCMV strains are located within lineage I, which contains all the US strains, with the exception of the virus isolate from Georgia in 1984, the sole member of lineage III (Figure 1). Lineage I includes the classic laboratory strains, WE and Armstrong, originally isolated in the 1930s. Notably, the sequences of the low passage WE strain obtained from virus stocks lyophilized in 1950 and 1960 were identical to one another, but statistically significantly different from the WE isolate currently in use and reported in GenBank (*29*). Another related strain of LCMV may have contaminated the virus stock during the passages in mice, and it is difficult to discern which virus represents the authentic WE. Although the stocks lyophilized in 1950s and 1960s were archived several decades ago, these viruses had been passaged in a laboratory in British Columbia before their return to New York.

Lineage I also includes viruses from France, Germany, and Slovakia. No obvious correlation could be seen between phylogenetic branching pattern and virus geographic origin. In addition, although this lineage contains viruses isolated during 1935–2008, no correlation was evident between phylogenetic position and date of virus isolation. These data are consistent with a long and complex evolutionary history with frequent movement of the rodent reservoir hosts during this lengthy period.

Lineage II appeared to only contain viruses from Europe. These included the LCMV M1 and M2 viruses, which had been isolated in Japan from a laboratory mouse colony established in Paris, France, from wild-caught *M. musculus musculus* that originated in Illmitz, Austria. In addition, lineage II contained the LE strain isolated from a patient in France and the Dandenong isolate obtained in Australia from a transplant recipient with a fatal LCMV infection. This patient had received organs from a donor with travel history to the Balkans before death and organ donation (*6*). Lineage IV was solely made up of viruses isolated in Spain from wild-caught wood mice (*Apodemus sylvaticus*) (*10*).

The L segment tree generated from analysis of 18 virus strains is shown in Figure 2. The overall lineage I, II, and III groupings are comparable to those observed in the S segment tree. Lineage IV is not observed because no L segment sequences were available from strains from Spain. Again, among the multiple virus representatives within lineage I, no clear correlation is apparent between phylogenetic pattern and geographic or temporal origin of the virus isolates. Most of the differences in tree topology seen between the S and L trees involve nodes, which are not strongly supported, and appear to mainly reflect the lack of resolution in the trees. In addition, the analyses do not include identical taxa sets (S and L segment data are not available for all of the viruses). Whether the observed differences reflect RNA segment reassortment or differences in evolutionary pressures cannot be discerned from the current analysis.

The Bayesian analysis enabled estimation of the rate of evolution of the 2 genome segments of the LCMV and Kodoko virus sequences. The molecular evolutionary rate for the S segment was estimated to be 3.3×10^{-4} substitutions/site/year with 95% highest posterior density of 1.4×10^{-4} to 5.2×10^{-4}. Similarly, the molecular evolutionary rate for the L segment was 3.7×10^{-4} substitutions/site/y (95% highest posterior density of 1.2×10^{-7} to 8.6×10^{-4}). These rates are similar to those found for other negative-stranded RNA viruses, such as the calculated rates for Rift Valley fever virus: 3.9×10^{-4}, 3.6×10^{-4}, and 2.8×10^{-4} for the S, M, and L segments, respectively (*30*).

The Bayesian analysis also addressed the question of how recently viruses may have shared a common ancestor.

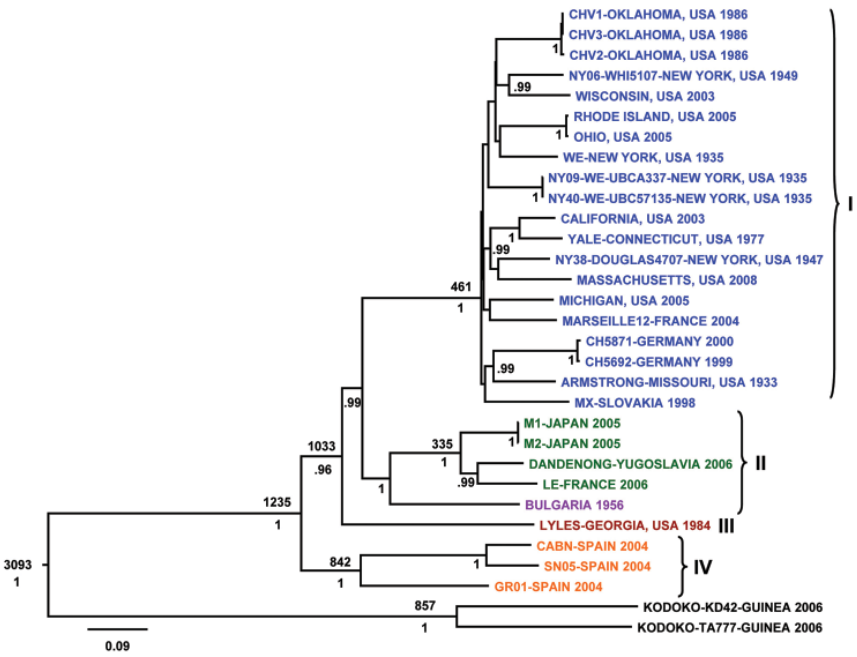

Figure 1. Bayesian coalescent analysis of lymphocytic chorio-meningitis virus (LCMV) based on the small (S) gene segment. The maximum clade credibility tree generated from analysis of available LCMV S segment sequences is shown. Branch lengths are proportional to the number of substitutions/site/year. Depicted at the main nodes are the time to most recent common ancestor estimates (TMRCA) based on Bayesian coalescent analysis of the virus sequences and isolation dates without inclusion of the Bulgarian strain for which no reliable isolation date was available. Posterior probabilities are listed below the branches for supported nodes. Scale bar indicates nucleotide substitutions per site.

The most recent common ancestor for LCMV S- and L- genome RNA segments was estimated to be 1,235 and 5,142 years ago, respectively. These data indicate that LCMV is quite ancient, and the extensive diversity of the virus has accumulated over the past 1,000–5,000 years. Also, despite similar rates of evolution, the evolutionary history of the L segment appears to be more complex and can be traced back substantially longer than that for the S segment.

The protein sequences and various motifs of the diverse LCMV strains were analyzed in detail. The N-terminal myristoylation site, the RING motif, and late domains in the Z protein are all highly conserved (*31*) (online Appendix Figure 1, www.cdc.gov/EID/content/16/7/1093-appF1.htm). The previously identified domains and catalytic core motifs of the LCMV L polymerase (*32*) and NP motifs (*33*) were also highly conserved among all strains analyzed (online Appendix Figure 2, www.cdc.gov/EID/content/16/7/1093-appF2.htm and online Appendix Figure 3, www.cdc.gov/EID/content/16/7/1093-appF3.htm). The GPC protein motifs initially identified in the LCMV Armstrong strain, such as the 2 hydrophobic domains found in the signal peptides, the myristoylation site G_2, and most of the predicted glycosylation sites found in other arenaviruses are well conserved (*34*) (online Appendix Figure 4, www.cdc.gov/EID/content/16/7/1093-appF4).

Discussion

The primary host of LCMV is thought to be the house mouse. Three house mouse complexes within the genus *Mus* (*castaneus, domesticus*, and *musculus*) are generally recognized; however, their taxonomic rank (i.e., species vs. subspecies) has been debated extensively (*35*). Recent

Table 2. Genetic diversity of lymphocytic choriomeningitis virus strains*

	RNA, nt		Proteins, aa			
Lineages	L	S	Z	L	GPC	N
Overall	22	19	18	13	10	6
Within lineage I	19	15	15	11	6	4
Within lineage II	25	15	34	18	7	3
Within lineage III†	NA	NA	NA	NA	NA	NA
Within lineage IV‡	NA	18	NA	NA	13	5
Between lineages I and II	27	22	27	19	11	6
Between lineages I and III	28	23	19	19	11	8
Between lineages I and IV	NA	24	NA	NA	19	9
Between lineages II and III	27	22	28	19	11	6
Between lineages II and IV	NA	24	NA	NA	18	10
Between lineages III and IV	NA	25	NA	NA	19	11

*nt, nucleotide; aa, amino acid; GPC, glycoprotein precursor; NA, not applicable. Pairwise genetic distances (uncorrected p values) were measured eliminating all positions containing alignment gaps and missing data.
†Only 1 sequence in lineage III.
‡No available L sequence in lineage IV.

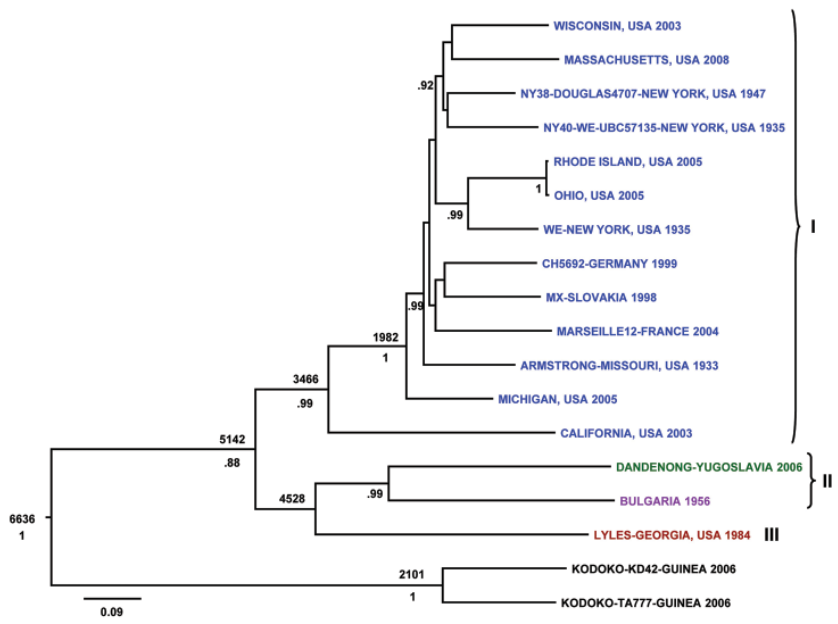

Figure 2. Bayesian coalescent analysis of lymphocytic chorio-meningitis virus (LCMV) based on the large (L) gene segment. The maximum clade credibility tree generated from analysis of available LCMV L segment sequences is shown. Branch lengths are proportional to the number of substitutions/site/year. Depicted at the main nodes are the time to most recent common ancestor estimates based on Bayesian coalescent analysis of the virus sequences and isolation dates without inclusion of the Bulgarian strain for which no reliable isolation date was available. Posterior probabilities are listed below the branches for supported nodes. Scale bar indicates nucleotide substitutions per site.

studies have led to the common view that house mice consist of 3 *M. musculus* subspecies, namely *M. m. musculus, M. m. domesticus, and M. m. castaneus*, that share a common ancestor ≈1 million years ago (*36*). Two of these, *M. m. domesticus* and *M. m musculus*, have been associated with LCMV infection.

Mus genus rodents have evolved over the past 7 million years during successive bursts of radiation and, on the basis of phylogeographic and fossil data, likely originated in Asia; the oldest *Mus* fossil recorded in Pakistan dates from the Late Miocene *37*). House mice spread to the Mediterranean Basin around 8,000 BC, and throughout the rest of Europe around 1,000 BC (*38*). The long history of association of house mice with human activity has led to their now global distribution by shipping and other commercial transport. House mice of *M. m. domesticus* descent (*35*) were first introduced to the Americas in the early 16th century aboard the ships of Spanish and Portuguese explorers and Conquistadors, and arrived in North America ≈100 years later with the French fur traders and the English colonists. Movement within the continental United States in more recent times was probably facilitated by modern means of trade and travel.

The high genetic diversity of LCMV and the lack of clear correlation of virus genetic lineages to particular geographic locations likely reflect the long and complex phylogeographic history of the house mouse host. Virus lineages I–III have all been associated with severe human disease, and lineages I and II have been directly linked to *M. musculus* rodents. Greater genetic diversity exists in Europe relative to the United States, with lineages I, II, and IV detected in Europe, but only lineages I and III in the United States. This finding may be the result of *M. musculus* mice, and presumably LCMV, having a much longer history in Europe (particularly around the Mediterranean) relative to the comparatively recent introduction of house mice into North America. In this context, it is noteworthy that LCMV viruses in lineage IV consist solely of isolates from wild-caught *Apodemus sylvaticus* mice from Spain (*10*). The close relationship between the *Mus* and *Apodemus* genera has been well-documented previously (*35*).

Given the proposed Asian origin of house mice, one would speculate that perhaps even greater LCMV genetic diversity may be found in mice of Middle Eastern or Asian origin. Unfortunately, no such LCMVs were available to test this hypothesis. Notably, the isolation of Kodoko virus from *M.* (*Nannomys*) *minutoides* (*9*) correlates with this conclusion because it forms a distinct phylogenetic lineage separate from the LCMV lineages. This species, in the subgenus *Nannomys,* is thought to be an offshoot of the Asian mice radiation that occurred around the beginning of the Pliocene ≈5 million years ago (*39*).

House mice have presumably been introduced into the United States on many occasions since the initiation of shipping traffic between the Old and New Worlds in the early 16th century. The lack of geographic correlation with LCMV genetic groupings, would suggest that traffic of LCMV-infected house mice has occurred frequently, both to the United States from the Old World and within the United States. This movement can also include the commercial traffic of LCMV-infected pet rodents (*5,40*). In the United States, no single genetic variant of LCMV dominates, although lineage I viruses appear to be the most frequently sampled. Similarly, there is no geographic clustering of different LCMV lineages within Europe consistent with extensive movement of the house mouse host.

Despite the generation of a large number of complete genome sequences for a diverse array of LCMV isolates, defining LCMV species solely on the basis of molecular data remains difficult. The current conservative approach is to consider all 4 identified lineages as being variants of LCMV. Although this approach would constitute a highly diverse species, it would be similar to the high genetic diversity observed within Lassa virus, another Old World arenavirus (*26*,*27*). Alternatively, it may be that the 4 LCMV lineages will be redefined as separate virus species; but that will require more detailed data regarding the virus host, serologic properties, and ecologic niche of these viruses. The diversity of LCMVs we describe has practical implications for the design of molecular diagnostic assays for screening of meningitis cases, tissue transplant materials, and the pet trade.

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Dr Albariño is currently a senior research fellow at the Centers for Disease Control and Prevention in Atlanta, Georgia. His research is focused on studying different aspects of RNA viruses with the goal of developing new diagnostic techniques and evaluating potential vaccines.

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Address for correspondence: Caesar G. Albariño, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G14, Atlanta, GA 30333, USA; email: calbarino@cdc.gov

Zoonotic Transmission of Avian Influenza Virus (H5N1), Egypt, 2006–2009

Amr Kandeel, Serge Manoncourt, Eman Abd el Kareem, Abdel-Nasser Mohamed Ahmed, Samir El-Refaie, Hala Essmat, Jeffrey Tjaden, Cecilia C. de Mattos, Kenneth C. Earhart, Anthony A. Marfin, and Nasr El-Sayed

During March 2006–March 2009, a total of 6,355 suspected cases of avian influenza (H5N1) were reported to the Ministry of Health in Egypt. Sixty-three (1%) patients had confirmed infections; 24 (38%) died. Risk factors for death included female sex, age ≥15 years, and receiving the first dose of oseltamivir >2 days after illness onset. All but 2 case-patients reported exposure to domestic poultry probably infected with avian influenza virus (H5N1). No cases of human-to-human transmission were found. Greatest risks for infection and death were reported among women ≥15 years of age, who accounted for 38% of infections and 83% of deaths. The lower case-fatality rate in Egypt could be caused by a less virulent virus clade. However, the lower mortality rate seems to be caused by the large number of infected children who were identified early, received prompt treatment, and had less severe clinical disease.

During January 2003–March 2009, a total of 417 human cases of avian influenza (H5N1) and 256 deaths (61%) were reported worldwide (*1*). Although human-to-human transmission has occurred (*2–4*), most human cases have been caused by zoonotic transmission from poultry (*5–8*). Investigations have emphasized the need for timely identification to determine demographic groups at risk and activities more likely to cause human infection so that control and prevention measures may be implemented. Such investigations may also determine whether the virus can cause pandemic disease.

Author affiliations: Ministry of Health, Cairo, Egypt (A. Kandeel, E.A. el Kareem, A.-N. M. Ahmed, S. El-Refaie, H. Essmat, N. El-Sayed); US Naval Medical Research Unit 3, Cairo (S. Manoncourt, H. Essmat, J. Tjaden, C.C. de Mattos, K.C. Earhart, A.A. Marfin); and US Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A.A. Marfin)

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Avian influenza (H5N1) in Egypt was first reported in February 2006 when outbreaks were observed in domestic poultry at commercial farms and in backyard flocks in 3 governorates in northern Egypt. Within the first month, avian influenza (H5N1) was detected in 12 other governorates. Despite control measures that included culling, disinfection, vaccination, and controlled poultry movement, epizootic transmission of avian influenza virus (H5N1) continues.

During February 2006–March 2009, avian influenza virus (H5N1) was detected on 907 commercial poultry farms and in 606 backyard flocks. In 2006, poultry farms accounted for 84% of 1,052 outbreaks. In 2007, backyard flocks accounted for 89% of 274 outbreaks. As of March 2009, nineteen of 29 governorates reported infected poultry. The poultry industry in Egypt produces ≈2 million birds per day. Social and economic consequences have been dramatic (losses of ≈2–3 billion US$). Backyard flocks are common; 4–5 million families (≈25 million persons) raise poultry at home. During February 2006–March 2009, a total of 3,941 asymptomatic persons exposed to avian influenza (H5N1) from a person with a confirmed case or from infected poultry were tested by using a real-time PCR; none were positive.

In March 2006, the first human case of avian influenza (H5N1) in Egypt was reported from Qalubiya Governorate (Figure 1). We report the first 63 human cases. We also describe affected demographic groups, illness, mortality rates, and specific events that contributed to transmission.

Materials and Methods

The study protocol (NAMRU3.2004.0023) was reviewed and approved by the Naval Medical Research Unit No. 3 Institutional Review Board in compliance with all applicable federal regulations governing protection of hu-

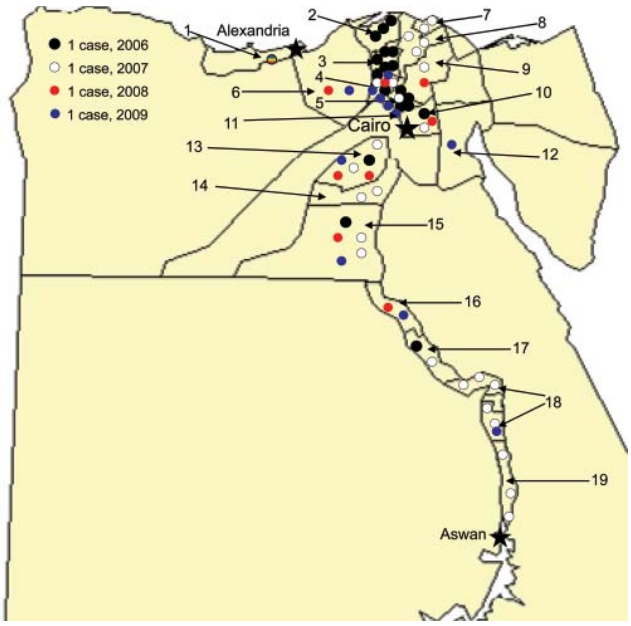

Figure 1. Residences of 63 case-patients with avian influenza virus (H5N1) infections, Egypt, 2006–2009. 1, Alexandria; 2, Kafr El Sheikh; 3, Gharbia; 4, Menofia; 5, Qalubiya; 6, Behera; 7, Damietta; 8, Dakahlia; 9, Sharkia; 10, Cairo; 11, 6th of October; 12, Suez; 13, Fayoum; 14, Benu Suef; 15, Menia; 16, Assyut; 17, Sohag; 18, Qena; 19, Aswan.

man subjects. Suspected cases of avian influenza (H5N1) in humans are reported from all districts in Egypt. A suspected case-patient is a person with influenza-like illness (fever ≥38°C and 1 of the following signs or symptoms [cough, sore throat, or shortness of breath]) and specific exposure to ill, dying, or dead poultry. All suspected case-patients are referred to specified fever or chest hospitals for testing and medical care. At these hospitals, oropharyngeal swab specimens and serum samples are obtained. Persons with suspected cases receive an initial dose of oseltamivir and are placed in respiratory isolation areas.

Swab specimens are sent to the Ministry of Health (MOH) Central Public Health Laboratory for real-time PCR testing for influenza A virus matrix and H5 genes by using primer–probe sets (*9*). Positive samples are sent to the US Naval Medical Research Unit 3, a World Health Organization H5 Reference Laboratory, for confirmation and virus isolation.

Most case-patients with confirmed avian influenza (H5N1) are transferred to Cairo, Egypt, for care at 2 tertiary hospitals. Data are obtained by healthcare providers who initially evaluate suspected cases and by MOH staff when cases are confirmed. Epidemiologic and clinical data are obtained for each patient with a confirmed case by using a standardized case investigation form. For this study, a medical record review was performed at tertiary care hospitals. Clinical and exposure data were not available for all 63 patients.

The number of patients for whom data were available is noted. Children are defined as persons <15 years of age, adults as persons ≥15 years of age, delayed hospitalization as >2 days between illness onset and hospitalization, and delayed oseltamivir as >2 days between illness onset and the first oseltamivir dose.

Univariate analyses were performed by using Epi Info version 3.4.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Unless otherwise noted, χ^2 or Fisher exact tests were used. Multivariate analysis to identify risk factors for death was performed by using a backward, stepwise logistic regression model starting with all variables (dichotomized at their median value) significant by univariate analyses. The Wald statistic and log-likelihood ratio were used to exclude variables. Multivariate analyses were performed by using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

Results

Overview

During March 2006–March 2009, a total of 6,355 suspected cases of avian influenza (H5N1) were reported, and samples were tested by the Central Public Health Laboratory. Of these, 63 (1%) cases were confirmed and 24 were fatal (case-fatality rate 38%) (Table 1). Among 63 case-patients, median age was 10 years (range 16 months–75 years), 24 (38%) were women ≥15 years of age, 5 (8%) were men ≥15 years of age, 16 (25%) were girls <15 years of age, and 18 (29%) were boys <15 years of age (Table 1). Two infected women were pregnant; both died of respiratory failure. Clinical or exposure data were not available for 2 case-patients (a 31-year-old man infected in Egypt who became ill and whose influenza was diagnosed in Jordan, and a 75-year-old woman who died within hours of hospitalization). Risk factor data on exposure to birds were available for 41 case-patients (Table 1). Confirmed cases were reported from 19 of 29 governorates in Egypt (Figure 1). During 2006, most case-patients were located in the Nile Delta region. In contrast, during 2007–2009, cases were distributed in northern and southern Egypt. Of 63 case-patients, 29 (46%) had illness onset in March (Figure 2).

Three family clusters were identified. The first cluster, reported in March 2006, was composed of 2 siblings (21 months and 6 years of age) in Kafr El Sheikh Governorate. The second cluster, reported in December 2006, was composed of 3 family members (a mother, her daughter, and the daughter's uncle) in Gharbia Governorate. The third cluster, reported in March 2007, was composed of 2 siblings (4 and 6 years of age) in Qena Governorate. In the first 2

Table 1. Demographic and exposure characteristics for persons with confirmed avian influenza (H5N1), Egypt, 2006–2009

Characteristic	No. (%) persons
Total confirmed cases	63
Deaths	24 (38.0)
Women	40 (63.5)
Age group, y	
0–4	23 (36.5)
5–14	11 (17.5)
15–49	27 (43.0)
≥50	2 (3.0)
Exposure (no. persons)*	
Exposure to a person with a confirmed case before illness (63)	4 (6.3)
Occupational (63)	4 (6.3)
Exposure to likely infected backyard flocks (63)	57 (90.5)
No known exposure (63)	2 (3.2)
Consumption of raw or undercooked poultry products (61)	0
Exposure to likely infected backyard flocks (41)	
Recently purchased domestic poultry from market/seller (41)	12 (29.2)
Recently purchased poultry became ill (12)	7 (58.3)
Noted illness or death among their birds (41)	33 (80.5)
Bred birds (27)	14 (51.8)
Slaughtered birds in past 10 d (27)	13 (48.1)
Defeathered birds in past 10 d (27)	13 (48.1)

*Denominators vary for each exposure because data were not available for all persons.

clusters, all shared a common exposure to likely infected poultry and became ill at the same time. In the third cluster, although illness onsets were separated by 4 days, an investigation showed that each child had 2 separate exposures to infected birds. Human-to-human infections were not identified. Household contacts were not given oseltamivir but were followed up closely for 10 days. Secondary infections were not found.

Clinical Manifestations

Median number of days between illness onset and hospitalization was 2 (range 0–12 days). Female patients and adults were ill longer before hospitalization than were male patients and children. Of 40 female patients, 23 (57.5%) had delayed hospitalization (>2 days after illness onset) compared with 7 (32%) of 22 males. Of 28 patients ≥15 years of age, 20 (71%) had delayed hospitalization compared with 10 (29%) of 34 patients <15 years of age ($p = 0.002$). Fever (97%) and cough (72%) were the most common clinical signs. Sore throat was reported by 45% of case-patients and shortness of breath by 25% (Table 2). Adults were more likely to have cough (81%), muscle and joint aches (46%), and shortness of breath (38%); sore throats were more common in children (50%) (Table 2).

Clinical Course of Disease

Forty-six (73%) of 63 case-patients were transferred to Cairo for definitive care. Of 17 case-patients not transferred to Cairo, 5 (29%) died <2 days after being admitted to a governorate hospital. Of 59 case-patients for whom data on complications were available, ≥1 secondary complication developed in 25 (42%). Sixteen case-patients had multiple complications (Table 3). The most common complications were acute respiratory distress syndrome (19 cases), shock (14 cases), renal failure (6 cases), and coagulopathy (4 cases). Mortality rate was higher for patients with a complication (n = 25); twenty (80%) died. Only 1 (3%) of 34 patients without complications died ($p<0.01$).

Of 19 case-patients with acute respiratory distress syndrome, 18 died. The only survivor was an 18-year-old woman who received intubation for 12 days and oseltamivir 2 days after illness onset. A complication was more likely to develop in adults; 20 (77%) of 26 had ≥1 complication compared with only 5 (15%) of 33 children ($p<0.001$).

Chest radiographs were reviewed for 58 patients. Twenty-five (43%) radiographs showed lobar infiltrates, of which 20 (80%) were bilateral. Of 33 radiographs for children, 27 (82%) showed no abnormalities. Abnormal laboratory test results included those for leukopenia (16/52, 31%), thrombocytopenia (13/49, 27%), and elevated levels of aspartate aminotransferase (23/46, 50%) and alanine aminotransferase (20/48, 42%).

Medication records were available for 60 patients who received antimicrobial drugs. Of 60 patients, 31 (52%) received oxygen, including 22 (37%) who received mechanical ventilation. Twenty (74%) of 27 adults received mechanical ventilation compared with only 2 (6%) of 33 children ($p<0.001$). Of 60 patients, 16 (27%) had received corticosteroids. Mortality rates did not differ between intubated patients who received corticosteroids and those who did not.

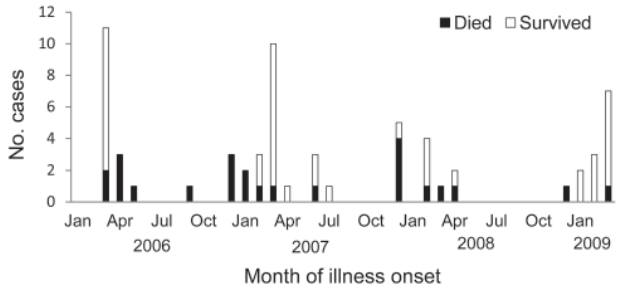

Figure 2. Illness onset for 63 case-patients with confirmed avian influenza (H5N1), by month, Egypt, 2006–2009.

Oseltamivir Treatment, Virus Isolates, and Oseltamivir Resistance

All 62 case-patients who became ill in Egypt received ≥1 dose of oseltamivir. Of 58 patients for whom complete data for oseltamivir was available, 25 (43%) received their first dose ≤48 hours after illness onset; all but 1 survived. Median duration of treatment was 8 days (range 1–37 days). The first dose of oseltamivir was more likely to be delayed for adults. Twenty (80%) of 25 adults had a delay before receiving oseltamivir compared with 13 (39%) of 33 children (p = 0.005).

Virus isolates were obtained from 34 (54%) of 63 case-patients. Sequencing of hemagglutinin and neuraminidase genes showed all viruses belonged to clade 2.2 and were closely related to isolates from birds in Europe and the Middle East (*10*). Drug sensitivity was determined for all isolates. Resistance to oseltamivir was confirmed in viruses from 2 patients in the same family; both died. Resistance was observed in the initial diagnostic sample and did not occur during treatment. A mutation at position N294S conferring a 12–15× reduction in drug susceptibility was identified in both isolates (*11*).

Mortality Rates

Of 63 case-patients with confirmed influenza, 24 (38%) died. Median time between onset of illness and death was 9 days (range 4–40 days). Ten (56%) of 18 ill patients died in 2006 compared with 9 (36%) of 25 ill patients in 2007 and 4 (50%) of 8 ill patients in 2008 (Figure 2). Mortality rates were higher for adults and female patients for whom hospitalization or oseltamivir administration were delayed (Table 4). Of 24 deaths, 22 (92%) were among adults and 21 (87.5%) were among female patients. Twenty-one (52.5%) of 40 female patients died compared with 3 (13%) of 23 male patients (p = 0.005). Two (6%) of 34 children died compared with 22 (76%) of 29 adults (p<0.001). Of 30 persons who were ill >48 hours before hospitalization, 20 (67%) died compared with only 4 (12.5%) of 32 persons hospitalized ≤48 hours after illness onset (p<0.001). Of 33 patients whose first oseltamivir dose was delayed, 19 (58%) died compared with only 1 (4%) of 25 patients who received oseltamivir ≤48 hours after illness onset (p<0.001).

Although adults were more likely than children to have a delay in hospitalization, age ≥15 years and delayed hospitalization were independently associated with higher mortality rates. Of 28 adults for whom hospitalization data were available, 12 (43%) were hospitalized in the first 48 hours of illness compared with 27 (79%) of 34 children (p = 0.007). Stratified analysis showed delayed hospitalization was a greater risk factor for death among adults than among children. Eighteen (86%) of 20 adults hospitalized >48 hours after illness onset died compared with 4 (50%) of 8 adults hospitalized ≤48 hours after illness onset (p = 0.04). None of 24 children hospitalized ≤48 hours after illness onset died compared with 2 (20%) of 10 children hospitalized >48 hours after illness onset. Nineteen (83%) of 23 female patients hospitalized >48 hours after illness onset died compared with 2 (12%) of 17 hospitalized ≤48 hours after illness onset (p<0.001).

Eighteen (90%) of 20 adults whose first oseltamivir dose was delayed died compared with 1 (20%) of 5 adults whose first oseltamivir dose was not delayed (p = 0.005). None of 20 children who received oseltamivir ≤48 hours of illness onset died compared with 1 (8%) of 13 children whose first dose was delayed.

Age, sex, delayed hospitalization, and delayed use of oseltamivir were included in multivariate analysis by using a logistic model to identify risk factors for death. Sex and delayed hospitalization did not contribute to the final model. Because of relatively few cases, high degree of covariance in age, and delayed use of oseltamivir, there was insufficient power to further develop this model. Despite this limitation, analysis showed that age ≥15 years and having received a first dose of oseltamivir >2 days after illness onset were likely independent risk factors contributing to death.

Table 2. Signs and symptoms at illness onset for 60 persons with confirmed avian influenza (H5N1), by age group, Egypt, 2006–2009*

	Age group, y, no. (%)		
Sign or symptom	<15, n = 34	≥15, n = 26	p value
Fever	34 (100)	24 (92)	>0.05
Cough	22 (65)	21 (81)	>0.05
Shortness of breath	5 (15)	10 (38)	<0.05
Sore throat	17 (50)	10 (38)	>0.05
Vomiting	3 (9)	7 (27)	>0.05
Diarrhea	2 (6)	4 (15)	>0.05
Muscle/joint pain	2 (6)	12 (46)	<0.001
Headache	1 (3)	6 (23)	<0.05
Alteration of consciousness	1 (3)	1 (4)	>0.05

*Data regarding signs and symptoms were available only for 60 of the 63 patients.

Table 3. Secondary complications in persons infected with avian influenza virus (H5N1), Egypt, 2006–2009*

No. secondary complications	No. persons	Type of complications (no. persons), outcome	Case-fatality rate by no. complications (%)
1	6	ARDS (n = 2), 2 died; bacteremia (n = 2), 2 alive; pneumonia (n = 1), 1 alive; shock (n = 1), 1 alive	2/6 (33)
2	11	ARDS/pneumonia (n = 3), 2 died, 1 alive; ARDS/shock (n = 5), 1 died; ARDS/ renal failure (n = 1), 1 died; ARDS/coagulopathy (n = 1), 1 died; pneumonia/toxic myocarditis (n = 1), 1 alive	9/11 (82)
3	5	ARDS/pneumonia/shock (n = 3), 3 died; ARDS/shock/renal failure (n = 1), 1 died; shock/renal failure/coagulopathy (n = 1), 1 died	5/5 (100)
4	2	ARDS/pneumonia/shock/renal failure (n = 1), 1 died; ARDS/shock/renal failure/coagulopathy. (n = 1), 1 died	2/2 (100)
5	1	ARDS/pneumonia/shock/ renal failure/coagulopathy (n = 1), 1 died	1/1 (100)

*ARDS, acute respiratory distress syndrome.

Exposure

Handling live domestic poultry likely infected with avian influenza virus (H5N1) was the primary source of exposure. Investigations showed that human-to-human transmission was unlikely; even clusters of case-patients had exposure to infected poultry. Of 63 case-patients, 4 (6%) were involved in poultry production or distribution (3 poultry farm workers and 1 seller), 2 (3%) had unknown poultry exposure, and 57 had direct contact with backyard poultry (Table 1).

Exposure data were available for 41 of 57 case-patients with exposure to backyard flocks. Of these case-patients, 33 (80%) reported having had ill birds in their egg-laying flocks and 12 (29%) had recently bought poultry. Of these 12 case-patients, 7 (58%) reported that purchased birds became ill after being brought home. Of 27 case-patients for whom information was documented, 13 (48%) slaughtered or defeathered birds (Table 1). No case-patients reported eating raw or undercooked animal products.

Discussion

In February 2006, avian influenza (H5N1) emerged among domestic poultry in the Nile Delta of Egypt. Within 4–5 weeks, it had affected commercial farms and backyard flocks throughout Egypt and resulted in zoonotic transmission to 10 persons in many governorates. Currently, Egypt has reported the third largest number of cases of avian influenza (H5N1) after Indonesia and Vietnam (*1*).

The mortality rate for avian influenza (H5N1) in Egypt (38%) is lower than that in other countries. As of March 2009, mortality rates were 82% in Indonesia, 50% in Vietnam, 66% in the People's Republic of China, and 68% in Thailand. Explanations for this observation include lower mortality rates for certain demographic groups, clinician awareness resulting in improved medical care, or less pathogenic virus. The most striking finding is the low mortality rate for children. Although children represent 54% of reported infections, they account for only 8% of deaths. This high survival rate is unlikely to be caused by young age alone. Children were hospitalized earlier in the clinical course of their illness, were more likely to receive oseltamivir within the first 2 days, and appeared to be less ill than adults, as noted by the high proportion of chest radiographs with no abnormal findings and the low proportion of children with respiratory failure. Differences in sensitivity of surveillance methods among countries must also be considered.

One must also consider whether the 2.2 virus clade is less virulent. This suggestion is not supported by a report of the 2005–2006 outbreak of clade 2.2 virus (H5N1) in Turkey, where of 8 patients 5–15 years of age, 4 (50%) died (*6*).

Despite overall low mortality rates, particularly among children, the mortality rate in women was >52%. This rate could be due to reasons that include receiving a higher

Table 4. CFRs for 63 persons infected with avian influenza virus (H5N1), Egypt, 2006–2009*

Characteristic	Total no.	No. died	CFR, %
Sex†			
F	40	21	52.5
M	23	3	13
Age group, y‡			
0–4	23	0	0
5–14	11	2	18
15–49	27	20	74
≥50	2	2	100
All ages	63	24	38
Days between illness onset and hospitalization§			
0–2	32	4	12.5
3–4	12	4	33
5–6	9	8	89
≥7	9	8	89
Days between illness onset and first oseltamivir dose¶			
0–2	25	1	4
3–4	14	3	21
5–6	7	5	71
≥7	12	11	92

*CFR, case-fatality rate.
†p = 0.0004, by χ^2 test for CFR for female patients vs. that for male patients.
‡p<0.001, by χ^2 test for patients <15 years of age vs. patients ≥15 years of age.
§p<0.001, by χ^2 test for patients hospitalized ≤2 d after illness onset vs. patients hospitalized after 2 d.
¶p<0.001, by χ^2 test for patients who received oseltamivir ≤2 d after illness onset vs. patients who received oseltamivir after 2 d.

virus inoculum to the lungs through activities associated with slaughtering and defeathering birds, a more profound proinflammatory cytokine response, or delay in receiving healthcare. Only delay in receiving healthcare was examined in this study. Women reported a longer time between illness onset and hospitalization and a longer time until the first dose of oseltamivir than men. Women and men who sought healthcare were admitted to the same facilities and received identical care.

More than 5,000 asymptomatic persons known to have been exposed to poultry infected with avian influenza virus (H5N1) or in contact with confirmed human case-patients were followed up clinically and tested by using real-time PCR. Although prophylaxis was not given, influenza-like illnesses were not observed and all persons showed negative results. Although serologic testing is needed to exclude infection with avian influenza virus (H5N1), it was unlikely that a large proportion of these persons with high-level exposures to infected birds or humans became infected and supports the decision of the MOH to discontinue testing asymptomatic persons. This finding is consistent with those of studies in Thailand (*12*) and Cambodia (*13*).

Although infection and illness do not develop in most persons exposed to infected poultry, all but 2 cases were attributed directly to exposure to poultry likely infected with avian influenza virus (H5N1). No illnesses were attributed to exposure to wild birds. Although 3 family clusters were identified, all 7 persons in these clusters had independent exposures. Many families in Egypt raise backyard flocks for eggs and purchase live poultry for meat. Among case-patients, the likely route of infection appears to be direct handling, slaughtering, or defeathering infected birds recently purchased for meat and mingling of recently purchased birds with egg-laying flocks. Recently purchased birds were frequently slaughtered before illness was noted, and purchase was often followed by illness and death among egg-producing flocks.

Contact between backyard flocks and wild infected birds could not be estimated, but exposure to feral poultry in canals and waterways near affected households was common. Because persons in Egypt rely on live poultry purchased at markets for dietary protein, the price of poultry influences poultry-buying practices of families. Women in several affected families noted exceptionally low prices for healthy looking birds. These prices indicated that they might be buying infected birds. This finding was true when prices of beef increased in response to decreased availability or increased demand. Despite this knowledge, most persons believed they would be able to slaughter and prepare birds before they became ill or died. This belief was true in most cases but recently purchased birds frequently infected egg-laying flocks, which died within days of exposure.

Despite knowledge of overall exposure patterns and identification of groups at risk for exposure, little detailed information on activities that result in infection is available. Although slaughtering and defeathering infected birds appear to be high-risk practices, there have likely been thousands of infected birds sold and slaughtered in homes in Egypt over the past 3 years. Despite this suggestion, we have reports of only 63 cases. Although exposure to avian influenza virus (H5N1) infection is necessary for infection, exposure is not sufficient to explain the epidemiology of cases of avian influenza (H5N1) in Egypt. Whether there is another unknown risk factor or variation in the way women slaughter poultry in Egypt is unclear.

Demographics of influenza cases in Egypt are different from those in other highly affected countries and are useful for determining exposures and activities that result in infection. Women appear to be at greater risk than men of becoming infected, and, once ill, at greater risk of death. In Egypt, the male:female ratio among patients is 1:1.7 and differs markedly from the 1:1 ratio seen globally (*14*,*15*). Caring for or slaughtering poultry is generally the responsibility of women and may explain a higher exposure rate for women. Similarly, age distribution of case-patients differs. In Egypt, 54% of case-patients were <15 years of age, compared with ≤35% in Indonesia, Vietnam, and China. In Egypt, small children follow their mothers during routine chores, such as feeding and slaughtering poultry. At other times, children will play with poultry, which roam freely around the home. There is a general belief that parents in Egypt will quickly seek medical care for their ill children. This belief is strongly suggested by the fact that children with fever and exposure to dead or ill poultry were consistently evaluated and hospitalized sooner than adults. In addition, many children had mild illness. Mild clinical illness may be caused by early hospitalization, early doses of oseltamivir, or a low virus inoculum.

This report describes 63 human cases of avian influenza (H5N1) in Egypt during March 2006–March 2009. During April–July 2009, a total of 20 additional cases were identified (83 cases by the end of July 2009) for which data were not available. Analysis of limited information reported to the World Health Organization showed a median age of 4 years (compared with 10 years for the 63 cases), a case-fatality rate of 15% (compared with 38%), and faster hospitalization after illness onset. Ongoing transmission in the summer of 2009 is indicative of persistent disease in poultry, and limited analysis reflects the high proportion of influenza in children. Thus, avian influenza virus (H5N1) remains endemic throughout Egypt. However, human infections are rare and disproportionately affect women and their children, who are responsible for caring for and slaughtering birds within the home. To reduce their risk, specific slaughtering practices and other transmission risk factors

should be identified and appropriate interventions implemented. In addition, emphasis on controlling domestic poultry populations and increased use of bird cages, hand washing, and other protective measures specific for women and children should continue.

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Dr Kandeel is the Undersecretary for Preventive Affairs at the Ministry of Health, Cairo. His primary research interest is avian influenza.

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Address for correspondence: Kenneth C. Earhart, US Naval Medical Research Unit 3, Cairo, Egypt; email: kenneth.earhart@gmail.com

Deforestation and Malaria in Mâncio Lima County, Brazil

Sarah H. Olson, Ronald Gangnon, Guilherme Abbad Silveira, and Jonathan A. Patz

Malaria is the most prevalent vector-borne disease in the Amazon. We used malaria reports for health districts collected in 2006 by the Programa Nacional de Controle da Malária to determine whether deforestation is associated with malaria incidence in the county (município) of Mâncio Lima, Acre State, Brazil. Cumulative percent deforestation was calculated for the spatial catchment area of each health district by using 60 × 60–meter, resolution-classified imagery. Statistical associations were identified with univariate and multivariate general additive negative binomial models adjusted for spatial effects. Our cross-sectional study shows malaria incidence across health districts in 2006 is positively associated with greater changes in percentage of cumulative deforestation within respective health districts. After adjusting for access to care, health district size, and spatial trends, we show that a 4.3%, or 1 SD, change in deforestation from August 1997 through August 2000 is associated with a 48% increase of malaria incidence.

Malaria risk in the Amazon and around the malaria belt is an integrated mix of environmental and sociodemographic risk factors (*1*–*3*). Despite >50 years of malaria control efforts from 1997 through 2006, on average, Brazil had ≈500,000 confirmed cases annually (*4*,*5*). Most malaria cases in Brazil occur in the Amazon Basin, where logging rates between 1999 and 2002 ranged from 12,000 to 20,000 km^2 per year, the sum of which would cover the country of Denmark (*6*).

The main vectors of malaria in the Amazon, *Anopheles darlingi* mosquitoes, seek out larval habitat in partially sunlit areas, with clear water of neutral pH and aquatic plant growth, and they are notably present and more abundant in altered landscapes (*7*–*9*). In Peru, *A. darlingi* mosquitoes are seldom observed in standing water bodies within undisturbed forests because they are shaded and soils are more acidic, and yet these forests remain abundant and rich in mosquito species that do not transmit malaria (*9*,*10*). Along the Iquitos–Nauta Road corridor entomologic risk factors of mosquito biting rate and larval count increase with more deforestation. The mean biting rate in areas with >80% deforestation was 8.33 compared with 0.03 per night for sites with <30% deforestation (*10*). Furthermore, the likelihood of finding *A. darlingi* larvae doubled in breeding sites with <20% forest compared with sites with 20%–60% forest, and the likelihood increased 7-fold when compared with sites with >60% forest (*8*). Human-altered landscapes provide a milieu of suitable larval habitats for *A. darling* mosquitoes, including road ditches, dams, mining pits, culverts, vehicle ruts, and areas of poor clearing.

The characteristics of these mosquitoes' preferred habitat and studies of human and entomologic malaria risk suggest that deforestation and land clearing contribute to the dynamic malaria patterns along the frontier of settlement. Frontier malaria theory explains this pattern in new settlements as follows: an initial epidemic occurs that abates to persistent low incidence and eventually eradication as the result of changing social, ecologic, and environmental relationships (*11*). For instance, from 1985 through 1995, malaria risk in Rondônia increased during the initial colonization phase due to ecosystem transformations that promoted larval habitats and then gradually subsided as urban area expanded, agriculture became established, settlers became more knowledgeable, access to healthcare increased, home construction improved, and suitable larval habitats declined, until, finally, malaria risk was mostly linked to human behavioral factors (*2*,*12*). Frontier malaria theory is further supported by research around the Granada area in Acre, where a population-

Author affiliations: University of Wisconsin, Madison, Wisconsin, USA (S.H. Olson, R. Gangnon, J.A. Patz); and Santo Antônio Energia, Porto Velho, Brazil (G. Silveira)

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based cohort study found land clearing activities and <5 years of residence associated with higher probability of PCR-confirmed malaria morbidity (*13*).

In this 2006 cross-sectional study, we examined the association of deforestation, socioeconomic and demographic factors, and malaria at the level of health districts (*localidades*) using a uniform surveillance tool implemented in 2003 by the Brazilian Ministry of Health's Programa Nacional de Controle da Malária (PNCM). This nationally standardized system covers 5.1 million km^2 of the malaria belt and reports monthly malaria statistics for >7,000 health districts. The surveillance system uses a 40-item questionnaire that includes items concerning patient demographics, diagnosis, and area of residence (*14*). The spatial, temporal, and overall quality of this surveillance program, combined with spatial mapping, presents an opportunity to identify ecologic risk factors within an extensive existing surveillance network. Our hypothesis was that deforestation is positively associated with higher malaria risk in health districts in Mâncio Lima, Acre State, Brazil (Figures 1, 2). We also examined the association of 2006 malaria incidence with socioeconomic and demographic factors, including age, access to care, method of surveillance, sex, and malaria type.

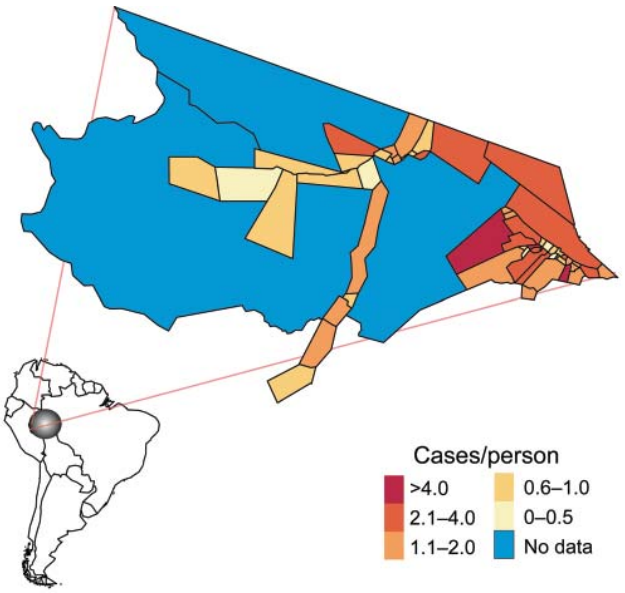

Figure 1. Mâncio Lima, the westernmost county in Brazil. The 2006 malaria incidence (cases/person) is mapped according to health districts (n = 54).

Materials and Methods

Study Area

Mâncio Lima (4,672 km^2) is situated in Acre State and is the westernmost county in Brazil, sharing a border with Peru to the west and Amazonas State to the north. Between 2000 and 2008, the population of the county increased 30% from 11,095 to 14,387. The county has 4% more men than women and a mixture of rural (48%) and urban (52%) households (*15*). The 67% of the territory that is considered uninhabited is made up of the Nukini and Poyanawa Indigenous Reserves and a portion of the Serra do Divisor National Park. The rural economy is based on agriculture and manioc flour production, and no areas have been licensed for mining exploration (*16*–*18*). Mâncio Lima has an average 4–30 cm monthly precipitation range and 19°C–32°C average monthly temperature range (*19*). The city of Mâncio Lima, which is the administrative and main population center, is connected by highway to Curzeiro do Sul, 24 km to the east. In 2006, Cruzeiro do Sul and Mâncio Lima ranked second and fourth highest, respectively, for malaria risk, and combined they reported 12.5% of all malaria cases in Brazil (*20*).

Health Data

Since 2003, PNCM has practiced a malaria control strategy that reports all suspected malaria cases, identified from both slide-confirmed passive and active surveillance, for local health districts, which are often points of care. For each case, the survey tool records date, age (<10 years), sex, whether or not care was received within 48 hours of symptom onset, malaria type (*Plasmodium vivax* or *P. falciparum*; we classified mixed infections as falciparum), and method of surveillance (passive or active). In addition, the malaria case report form includes voluntary questions on education level and occupation type (*14*). We screened for patients who were residents of the health district in which they sought treatment and extracted monthly and

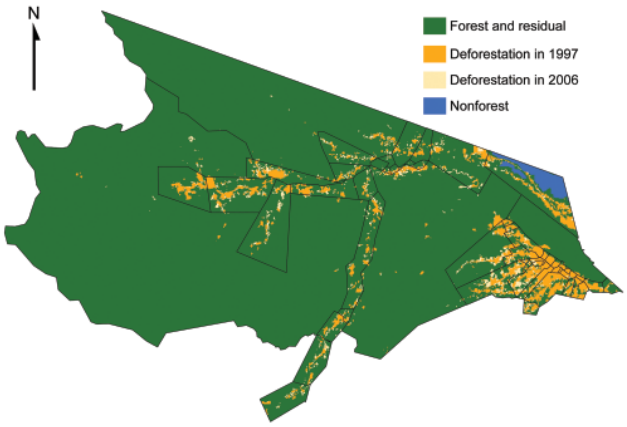

Figure 2. Deforestation trends in Mâncio Lima, Brazil, based on PRODES (Programa de Cálculo do Deflorestamento da Amazônia) 60 × 60-meter classified satellite imagery. The health districts are outlined in black. Baseline deforestation that occurred in 1997 is orange, deforestation that occurred between 1997 and 2006 is light brown, nonforested land is blue, and forested land is green.

annual percentages of these records from the Information System of Epidemiologic Surveillance of Malaria (SIVEP MALÁRIA) for the county of Mâncio Lima using Tableau 4.0 (www.tableausoftware.com) and Excel version 11.3 (Microsoft Corp., Redmond, WA, USA).

Remote Sensing

In 2006, health district boundaries in Mâncio Lima were initially drawn by health district field staff and then mapped in real time with a GPS Garmin 12XL (Garmin International, Inc., Olathe, KS, USA). Then Track Maker 13.0 (www.gpstm.com) and ArcView 3.2 (www.esri.com) software were used to convert the paths into 54 health district polygons, and the population of each health district was enumerated. Next, the geographic data of each health district was linked to SIVEP MALÁRIA data. The uninhabited portion of the county, including the indigenous reserves and the national park, was divided into 3 geographic areas and excluded from data analysis (*21*).

Classified deforestation estimates at 60 × 60–meter resolution from 1997, and 2000 through 2006 were downloaded from the Programa de Cálcudo do Deflorestamento da Amazônia (PRODES) in the National Institute for Space Research (*22*) (Figure 2). PRODES processes photographic images and Landsat imagery acquired at 30 × 30–meter resolution and is considered the gold standard reference for spatial deforestation data (*23*,*24*). The classification of deforestation in PRODES is cumulative; once a unit is deforested, it does not revert back to forest (*25*). Subsequently, in our analysis, we do not consider the effects of regrowth. ArcMap version 9.3 (www.esri.com) was used to calculate the health district geometric center and the amount of deforestation observed in 1997 and during 2000–2006.

Analysis and Modeling

Summary statistics of variables from the SIVEP-MALÂRIA database for 2006 and deforestation data were calculated and mapped according to the geographic boundaries of the health districts. Numbers of malaria cases in 2006 for each health district were modeled by using a negative binomial (overdispersed Poisson) generalized additive regression model with a log-link function. For each health district, (log) census population for 2006 was included in the model as an offset term. Geographic location (latitude/longitude) was included in all models as a penalized 2-dimensional thin plate regression spline with smoothing parameter chosen by generalized cross-validation (*26*). Initial models considered deforestation and social or demographic variables to the above model individually.

A multivariable model was constructed in a stepwise fashion based on optimizing Akaike information criterion (AIC) (*27*). As before, the dependent variable was the number of malaria cases in 2006 for each health district and (log) census population for 2006 was included as an offset term. Deforestation variables of interest included absolute deforestation in 1997, absolute deforestation in 2006, percentage of deforestation in 1997, percentage of deforestation in 2006, and percentage change in deforestation during 1997–2006, 1997–2005, 1997–2004, 1997–2003, 1997–2002, 1997–2001, 1997–2000, 2001–2006, 2002–2006, 2003–2006, 2004–2006, and 2005–2006. Percentage of deforestation change was calculated by obtaining the percentage difference of deforestation in each health district between 2 time points. Social and demographic risk factors included percentage of malaria cases detected through active surveillance, percentage of malaria patients who received access to care in <48 hours of symptom onset, percentage of malaria patients <10 years of age, percentage of malaria patients who are male, and percentage of falciparum malaria cases. Interactions between variables selected as main effects in the stepwise model were also considered. Relative risks are presented for a ±1 SD change in the risk factor. Residual spatial autocorrelation was assessed by using Moran I with k = 4 distance-based neighbors. A 2-sided p value of 0.05 was considered to be statistically significant. Maps, statistical analysis, and figures were completed in R version 2.9.2 and Adobe Illustrator version 10.0.3 (www.adobe.com) (*28*).

Results

Fifty-four health districts occupy 27% (1,270/4,760 km^2) of Mâncio Lima and spatially reflect the population settlements along 2 dominant river channels and in the urban zone around the city of Mâncio Lima. In 2006, the health districts reported a total of 15,437 slide-confirmed malaria cases, a mixture of both falciparum (41%) and vivax (59%) malaria. Most malaria patients across health districts were males (56%) >10 years of age (72%); the cases were identified by active surveillance (65%), and the patients received access to care within 48 hours of symptom onset (71%). The average incidence rate of the malaria epidemic was 1.16 cases/person, but within individual districts, the incidence was 0.4–12 cases/person (Figures 1, 3). We were unable to analyze answers to categorical questions on the education level and activities of case-patient activities within the previous 2 weeks because the response rates were insufficient. Choropleth maps depicting population distribution, access to care, malaria incidence, and percentage of deforestation change during 1996–2006 are shown in Figure 4.

Baseline deforestation in 1997 was concentrated within and near the city of Mâncio Lima, with varying degrees of deforestation found in the health districts along the river ways. The most deforestation change between 1997 and 2006 was located just west and south of the city. Over this period, percentage of deforestation in health districts increased, on average, 6.6%–26%. The SD of this increase

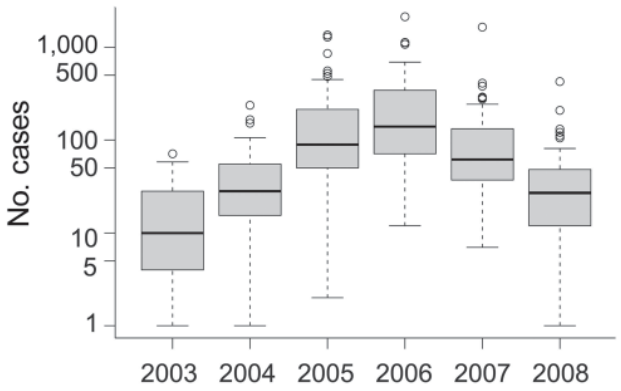

Figure 3. Box-and-whisker plots of slide-confirmed malaria cases on a logarithmic scale by health districts in Mâncio Lima, Brazil, 2003–2008. Error bars indicate interquartile ranges, and thick horizontal bars indicate the median.

is 5.9% (Table). Notably, a large wetland area northeast of the city limited the amount of land clearing taking place in that area (Figures 2,4).

The univariate analysis adjusts for variability between the health districts, and spatial trend. We show the influence of ecologic deforestation and social demographic risk factors on malaria incidence. Percentage of deforestation during 1997–2000 is the factor most predictive of malaria risk in the health districts on the basis of model AIC. Health districts that deforested 4.3% (±1 SD) from 1997 to 2000 are associated with 1.33 (95% confidence interval [CI] 1.12–1.58) increase in malaria risk. Historic baseline deforestation in 1997 is not significant, but malaria risk and percentage deforestation from 1997–2002, 1997–2001, and 1997–2000 are significant and positively correlated. More recent percentage deforestation changes from 2001 through 2006 are not associated with malaria risk, along with 1997 and 2000–2006 measures of absolute deforestation and cumulative percent deforestation (Table).

Although these results were not quite significant in the univariate analysis, the risk of malaria is 1.27 (95% CI 0.97–1.66) when active surveillance increases by 19% within a health district. Malaria risk is 1.18 (95% CI 0.87–1.59) when 14% of more cases obtain care within the first 48 hours of symptoms. The spatial size of health districts is also nearly significant as the relative malaria risk is 1.20 (95% CI 0.97–1.48) for a 32 km^2 increase in health district size. Associations with malaria risk based on age, sex, or malaria type are not significant (Table).

The multivariate analysis shows a 4.3% increase in the percentage of deforestation between 1997 and 2000 is associated with a malaria risk of 1.48 (95% CI 1.26–1.75) after access to care and the spatial area of the health districts are adjusted for (Table). Figure 5 shows the interaction and

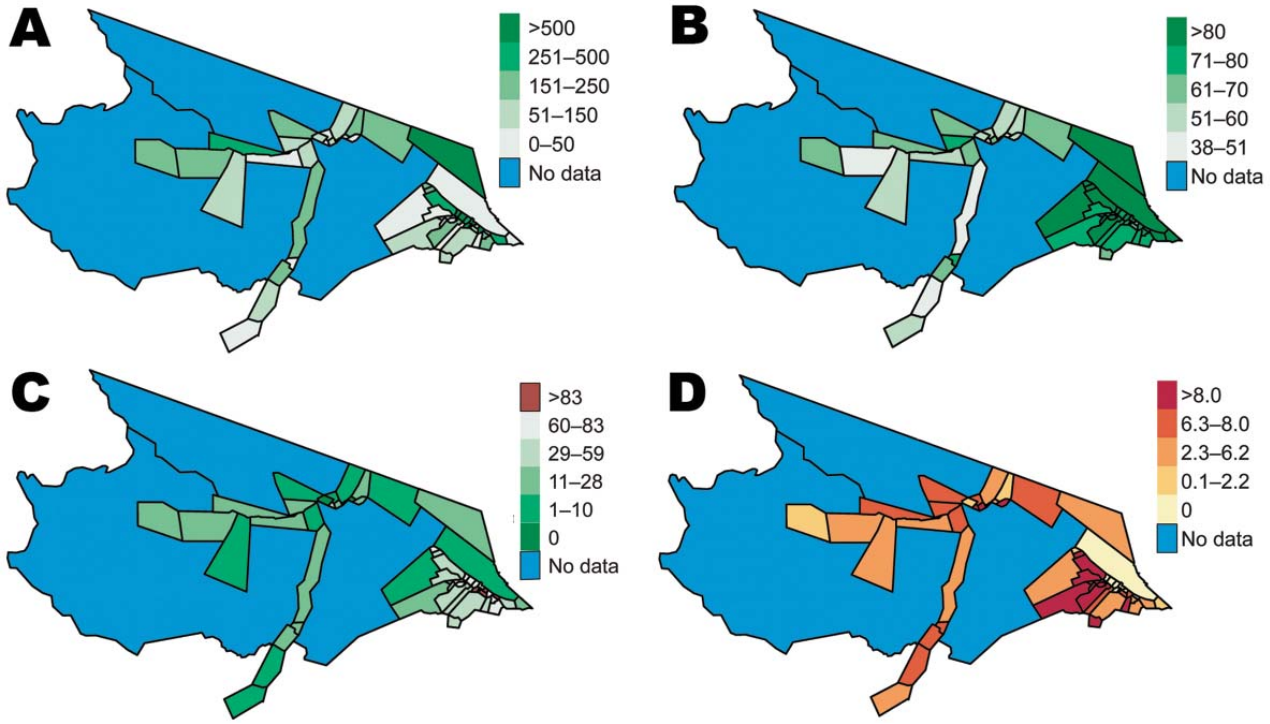

Figure 4. Cloropleth maps of selected malaria risk factors for health districts in Mâncio Lima, Brazil. A) Resident population in health districts in 2006. B) Percentage of slide-confirmed malaria cases receiving access to care within the first 48 hours of symptom onset in 2006. C) Percentage of 1997 deforestation in each of the health districts calculated from 60 × 60-meter resolution classified PRODES data. D) Cumulative percentage change in deforestation by health district from 1997 to 2006. Uninhabited areas are excluded from the analysis.

Table. Influence of ecologic deforestation and social demographic risk factors on malaria incidence in health districts, Mâncio Lima County, Brazil, 1997–2006*

	2006 health district summary		Univariate analysis†			Multivariate analysis†	
Variable	Mean	SD	RR	95% CI	AIC	RR	95% CI
Ecologic							
Deforested in 1997, %	40.2	32.5	0.84	0.64–1.09	671.1		
Deforested 1997–2002, %	3.2	4.3	1.31	1.11–1.56	664.0		
Deforested 1997–2001, %	2.7	4.2	1.32	1.11–1.57	663.8		
Deforested 1997–2000, %	2.3	4.3	1.33	1.12–1.58	663.6	1.48	1.26–1.75
Deforested 2001–2006, %	3.4	3.6	1.03	0.85–1.23	672.7		
Social demographic							
Active surveillance, %	64.9	19.3	1.27	0.97–1.65	669.6		
Access to care <48 h, %	70.6	13.5	1.18	0.87–1.59	671.4	0.92	0.72–1.17
Case-patients <10 y, %	27.6	9.3	1.18	0.94–1.46	669.9		
Case-patients, male, %	55.9	7.8	1.07	0.88–1.31	672.1		
Falciparum cases, %	41.3	10.1	1.11	0.87–1.41	671.0		
Spatial							
Area, km^2	23.9	33	1.20	0.99–1.46	669.1	1.26	1.06–1.49
Interaction‡						1.20	1.05–1.39

*Summary statistics of variables, relative risks (RR), and 95% confidence intervals (CIs) for the univariate and multivariate negative binomial generalized additive models with integrated smoothness estimation of spatial correlation. The SD is used as the unit of analysis for all risk factors and the Akaike information criterion (AIC) values for the univariate models are shown. The AIC for the multivariate model is 655.9.
†Models adjusted for spatial trend
‡Area × access to care.

joint relative risk of percentage access to care and the spatial area on malaria incidence within each health district adjusted for percentage deforestation from 1997 through 2000. In Mâncio Lima, malaria risk decreases as the percentage of access to healthcare increases for health districts <23.9 km^2 (mean value). The pattern of relative risk in health districts of larger size is less clear, due to a shortage of observations. A map of the model residuals did not show any spatial trends; the global spatial autocorrelation of the residuals based on Moran's I is –0.12 and not significant (p = 0.90) (Figure 6).

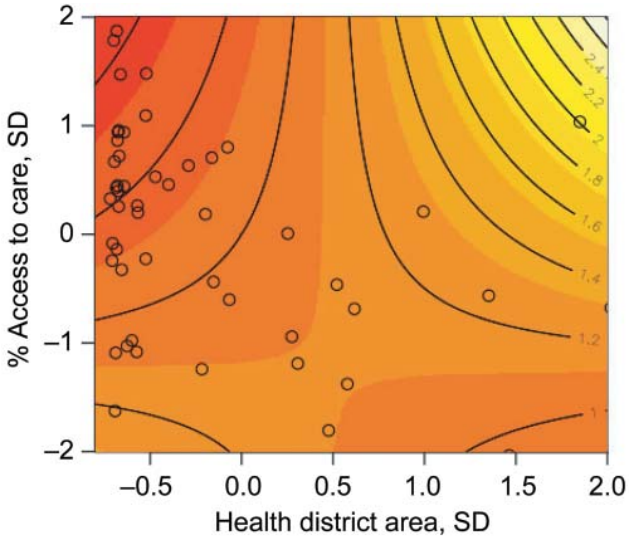

Figure 5. Joint relative risk plot of access to care and health district spatial area, Mâncio Lima, Brazil. Contour lines indicate the joint relative risk for standard deviation changes in percentage access to care and health district spatial area. Open circles are the observed percentage access to care and health district spatial area size data pairs for the 54 health districts. The contour line increment of relative risk is 0.2, increasing with the shading from red to white.

Discussion

We based our investigation of environmental and sociodemographic malaria risk factors on an existing surveillance system and estimated the relative risk for these factors at the health district level. Malaria surveillance in Brazil is unprecedented in scale and uniformity. Focusing on 1 county linked to global information systems health district level data, we report the characteristics of the health districts, map the distribution of risk factors, and find significant associations between deforestation and malaria incidence. Adjusting for population, access to care, and district size, we found that malaria risk increased ≈50% in health districts when 4% of the area underwent deforestation in 1997–2000.

Our approach shows the relative associations of malaria incidence and deforestation patterns across space, rather than a trend of malaria incidence and deforestation across time. The model assumes annual regional variability in temporal risk factors, such as climate and intervention measures, was uniform for the 2006 observations. We examined measures of deforestation before 2006 to model the pattern of malaria observed in 2006. However, given the cross sectional design, the association of malaria incidence to prior deforestation does not necessarily imply a causal trajectory of increased deforestation and elevated malaria incidence.

We found that the univariate models predict that a higher malaria risk is associated with more active sur-

veillance and access to care. This seems counterintuitive, as active surveillance generally identifies cases quickly, which leads to faster treatment and lower disease risk, and access to care is a variable reliably associated with lower disease rates. For example, in the Indian state of Assam, malaria incidence is consistently lower in villages within 5 km of healthcare facilities (*29*). The univariate relative risks suggest that increased active surveillance and access to healthcare during the epidemic led to the identification of cases that normally would have gone unreported. The significant interaction of health district size and access to care improved the performance of the multivariate model for percentage of deforestation from 1997 through 2000, health district size, and access to care. The interaction and joint relative risk show that increased surveillance in health districts <23 km^2 is protective against malaria risk, after adjusting for percentage of deforestation.

The landscape establishes local ecology and biodiversity, and our results confirm that cleared land is associated with a higher malaria risk. This association has been identified in previous research, but here we link the ecologic observations of the habitat preference of *A. darlingi* mosquitoes for deforested areas to an existing malaria surveillance program. Moreover, we found that human malaria risk is specifically associated with deforestation 5–10 years previously. We did not find an association with deforestation before or after that time frame. These findings seem to agree with other research that observed that shrub land cover, which develops 5 years after deforestation and becomes classified as secondary growth ≈15 years after deforestation, has significantly greater abundance of *A. darlingi* larvae than does forested land (*8*). Together these findings suggest that entomologic risk is based on the fate of cleared land.

The study is limited by several factors. The malaria data are based on annual percentage measures derived from the PNCM malaria surveillance questionnaire. Each health district was the unit of analysis, so we were unable to adjust for risk factors at the individual case level. The categorical data structure was restrictive and there was insufficient reporting on voluntary portions of the survey that limited our ability to adjust for socioeconomic drivers. The frequency of double reporting is unknown, but we have filtered the data for only those patients who reported living within the health district where they sought treatment. Temperature is nearly always suitable for malaria transmission in the Amazon Basin, but any variability of rainfall and hydrologic characteristics in Mâncio Lima may also be a confounding factor (*30*).

Another consideration is the absence of current immigration information, but several observations suggest that migration is an unlikely factor in explaining malaria patterning in this study site. In 2006 there were a total of

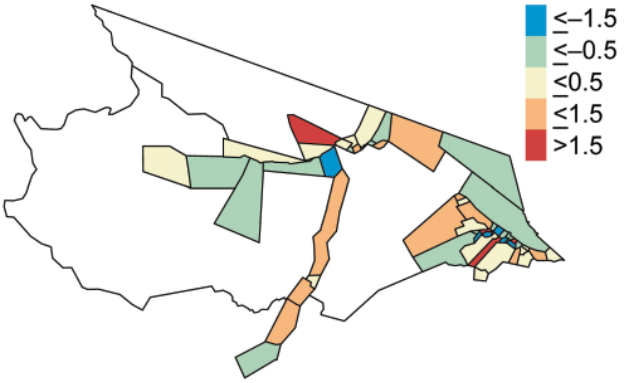

Figure 6. Multivariate model residual map, Mâncio Lima, Brazil.

750,000 emigrants living in the Northern Region, which encompasses the states of Acre, Amazonas, Anapá, Roraima, Rondônia, Pará, and Tocantins. This represents just 4% of all emigrants to new regions within Brazil based on place of birth. More locally in 2000, Mâncio Lima recorded an influx of just 29 emigrants ≥5 years of age since 1995 from areas outside of Acre, or just 0.2% of all migration to Acre (*15*). These trends suggest that a minimal amount of migration to Mâncio Lima occurred before 2006.

The emerging local aquaculture industry is an important concern that might also be correlated with the deforestation patterns in health districts. Pond, wells, or fish farms >50 m in circumference significantly increase the abundance of *A. darlingi* larvae (*9*). Mâncio Lima's aquaculture production has been growing since this association was established in 2003. The ponds ranged size from 5 ha to 175 ha, and the April 2007 harvest yielded 30,000 kg of fish, valued at R$216,000 or US$106,000 (*31*). Taking the 2007 harvest at a yield obtained in the neighboring state of Amazônia of ≈70 kg/ha/year, suggests that ≈430 ha of aquaculture existed in Mâncio Lima in 2006 (*32*). Fish farms are often located in degraded and deforested lands, yet this practice maybe leading to more mosquito larval habitat and higher malaria incidence. Further investigation is needed to differentiate deforestation from the effects of fish farming.

Our models assume that environmental exposures occurred in the health district in which a patient claims residency. If persons slept and worked in different areas, we could not directly associate exposure with environmental variables within the health district. However, the diurnal biting pattern of *A. darling* mosquitoes, which generally peaks in the evening and sometimes in the early morning, means most exposure will occur near the home (*7*,*10*,*33*). Additionally, we were not able to adjust for the presence or absence of the agent, plasmodium sporozoites, yet the county was saturated with malaria at the peak of an epi-

demic, which increased the probability of widespread malaria exposure. In a scenario in which *A. darlingi* mosquitoes are very abundant but the parasite is absent, once the malaria sporozoite is introduced, malaria should spread.

We showed how the framework of health districts can link landscape and disease risk, but the overall generalizability of our findings is limited. In the Amazon, patterns of malaria risk factors are known to change from 1 community to the next. We found that age and sex were not associated with malaria risk in Mâncio Lima, yet men carry double the risk of women in some communities, and in others, gold miners have a risk 3 times higher than that of urban residents (*34*). Another community has no age-specific, occupational, or gender risks, but activities such as strolling outdoors after 6:00 PM and waking before 6:00 AM for adults, and attending church services in the evening for children are significantly associated with malaria risk (*35*). Beyond the Amazon, evidence has shown that mosquito survival can depend on slight variations in temperatures, humidity, and sunlight as a result of deforestation (*36*,*37*). Even though eradication of malaria is a reemerging priority of the global health community, no spatially standardized approach has been developed that can monitor patterns of malaria at the clinic or treatment unit (*38*).

At present, policy makers and epidemiologists continue to speculate about the regional and local variation of malaria and malaria risk factors. But policy makers also know that, "policies are sometimes applied more broadly than appropriate to large regions when it may actually only be relevant to a particular setting within the region … (and) policies often need to be specific to be useful" (*38*, p. 95). Currently, beyond our findings in Mâncio Lima and a few isolated studies, the ultimate relationship and geographic extent of the malaria incidence and deforestation process are unknown.

Our findings illustrate the importance of relative deforestation between health districts in the county of Mâncio Lima, but they do not necessarily explain the overall intensity of the 2006 epidemic (Figure 3). The epidemic is likely a result of a combination of forces with deforestation (and perhaps aquaculture) creating the landscape conditions more suitable to *A. darlingi* mosquitoes. The extensive drought of 2005 may also have contributed to higher mosquito populations and malaria risk (*39*). This conclusion is supported by studies of El Niño events, caused by warming sea surface temperatures, which decreased precipitation in the region and are associated with higher malaria incidence the following year in Venezuela and Guyana (*40*). Here we show that deforestation significantly affects malaria risk, which suggests that land use measures may be 1 method to employ in malaria control.

In summary, we show that focused monitoring and high resolution spatial mapping of health districts can identify ecologic associations between malaria incidence and deforestation. Other human health and ecology linkages may be discernable with similar high resolution and spatially explicit data.

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Dr Olson recently completed a joint PhD from the University of Wisconsin–Madison in population health from the School of Medicine and Population Health and in environment and resources from the Nelson Institute. Her research addresses regional landscape and climate links in the ecology of vector-borne infectious diseases.

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Address for correspondence: Sarah H. Olson, University of Wisconsin, 1710 University Ave, Madison, WI 53726, USA; email: sholson1@wisc.edu

Fatal Babesiosis in Man, Finland, 2004

Karita Haapasalo, Pekka Suomalainen, Antti Sukura, Heli Siikamäki, and T. Sakari Jokiranta

We report an unusual case of human babesiosis in Finland in a 53-year-old man with no history of splenectomy. He had a rudimentary spleen, coexisting Lyme borreliosis, exceptional dark streaks on his extremities, and subsequent disseminated aspergillosis. He was infected with *Babesia divergens*, which usually causes bovine babesiosis in Finland.

Babesiosis is an arthropod-transmitted infection caused by an apicomplexan parasite. Most zoonotic cases in humans have been reported from the eastern coast of the United States, where the causative agent is *Babesia microti*, which is transmitted from white-footed mice to humans by *Ixodes scapularis* ticks (*1*,*2*). Rare cases of human babesiosis caused by *B. divergens* have been reported in Europe. *B. divergens* is a bovine parasite transmitted mainly by *I. ricinus* ticks. Both *Ixodes* spp. ticks also transmit *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis. In certain areas of the United States, >10% of patients with a diagnosis of Lyme disease are co-infected with *B. microti* (*3*). Co-infections with *Borrelia* spp. and *B. divergens* infections have been documented only serologically (*4*).

Babesia sporozoites are transmitted to the vertebrate host by a tick bite (*5*). The sporozoites invade erythrocytes and transform into ring-form trophozoites and typical Maltese cross assemblies of merozoites. Parasites lyse infected erythrocytes, which release merozoites that can invade new erythrocytes (*6*,*7*). Trophozoites can alternatively develop into gametocytes, enabling continuation of the life cycle in the tick after it has had a blood meal.

Most disease manifestations of human babesiosis are related to hemolysis (*1*). Symptoms are anemia, malaise, fever, chills, myalgia, and fatigue. High parasitemia levels can cause massive hemoglobinuria, acute renal tubular necrosis, and renal failure. In addition to hemolysis-associated manifestations, acute respiratory distress syndrome may occur as a complication and lead to death (*8*). *B. divergens* infections in Europe have been severe, and the mortality rate is high (42%) compared with that of *B. microti* infections in the United States (5%–20%) (*1*,*2*,*9*). All 22 published cases of *B. divergens* infection in Europe have occurred in patients who have undergone splenectomy (*2*). We report an unusual case of human babesiosis in Finland.

Author affiliations: University of Helsinki, Helsinki, Finland (K. Haapasalo, A. Sukura, T.S. Jokiranta); Hospital District of Helsinki and Uusimaa, Helsinki (K. Haapasalo, T.S. Jokiranta); South Karelia Central Hospital, Lappeenranta, Finland (P. Suomalainen); and Helsinki University Central Hospital, Helsinki (H. Siikamäki)

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The Patient

A 53-year-old man was admitted to South Karelia Central Hospital in Lappeenranta, Finland, in September 2004 with septic shock. The patient had a history of severe alcohol-induced pancreatitis (in 1993), subsequent type 1 diabetes mellitus, *Staphylococcus aureus* septicemia (in 2000), and atherosclerosis. He had had fever, back pain, and fatigue for a week before admission and reported that his urine was darker and lower in volume than usual for several days. The patient had no history of travel abroad in the past 5 years.

At admission, the patient was conscious. Physical examination and intensive care unit monitoring indicated septic shock and lactate acidosis. The Physiology and Chronic Health Evaluation score of the patient was 60%, and the Simplified Acute Physiology score was 50%. The patient was treated with routine intensive care measures, which were started at admission.

Physical examination showed a wide (maximum diameter 10 cm) erythema chronicum migrans (ECM) lesion on the left thigh (Figure 1, panel A) and dark streaks on both legs and the right arm (Figure 1, panels B, C). During the first 24 hours, laboratory analyses showed massive hemolysis (plasma hemoglobin 2,650 mg/dL, blood hemoglobin 5.7 g/dL, haptoglobin 30 mg/dL, lactic dehydrogenase 11,240 U/L, bilirubin 213 μmol/L), thrombocytopenia (thrombocyte count 56,000/μL), an elevated C-reactive protein level (13.9 mg/dL at admission), and an elevated aspartate aminotransferase level (9,559 U/L). Ultrasound showed a low signal from the spleen. Microscopic examination of a blood smear was conducted 25 hours after admission because of suspicion of hemolytic uremic syndrome and showed intraerythrocytic structures typical of babesiosis with a parasitemia level of ≈10% (Figure 2).

The patient was treated with intravenous quinine (1,000 mg 3×/d for 12 days), clindamycin (600 mg 4×/d for 12 days), and cefotaxime (2 g/d for 12 days). At the end of day 2, a 9-liter blood exchange was performed. Despite these procedures and reduction of the high level of parasitemia (to 1% within 2 days), the patient had hypotension and was treated in the intensive care unit in a respirator under propofol-based sedation. At day 9, he had pulmonary aspergillosis (infection with *Aspergillus fumigatus*), *S. epidermidis* in blood cultures, and *Candida albicans* cultured from the tip of his dialysis catheter. After the patient received a diagnosis of multiple organ failure, treatment was terminated on day 12; he died the same day. Histologic

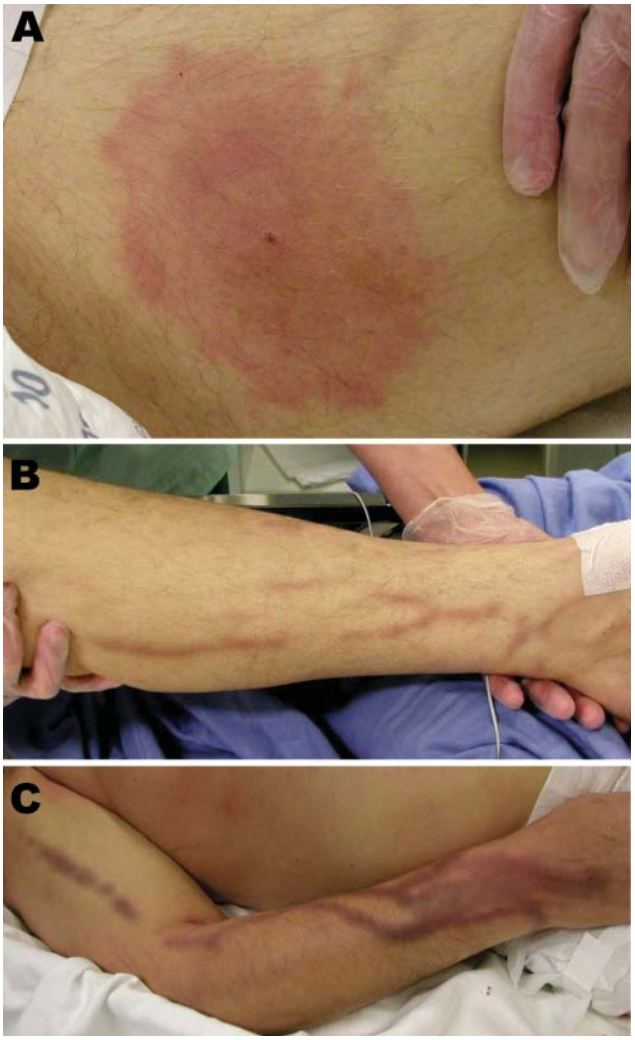

Figure 1. Lesions of the patient infected with *Babesia divergens* 1 day after hospitalization, Finland, 2004. A) Left thigh showing a classical erythema chronicum migrans lesion; B) left leg and C) right arm showing dark purple streaks.

analysis of autopsy specimens showed aspergillosis in the lungs, heart, and kidneys. The spleen was rudimentary, and no malignancy was found. The patient was HIV negative.

To identify *Babesia* spp., DNA was extracted from anticoagulated venous blood samples obtained on day 2. DNA was amplified and the PCR product was sequenced as described (*10*). The sequence obtained (GenBank accession no. GU945501) was 100% identical to previously a reported *B. divergens* 18S rDNA sequence (GenBank accession no. AY789076.1).

Conclusions

We report an unusual human case of babesiosis and provide molecular evidence that the causative organism was *B. divergens*. Human babesiosis has not been previously reported in Finland. Eight fatal cases of babesiosis have been described in Europe (1 each in the former Soviet Union, France, Spain, and the former Yugoslavia, and 4 in the British Isles) (*2*,*11*). All cases were *B. divergens* infections in patients who had undergone splenectomy. This species has been identified by inoculation into animals or by serologic analysis.

Bovine babesiosis is endemic to Finland, although its incidence has dramatically decreased over the past 2 decades.

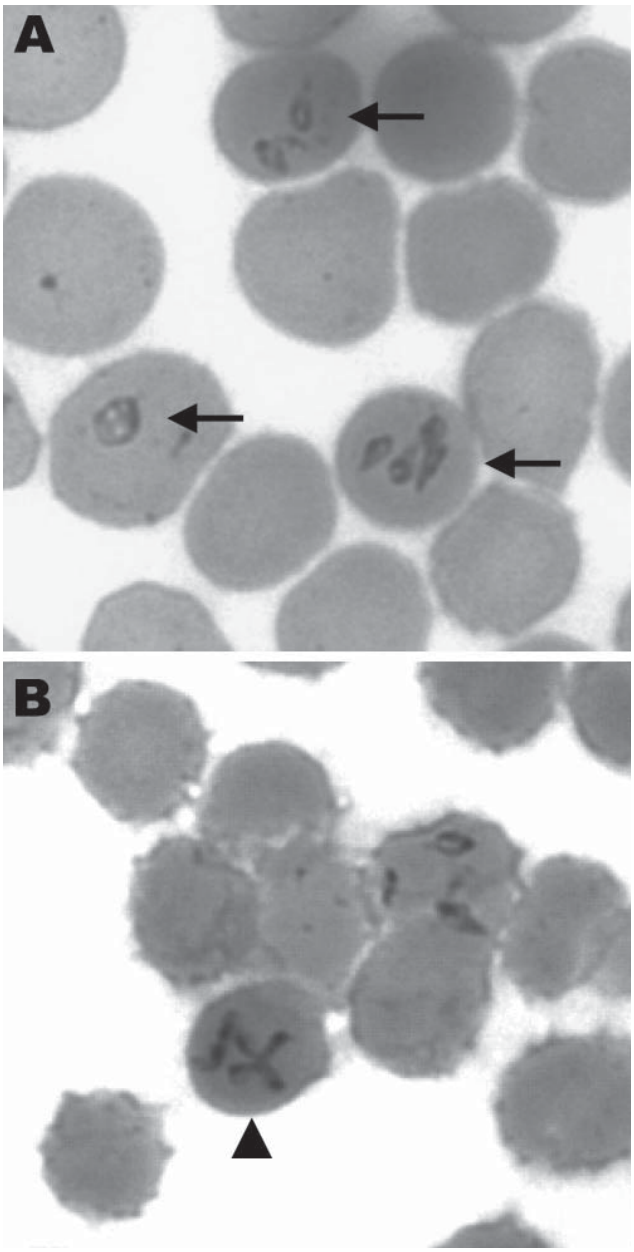

Figure 2. Giemsa-stained smears of peripheral blood of the patient infected with *Babesia divergens*, Finland, 2004. Intraerythrocytic parasites are indicated by arrows (A), and a representative Maltese cross form of the parasite is indicated by an arrowhead (B). (Original magnification ×750.)

Table. Incidence of cattle babesiosis, Finland*

	Year						
District	1965	1975	2000	2002	2002	2003	2004
Southern	–	–	21	7	23	6	11
Eastern†	–	–	39	31	27	28	18
Oulu	–	–	0	0	0	0	0
Lapland	–	–	0	0	0	0	0
Ahvenanmaa	–	–	18	12	18	2	0
Western	–	–	1	1	6	6	2
Total	4,796	2,524	79	51	74	42	312

*–, no district data were available.
†Region where the index case-patient lived.

Monthly data for bovine babesiosis in Finland are obtained by municipal veterinarians who send reports to the Ministry of Agriculture and Forestry (T. Aaltonen, unpub. data). Three cases of bovine babesiosis were reported to health authorities in the municipality where the patient lived in the same year he was infected and became ill (online Appendix Figure, www.cdc.gov/EID/content/16/7/1116-appF.htm). The patient lived in the country next to a cattle farm. He took walks and worked in the forest nearby. Because the patient had no travel history, we conclude that babesiosis was acquired in Finland, and, most likely, locally from the zoonotic reservoir of *B. divergens* in cattle through a tick bite (Table). This conclusion is supported by the presence of ECM and a tick bite on the thigh of the patient. Infection with *Babesia* sp. and *Borrelia* sp. from the same tick bite is likely because ECM and babesiosis caused by *B. divergens* are manifested 3–30 days and 7–21 days, respectively, after a tick bite.

The poor outcome of the patient can be explained by several factors. His immune system was not intact. He also had diabetes, which impaired his general immunity. Although he had not undergone splenectomy, he was also considered asplenic because only a rudimentary spleen was found by autopsy. The rudimentary spleen may have been caused by severe pancreatitis or by high consumption of alcohol (*12*). Invasive aspergillosis, which is usually seen only in severely immunocompromised patients, also developed in the patient. The origin of dark streaks on his arms and legs is not clear but was probably caused by massive intravascular hemolysis, which was also suggested by dark urine.

Co-infection with *Babesia* sp. and *Borrelia* sp. has been shown to cause disease with additional symptoms and a more persistent illness than infection with *B. microti* (*3*). This co-infection could have caused the severe and fatal disease in our patient. Thus, clinicians should be aware of co-infection with *B. divergens* and *Borrelia* sp.

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Ms Haapasalo is a scientist at the Haartman Institute, University of Helsinki, Helsinki, Finland. Her research interest is microbial pathogenesis, specifically the role of innate immunity in parasitic and bacterial infections.

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Address for correspondence: T. Sakari Jokiranta, Haartman Institute, University of Helsinki, Haartmaninkatu 3, PO Box 21, Helsinki, Finland; email: sakari.jokiranta@helsinki.fi

Postexposure Treatment of Marburg Virus Infection

Thomas W. Geisbert, Lisa E. Hensley, Joan B. Geisbert, Anders Leung, Joshua C. Johnson, Allen Grolla, and Heinz Feldmann

Rhesus monkeys are protected from disease when a recombinant vesicular stomatitis virus–based vaccine is administered 20–30 min after infection with Marburg virus. We protected 5/6 monkeys when this vaccine was given 24 h after challenge; 2/6 animals were protected when the vaccine was administered 48 h postinfection.

The filoviruses, Marburg virus (MBGV) and Ebola virus (EBOV), have been associated with sporadic episodes of hemorrhagic fever (HF) in Central Africa that produce severe disease and high mortality rates among infected patients (*1*). MBGV and EBOV are also considered potential biological weapons. No approved active or passive therapeutic modalities exist for filovirus infections. Although much progress has been made in developing preventive vaccines that can protect nonhuman primates against lethal challenge with MBGV and EBOV, advances in development of postexposure interventions against the filoviruses have not kept pace. Some degree of success has been achieved by using strategies that mitigate the coagulation abnormalities characterizing filoviral infection (*2*,*3*). Also, new postexposure treatment approaches, based on small interfering RNA (*4*) and antisense oligomers (*5*,*6*), have shown promising results in rodent models, but no reports have been published of evaluations of either strategy in the more stringent macaque models.

Recently, we showed the first complete postexposure protection of nonhuman primates against a filovirus by administering a live-attenuated recombinant vesicular stomatitis virus (rVSV) vaccine vector expressing the MBGV glycoprotein (GP) (VSVΔG MBGV GP) shortly after a high-dose MBGV challenge (*7*,*8*). We demonstrated that an rVSV vector, expressing the Zaire EBOV (ZEBOV) GP, protected 50% of rhesus macaques when administered shortly after a high-dose ZEBOV challenge (*9*). We further showed that an rVSV vector expressing the Sudan EBOV GP completely protected rhesus monkeys from a lethal challenge with this virus when administered shortly after exposure (*10*). All animals in these 3 studies were treated once with rVSV vectors 20–30 min after filovirus challenge. The primary question raised from these investigations is how far out treatment can be delayed before there is no survival or beneficial effect. Using a homologous VSVΔG MBGV GP vector, we have delineated a window of opportunity for treatment of MBGV-infected rhesus macaques.

The Study

Animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. Fifteen healthy, filovirus-seronegative rhesus macaques (each weighing 4 kg–7 kg) were randomized into 2 experimental groups of 6 monkeys per group and 3 control groups of 1 animal per group. All 15 animals were challenged by intramuscular (IM) injection with 1,000 PFU of MBGV (Musoke strain). Approximately 24 h after MBGV challenge, animals in experimental group 1 received a single IM injection of VSVΔG MBGV GP (≈2 × 10^7 PFU) (*8*), and the animal in control group 1 received an equal dose of a VSV vector encoding a nonrelated GP (VSVΔG/LassaGPC). Approximately 48 h after MBGV challenge, animals in experimental group 2 received a single IM injection of VSVΔG MBGV GP (≈2 × 10^7 PFU), and the animal in control group 2 received an equal dose of VSVΔG/LassaGPC. The animal in control group 3 was not treated. Blood samples for viral infectivity titration, reverse transcription–PCR (RT-PCR), hematologic analysis, serum biochemical analysis, and immunoglobulin (Ig) G were collected before MBGV challenge and on days 3, 6, 10, 14, and 31–35 after MBGV challenge.

Five of the 6 animals treated with VSVΔG/MBGV GP 24 h after MBGV challenge (animals 1, 2, 4–6) and 2 of the 6 animals treated with VSVΔG/MBGV GP 48 h after MBGV challenge (animals 7 and 10) survived (Figure; online Appendix Table, www.cdc.gov/EID/content/16/7/1119-appT.htm). In contrast, symptoms consistent with MBGV HF developed in 1 of the 6 macaques treated with VSVΔG/MBGV GP at 24 h (animal 3) and in 4 of the 6 animals treated with VSVΔG/MBGV GP at 48 h (animals 8, 9, 11, and 12); these included anorexia and a macular rash (Table 1). The 5 animals in which macular rash developed (animals 3, 8, 9, 11, and 12) also had plasma viremias

Author affiliations: National Emerging Infectious Diseases Laboratories Institute, Boston, Massachusetts, USA (T.W. Geisbert, J.B. Geisbert); US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA (L.E. Hensley, J.C. Johnson); Public Health Agency of Canada, Winnipeg, Manitoba, Canada (A. Leung, A. Grolla); and National Institutes of Health, Hamilton, Montana, USA (H. Feldmann)

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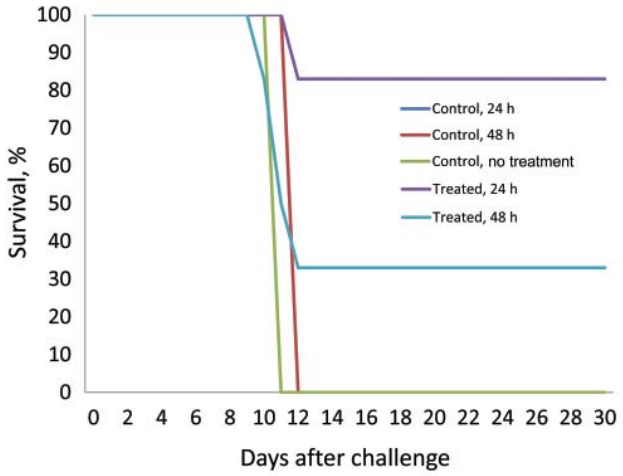

Figure. Survival curves for Marburg virus–infected rhesus macaques treated 24 or 48 h after challenge with a recombinant vesicular stomatitis virus vaccine.

>6.0 $\log_{10}$ PFU/mL by day 10; all 5 animals died during days 10–12 (Figure; Table 1; online Appendix Table). Symptoms developed in control animals 1–3 consistent with MBGV HF; each had plasma viremia levels >7.0 $\log_{10}$ PFU/mL by day 10 and died on days 12, 12, and 11, respectively (online Appendix Table).

Two of the 6 animals that survived MBGV challenge (animals 1 and 6) showed no change in appearance or behavior that indicated overt illness. Changes in hematologic results and/or blood parameters were observed in 5 of the surviving animals (2, 4, 5, 7, and 10) during the course of the study (online Appendix Table). Plaque assay and RT-PCR were unable to detect any evidence of MBGV in the plasma of 6 of the 7 surviving animals (1, 2, 4–6, and 10). However, RT-PCR showed evidence of MBGV in peripheral blood mononuclear cells of 2 of these surviving animals (1 and 2) at day 10 (Table 2). Viremia of 4.2 $\log_{10}$ PFU/mL developed on day 10 in 1 surviving animal (7) treated 48 h after infection, and RT-PCR showed evidence of MBGV in peripheral blood mononuclear cells of this animal on days 6 and 10. Viremia in plasma was cleared, and the animal showed little evidence of illness by day 14. The serologic response profile of MBGV infection after treatment was evaluated by IgG ELISA. All 7 animals that were treated with VSVΔG/MBGV GP and survived infection showed moderate to high levels of IgG by day 14 (320–1,000); humoral response against MBGV was not detectable in the treated animals that died or in the control animals (Table 2).

Conclusions

This rhesus macaque model represents a worse-case scenario such as an accidental needle-stick exposure of a laboratory worker or first responder to a high infectious dose of a filovirus. Accidents such as these have occurred several times over the past 5 years (*11*–*13*). Of direct relevance to our study was a recent laboratory accident in which an rVSV vector expressing the ZEBOV GP, which had been used successfully in postexposure treatment of experimentally infected nonhuman primates (*9*), was administered to a human ≈40 h after a ZEBOV needle-stick exposure (*13*). The patient received a dose of ≈5×10^7 PFU of the VSV ZEBOV GP vaccine, which is consistent with doses used in nonhuman primate studies (*7*,*9*,*10*). Fever, headache, and myalgia developed in the patient hours after injection but were successfully controlled with analgesics and antipyretics. Other adverse effects were not reported,

Table 1. Viral load in rhesus monkeys after Marburg virus challenge*

Animal no.	Group	Treatment	Time of treatment after challenge, h	Plasma†			PBMC		
				Day 6	Day 10	Day 14	Day 6	Day 10	Day 14
1	Exp 1	VSV-Marburg	24	0 (–)	0 (–)	0 (–)	NT (–)	NT (+)	NT
2	Exp 1	VSV-Marburg	24	0 (–)	0 (–)	0 (–)	NT (–)	NT (+)	NT (–)
3	Exp 1	VSV-Marburg	24	3.76 (–)	6.19 (+)		NT (–)	NT (+)	
4	Exp 1	VSV-Marburg	24	0 (–)	0 (–)	0 (–)	NT (–)	NT (–)	NT (–)
5	Exp 1	VSV-Marburg	24	0 (–)	0 (–)	0 (–)	NT (–)	NT (–)	NT (–)
6	Exp 1	VSV-Marburg	24	0 (–)	0 (–)	0 (–)	NT (–)	NT (–)	NT (–)
Control 1	Cont 1	VSV-Lassa	24	3.76 (–)	7.33 (+)		NT (–)	NT (+)	
7	Exp 2	VSV-Marburg	48	0 (–)	4.20 (+)	0 (–)	NT (+)	NT (+)	NT (–)
8	Exp 2	VSV-Marburg	48	0 (–)	7.27 (+)		NT (+)	NT (+)	
9	Exp 2	VSV-Marburg	48	3.76 (–)	7.25 (+)		NT (+)	NT (+)	
10	Exp 2	VSV-Marburg	48	0 (–)	0 (–)	0 (–)	NT (–)	NT (–)	NT (–)
11	Exp 2	VSV-Marburg	48	5.24 (+)	7.35 (+)		NT (+)	NT (+)	
12	Exp 2	VSV-Marburg	48	3.76 (–)	6.81 (+)		NT (–)	NT (+)	
Control 2	Cont 2	VSV-Lassa	48	4.05 (–)	7.24 (+)		NT (+)	NT (+)	
Control 3	Cont 3	None	NA	5.07 (+)	7.15 (+)		NT (+)	NT (+)	

*PBMC, peripheral blood mononuclear cells; Exp, experimental; VSV, vesicular stomatitis virus; NT, not tested; Cont, control; (+), sample positive for Marburg virus by reverse transcription–PCR (RT-PCR); (–), sample negative for Marburg virus by RT-PCR; NA, not applicable.
†$\log_{10}$ PFU of Marburg virus per milliliter of plasma.

Table 2. Serologic response profiles of Marburg virus–infected rhesus monkeys after treatment with VSVΔG/Marburg virus glycoprotein vectors*

Animal no.	Group	Treatment	Time of treatment after challenge, h	Serum anti–Marburg virus IgG†		
				Day 6	Day 10	Day 14
1	Exp 1	VSV-Marburg	24	0	320	1,000
2	Exp 1	VSV-Marburg	24	0	100	1,000
3	Exp 1	VSV-Marburg	24	0	0	NA
4	Exp 1	VSV-Marburg	24	0	100	320
5	Exp 1	VSV-Marburg	24	0	1,000	1,000
6	Exp 1	VSV-Marburg	24	0	320	320
Control 1	Cont 1	VSV-Lassa	24	0	0	NA
7	Exp 2	VSV-Marburg	48	0	320	1,000
8	Exp 2	VSV-Marburg	48	0	0	NA
9	Exp 2	VSV-Marburg	48	0	0	NA
10	Exp 2	VSV-Marburg	48	0	320	1,000
11	Exp 2	VSV-Marburg	48	0	0	NA
12	Exp 2	VSV-Marburg	48	0	0	NA
Control 2	Cont 2	VSV-Lassa	48	0	0	NA
Control 3	Cont 3	None	NT	0	0	NA

*VSV, vesicular stomatitis virus; Ig, immunoglobulin; Exp, experimental group; Cont, control group; NA, not applicable because animal had died; NT, not treated.
†Endpoint dilution titers.

but whether treatment was effective or whether the patient never became infected remains uncertain.

MBGV infection of humans normally progresses at a slower rate than does MBGV infection of macaques, with case-fatality rates in humans of 23%–90% (*1*) suggesting that the therapeutic window may be larger for humans than for infected macaques. In addition, the challenge dose that we employed in the rhesus monkey model of MBGV HF of 1,000 PFU represents >10,000 LD_{50} doses (*14*), again showing that this is a robust challenge model. In the current study, we achieved near complete protection from death when treatment with a single-dose regimen was delayed 24 h and 33% protection when treatment was delayed 48 h postexposure. Because no approved treatments exist for exposure to infectious filoviruses, the rVSV vectors described in the current study merit consideration for treating potential exposures and for further development for human use.

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Dr Geisbert is the Associate Director of the National Emerging Infectious Diseases Laboratories Institute at Boston University, Boston, Massachussetts. His research focuses on emerging viruses with an emphasis on viruses causing hemorrhagic fever, including Ebola, Marburg, and Lassa viruses.

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Address for correspondence: Thomas W. Geisbert, Boston University School of Medicine, National Emerging Infectious Diseases Laboratories Institute, 620 Albany St, Rm 401B, Boston, MA 02118, USA; email: geisbert@bu.edu

Detection of Lassa Virus, Mali

David Safronetz, Job E. Lopez, Nafomon Sogoba, Sékou F. Traore', Sandra J. Raffel, Elizabeth R. Fischer, Hideki Ebihara, Luis Branco, Robert F. Garry, Tom G. Schwan, and Heinz Feldmann

To determine whether Lassa virus was circulating in southern Mali, we tested samples from small mammals from 3 villages, including Soromba, where in 2009 a British citizen probably contracted a lethal Lassa virus infection. We report the isolation and genetic characterization of Lassa virus from an area previously unknown for Lassa fever.

Lassa fever is an acute viral infection associated with a wide spectrum of disease manifestations, which range from mild to hemorrhagic fever characterized by multiorgan failure (*1*). The etiologic agent of Lassa fever is Lassa virus (LASV, family *Arenaviridae*, genus *Arenavirus*), which is maintained in its natural rodent reservoir, the multimammate rat (*Mastomys natalensis*) (*1*–*3*). Although *M. natalensis* rats are ubiquitous in many parts of sub-Saharan Africa, infected rodents have only been reported in West African countries, most commonly Nigeria, Sierra Leone, and Guinea (*3*–*5*). Consequently, cases of Lassa fever mainly occur in regions in which the virus is endemic, consisting of those 3 countries and Liberia, with an annual incidence ranging from 300,000 to 500,000 cases and ≈5,000 deaths (*6*). Serologic evidence of LASV infections has also been reported from other West and Central African countries (*5*,*7*–*9*). Additionally, LASV has been introduced into Europe and North America several times, making Lassa fever one of the most prominent imported exotic viral hemorrhagic fevers, which strongly affects public health systems (*9*).

In 2009, Lassa fever was diagnosed postmortem in a young man with a 10-day history of fever who had been evacuated from Mali to London (*10*). The patient had no travel history to any LASV-endemic region, which suggests that he contracted the infection in Mali, most likely while working in the village of Soromba (Figure 1). In this report, we provide evidence that LASV is circulating in *M. natalensis* in southern Mali, thereby expanding the geographic distribution of LASV in West Africa and posing a risk to humans in an area previously unknown for Lassa fever. The study was carried out in accordance with a protocol approved by an Institutional Animal Care and Use Committee of the US National Institutes of Health.

Author affiliations: National Institutes of Health, Hamilton, Montana, USA (D. Safronetz, J.E. Lopez, S.J. Raffel, E.R. Fischer, H. Ebihara, T.G. Schwan, H. Feldmann); University of Bamako, Bamako, Mali (N. Sogoba, S.F. Traore'); Tulane School of Medicine, New Orleans, Louisiana, USA (L. Branco, R.F. Garry); and Autoimmune Technologies LLC, New Orleans (L. Branco)

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The Study

From June 5 through 14, 2009, a total of 103 small mammals (trap success rate of 17.6%) were live trapped and sampled from 3 villages in Mali (Figure 1; Table): N'Tessoni (11°2′0″N, 5°59′0″W), Soromba (10°35′0″N, 7°9′0′W), and Doneguebougou (12°48′21″N, 7°59′0″W). Pertinent information was recorded for each animal, and ear punch, heart, blood, lung, and liver samples were collected. *Mastomys* spp. rats were the predominant rodent captured (82/103, 79.6%), with all but 4 of these animals identified as *M. natalensis* rat by cytochrome B sequence analysis of DNA isolated from ear punch specimens (*11*; GenBank accession nos. HM130517–HM130519).

Total RNA was extracted from tissue specimens and blood by using RNeasy or QIAamp viral RNA kits (QIAGEN, Valencia, CA, USA), respectively, and screened for the presence of LASV RNA by using a SYBR-green based, real-time reverse transcription–PCR assay that amplifies a 195-bp portion of the small genomic segment (primers Gc656s: 5′-ATTGCTCTTGACTCAGGCC-3′ and Gc851as: 5′-GTGTCCATGTGAATGTGCCTA-3′; TIB Molbiol GBH, Adelphia, NJ, USA). Six infected rodents were identified, each of which had LASV-positive lung, liver, and blood specimens. Positive rodents were genetically identified as *M. natalensis*, and all were captured from Soromba, for a village prevalence of 24% (6/25, Table). Four (66.7%) of 6 infected rodents were male and

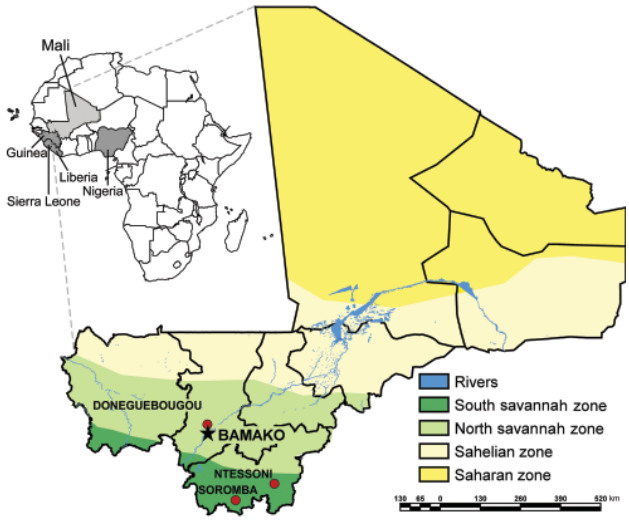

Figure 1. Ecozones of Mali and locations where small mammals were trapped in June 2009. Inset shows location of Mali in relation to countries where Lassa virus is endemic (shaded).

Table. Results of investigation of small mammals trapped in 3 villages in Mali, 2009

Village	No. total captures (trap success, %)*	No. non–*Mastomys* spp. rat captures	No. *Mastomys* spp. rat captures (trap success, %)	No. (%) Lassa-positive *M. natalensis* rats
N'Tessoni	25 (14.9)	8†	17 (10.1)‡	0
Soromba	25 (15.0)	0	25 (15.0)	6 (24)
Doneguebougou	53 (21.1)	13†	40 (15.9)‡	0

*Trap success is defined as the total number of individual rodents captured divided by the cumulative total of traps set at each location, multiplied by 100.
†Non-*Mastomys* species captured included 1 *Praomys daltoni* (Dalton's mouse), 3 *Mus musculoides* (Temminck's mouse), 3 *Crocidura olivieri* (giant shrew), and 1 *Arvicanthis niloticus* (grass rat) from N'Tessoni and 1 *Praomys daltoni*, 1 *Rattus rattus* rat, 1 *Arvicanthis niloticus*, 3 *Crocidura viaria* (savannah path shrew), and 7 *Crocidura olivieri* from Doneguebougou.
‡Two *Mastomys* spp. rodents captured in N'Tessoni and Doneguebougou were identified as *M. erythroleucus* by cytochrome B sequencing.

5 (83.3%) of 6 were adult. All 6 infected animals were captured indoors.

Tissue homogenates were prepared from selected LASV-positive animals and passaged twice on subconfluent monolayers of Vero E6 cells. After 2 passages, no discernible cytopathic effect was observed, although LASV RNA was detected by reverse transcription–PCR in cells and supernatant. Virus isolation was confirmed by immunoblot analysis in infected cells and supernatant by using a LASV nucleoprotein-specific monoclonal antibody, which detected a 50–55-kDa protein consistent in size with the LASV nucleoprotein (*12;* online Appendix Figure, panel A, www.cdc.gov/EID/content/16/7/1123-appF.htm). Furthermore, electron microscopy performed on infected cells showed viral particles consistent in size and morphologic features with an arenavirus (online Appendix Figure, panel B).

An ≈800-nt fragment of the LASV polymerase gene was amplified from the isolated virus and the tissues of infected rodents by using a pan–Old World arenavirus assay (*13*). Additionally, an 873-bp fragment of the LASV glycoprotein gene, corresponding to the sequence from a British Lassa fever patient (*10*), was amplified from the same samples by using primers Gc F1 5′-GCATTTTAATTCAGCCTCAATTAAC-3′ and Gc R1 5′-ATGGGGCAGATTGTGACATTCTTTC-3′. Amplicons were sequenced (GenBank accession nos. GU573541–GU573546 and GU573547–GU573552 for polymerase and glycoprotein fragments, respectively) and aligned with previously described arenavirus sequences by using ClustalX version 2.0.10 software (www.clustal.org). Nucleotide sequences from the isolated LASV were indistinguishable from the sequences generated from the infected rodent tissues from which they were derived (data not shown). Phylogenetic analysis of the glycoprotein sequences confirmed that all rodent-derived LASV sequences belonged to the same genetic clade as the sequence of the imported Lassa fever case in the United Kingdom. Polymerase fragment sequences confirmed that this clade is most closely related to the previously described AV strain of LASV that originated from the neighboring countries of Côte d'Ivoire, Burkina Faso, or Ghana (*14*; Figure 2).

Conclusions

This study demonstrates that *M. natalensis* rats in Mali carry a genetically unique strain of LASV (proposed name Soromba-R). Genetically, Soromba-R is nearly indistinguishable from the nucleotide sequence generated from a lethal case of Lassa fever imported from Mali to the United Kingdom, which supports the epidemiologic data that the infection occurred in the village of Soromba in southern Mali (*10*). The Soromba-R strain of LASV is genetically closely related to the AV strain, which was isolated from an imported lethal Lassa fever case in a patient who contracted the infection while traveling in neighboring countries (*14*). Thus, this study provides evidence for an expanded region of LASV endemicity in West Africa.

On the basis of Mali's geographic proximity to countries where LASV is endemic, it should not be surprising that LASV is circulating there. Cases of Lassa fever are diagnosed annually in Guinea, and sporadic cases have been diagnosed in Burkina Faso and Côte d'Ivoire, all of which border Mali (*5*,*14*,*15*). Furthermore, although to date only 1 laboratory-confirmed Lassa fever case has been diagnosed from Mali, there is serologic evidence of a LASV infection in 1971 in a missionary stationed in Mali (*8*). Considering this probable case and the identification of a genetically unique strain of LASV in rodents and a human case from Mali, it is likely that LASV has been present in Mali yet undetected for decades. The nonspecific clinical signs and symptoms of a large proportion of LASV infections, combined with the unfamiliarity of Malian physicians with Lassa fever, suggest that the infection could easily be misdiagnosed. Currently, Lassa fever is not considered in the differential diagnosis for febrile illness in Mali. This report, however, provides evidence of an emerging infectious disease problem with public health effects; thus, Lassa fever diagnostics and surveillance should be implemented at least in the southern regions of Mali.

Soromba and the surrounding area may represent an enzootic hotspot for LASV-infected rodents. However, a greater distribution of LASV in Mali should not be ruled out, particularly in the southern regions of the country where the climate and geography are similar to conditions found in many parts of Guinea and Sierra Leone (Figure

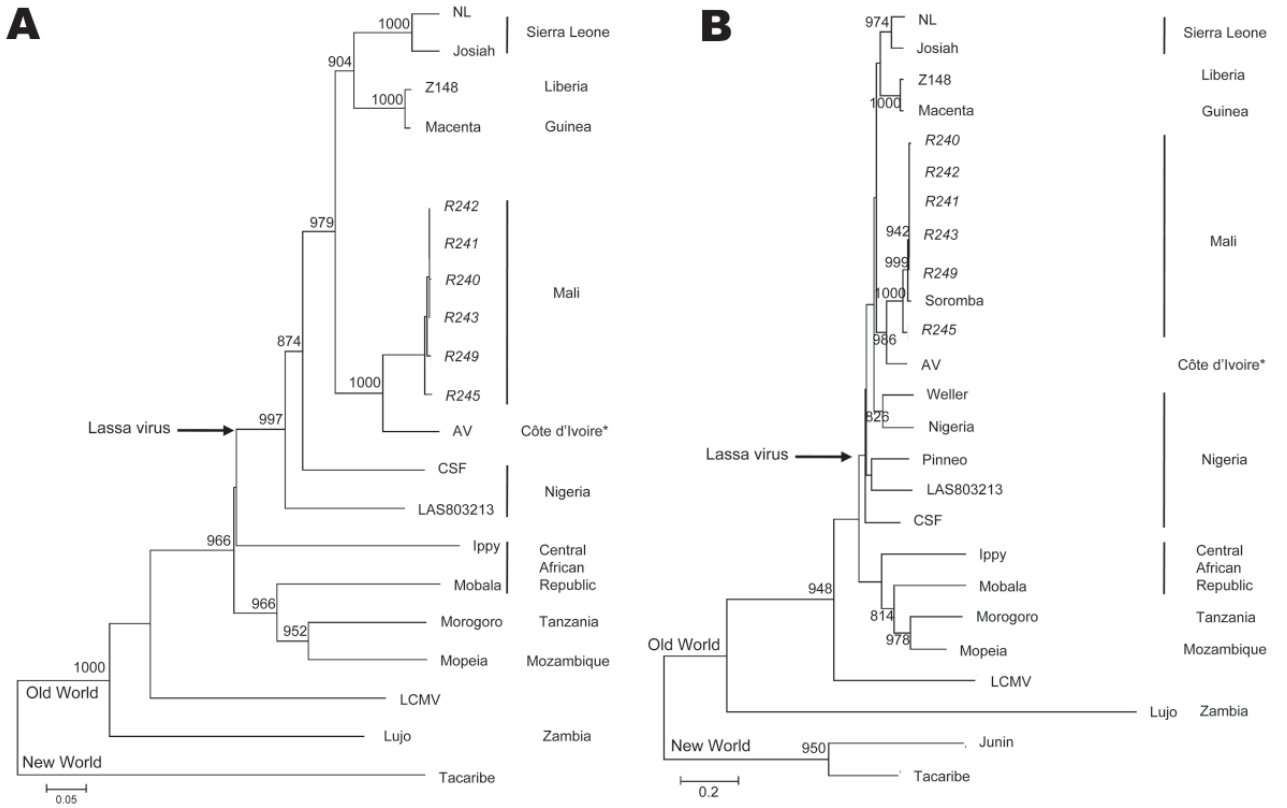

Figure 2. Phylogenetic analysis of Lassa virus conducted on A) a 754-bp fragment of the polymerase gene (large genomic segment nucleotide positions 3427–4180) and B) a 771-bp fragment of the glycoprotein precursor (small genomic segment nucleotide positions 2526–3296). The fragments were amplified from infected rodent tissues with sequence analysis accomplished with PHYLIP version 3.69 software (http://evolution.genetics.washington.edu/phylip.html) by using the neighbor-joining method with 1,000 replicates of bootstrap. Sequences were compared with the following arenavirus sequences (small and large segment GenBank accession nos.): Tacaribe (NC_004293, NC_004292), Lujo (NC_012776, NC_012777), Lymphocytic choriomeningitis virus (LCMV, strain Armstrong, AY847350,J04331), Ippy (NC_007905, NC_007906), Mobala (NC_007903, NC_007904), Morogoro (NC_013057, NC_013058), Mopeia (NC_006575, NC_006574), and Lassa virus strains 803213 (AF803213, AY693640), CSF (AF333969, AY179174), NL (AY179173, AY179172), Josiah (AY628203, U63094), Z148 (AY628205, AY628204), Macenta (AY628201, AY628200), and AV (AF246121, AY179171). Small segment analysis also included Lassa virus strains LP (AF18185), Weller (AY628206), Nigeria (M36544), the sequence generated from the imported case of Lassa fever from Mali (FJ824031), and Junin virus (NC_005081). Bootstrap values >800 are shown. *Whether the AV strain of Lassa virus originated from Côte d'Ivoire, Burkina Faso, or Ghana is not clear. Italicized names represent sequences generated as part of these studies. Scale bars indicate 5% (A) or 20% (B) nucleotide divergence.

1). Combined with our findings that *M. natalensis* was the predominant species of rodent captured in all 3 villages in Mali suggests that conditions favor LASV circulation. Furthermore, on the basis of the timing of the peak incidence of Lassa fever cases, which typically corresponds with the dry season (*15*), we believe that the proportion of LASV-infected rodents may have been low at the time of these studies and beyond the sensitivity of the limited sampling effort conducted here. Future studies need to address these concerns as well as determine the geographic distribution of LASV-infected rodents in Mali to help focus public health preparedness efforts.

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Dr Safronetz is a visiting fellow in the Disease Modeling and Transmission Section of the Laboratory of Virology at the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. His research interests include the ecology, epidemiology, and pathogenesis of rodent-borne viruses, including hantaviruses and arenaviruses.

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Address for correspondence: Heinz Feldmann, Rocky Mountain Laboratories, National Institutes of Health, 903 S 4th St, Hamilton, MT 59840, USA; email: feldmannh@niaid.nih.gov

Septicemia Caused by Tick-borne Bacterial Pathogen *Candidatus* Neoehrlichia mikurensis

Jan S. Fehr,[1] Guido V. Bloemberg,[1] Claudia Ritter, Michael Hombach, Thomas F. Lüscher, Rainer Weber, and Peter M. Keller

We have repeatedly detected *Candidatus* Neoehrlichia mikurensis, a bacterium first described in *Rattus norvegicus* rats and *Ixodes ovatus* ticks in Japan in 2004 in the blood of a 61-year-old man with signs of septicemia by 16S rRNA and *groEL* gene PCR. After 6 weeks of therapy with doxycycline and rifampin, the patient recovered.

Since the novel bacterial genus *Neoehrlichia* was first described in 2004, its pathogenic role in humans has remained unexplained (*1*). Related bacteria such as *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* are emerging tick-borne human pathogens that cause monocytic and granulocytic ehrlichiosis, respectively. These tick-borne diseases manifest themselves as febrile illness, mild transient hepatitis, transient thrombocytopenia, and occasionally as a rash. The family *Anaplasmataceae* compromises the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Aegyptianella*, and the proposed genus *Neoehrlichia.* These are all obligate intracellular bacteria, which currently are difficult or impossible to isolate and culture (*2*). Infections caused by agents of this bacterial family have been recognized as an emerging problem in the past 2 decades, possibly due to ecologic changes and the resulting expansion of tick populations (*3*).

Case Report

In August 2009, a 61-year-old Caucasian man who lived in Switzerland sought treatment at the emergency department of University Hospital in Zurich, reporting a 10-day history of malaise, temperature as high as 39.5°C, chills, and moderate dyspnea. Six weeks previously, he had undergone coronary artery bypass graft surgery and mitral valve reconstruction for which prosthetic material was used. The patient had not noticed tick bites or a skin rash; neither did he recall a rodent bite. A pet dog and cat lived in his household.

Physical examination showed a reduced general health condition and a temperature of 38.5°C. Blood pressure was 109/68 mm Hg, heart rate was 86 beats/min, and oxygen saturation was 95% with 2 L nasal oxygen. No murmur was detected on cardiac auscultation. No skin or joint abnormalities were found. Laboratory tests showed elevated leukocytes (12.9 × 10^3 cells/μL), with a high fraction of neutrophils (10.1 × 10^3 cells/μL) and thrombocyte count within reference range (277 × 10^3 cells/μL); aminotransferase levels within reference ranges (aspartate aminotransferase 18 U/L, alanine aminotransferase 20 U/L); and an elevated C-reactive protein (CRP) of 68 mg/L (reference range <5 mg/L). Chest radiograph showed no signs of cardiac decompensation or of pulmonary infiltrates. Transthoracic echocardiograph showed only minor insufficiency of the aortic and tricuspid valves. In addition, degenerative alterations of aortic valve, but no vegetations, were noted with comparable findings in the follow-up echocardiograph 1 week later.

At the follow-up visit, no hints of infectious foci were found. Five sets of blood cultures were drawn with >12 h difference between the first and the last set. Antimicrobial drug treatment for endocarditis with prosthetic material, consisting of vancomycin, gentamicin, and rifampin, was initiated.

Blood cultures remained negative for microbial growth, even after extended incubation. Serologic tests for agents of culture-negative endocarditis and tick-borne diseases were performed. Enzyme immunoassays (EIAs) were positive for immunoglobulin (Ig) G antibodies reactive to *Bartonella henselae* (512) and *B. quintana* (1,024), *Coxiella burnetii* (phase II IgG titer 160), *Rickettsia rickettsii/conorii* (IgG 256), and *Rickettsia typhi* (IgG 128), *Mycoplasma pneumoniae* (index 2.7). IgM was positive only for *A. phagocytophilum* (512, atypical fluorescence pattern), presenting a low titer of IgG at this stage. Serologic test results for *Brucella* spp., *Chlamydia trachomatis, Chlamydia pneumoniae,* and *Borrelia burgdorferi* were negative. Species-specific PCRs for *A. phagocytophilum, Tropheryma whipplei*, *B. henselae*, *B. quintana*, *Legionella* spp., and *L. pneumophila* were negative.

Bacterial broad-spectrum 16S rRNA gene PCR, followed by sequence analysis, identified *Candidatus* Neoehrlichia mikurensis in 4 of 8 sequential blood samples; the 4 samples that tested positive were collected before (day 0) and during the initial phase (days 7 and 13) of an effective course of antimicrobial drug therapy (Figure 1). For 16S rRNA gene amplification, DNA was extracted and ampli-

Author affiliations: University Hospital, Zurich, Switzerland (J.S. Fehr, T.F. Lüscher, R. Weber); and University of Zurich, Zurich (G.V. Bloemberg, C. Ritter, M. Hornbach, P.M. Keller)

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[1]These authors contributed equally to this article.

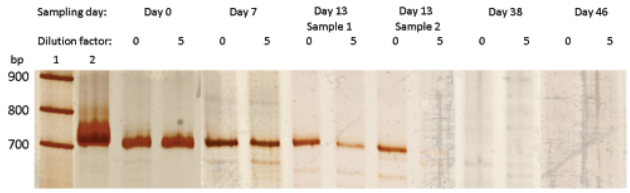

Figure 1. Polyacrylamide gel electrophoresis analysis of broad range 16S rRNA gene PCR products obtained from blood samples. Lane 1, marker, 100 bp DNA ladder (Roche DNA Marker XIV); lane 2, positive control, *Escherichia coli*; following lanes, PCR products obtained from blood specimens arranged by date of collection. For each specimen PCR products are shown obtained with undiluted (0) and 5×-diluted (5) DNA extracts. The 2 last negative samples are not shown.

fied from anticoagulated blood (4-mL EDTA tubes), uncoagulated blood from a BacT/ALERT SA aerobic blood culture flask (bioMérieux SA, Geneva, Switzerland), and coagulated blood (online Technical Appendix, www.cdc.gov/eid/content/16/7/1127-Techapp.pdf) as described (*4*). Sequences derived (GenBank accession nos. GQ501089-GQ501091) were analyzed by SmartGene IDNS software (Zug, Switzerland). We amplified and sequenced (1,150 bp; GenBank accession no. HM045824) an alternative target gene (*groEL*) with primers (online Technical Appendix) derived from published sequences (*1*), which showed 98% homology to previously published *Candidatus* Neoehrlichia mikurensis *groEL* sequences (Figure 2, panel B).

After being treated with vancomycin, gentamicin, and rifampin for 10 days, the patient became afebrile, and clinical symptoms improved. Leukocyte count was within reference range, and CRP dropped from 68 mg/L to 23 mg/L. At this time, *Candidatus* Neoehrlichia mikurensis was detected in the first blood sample. Rifampin (450 mg 2×/d) was continued, and vancomycin and gentamicin were switched to oral doxycycline (100 mg 2×/d). Three weeks later, CRP was 1 mg/L, body temperature was within the normal range, and treatment was continued to finish a 6-week course. Two weeks after the end of treatment, the patient was seen for a follow-up visit. Neither clinical nor laboratory results raised any concern of relapse. Results of broad-range PCR of the 16S rRNA gene to detect *Candidatus* Neoehrlichia mikurensis were negative for the first time in 5 weeks since treatment began initiation and remained negative at the follow-up visit 2 weeks after the end of treatment.

Candidatus Neoehrlichia mikurensis was previously found in *Rattus norvegicus* rats and *Ixodes ovatus* ticks in Japan (*1*), in *R. norvegicus* rats in China (*5*), and in *I. ricinus* ticks in the Netherlands (*6*,*7*), Slovakia (*8*), and the Asian part of Russia (9). Closely related rickettsial bacteria (Figure 2) have been identified in *Procyon lotor* raccoons in the Piedmont region of Georgia, USA (*10*). Another closely related species (*Candidatus* Ehrlichia walkeri) has been detected in *I. ricinus* ticks collected from humans in northern Italy (*11*). The geographic distribution of the tick population has also been studied (*12*).

Our patient lives in a high-risk area for ticks in Switzerland. *I. ricinus* is the main tick species in this region. A tick-borne disease appears epidemiologically possible in this patient, who is a golfer and the owner of a large garden and thus is repeatedly exposed to the habitat of the potential vector, *I. ricinus*, even though he remembered no tick bites. Of note, only 50%–70% of patients with Lyme disease remember receiving a tick bite (*13*). Blood of the patient's pet animals (dog and cat) was examined by broad-range 16S rRNA gene PCR to exclude presence of bacterial pathogens.

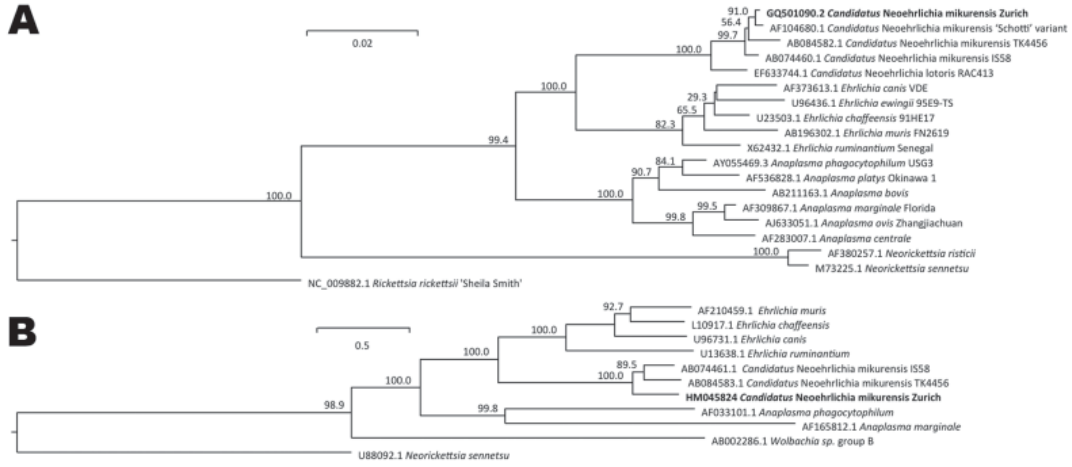

Figure 2. A) Phylogenetic tree based on the 16S rRNA gene sequences of *Candidatus* Neoehrlichia mikurensis GQ501090.2 (our patient's isolate, herein termed Zurich and indicated in **boldface**) and related organisms. The number at nodes indicates percentages of bootstrap support based on 10,000 replicates. Scale bar indicates 0.02 substitutions per nucleotide position. B) Phylogenetic tree based on the *groEL* sequences. Scale bars indicate 0.05 substitutions per nucleotide position.

In Wister rats, *Candidatus* Neoehrlichia mikurensis has been shown to infect spleen sinus endothelial cells, forming intracellular inclusions on the side of the endosome (by electron microscopy 60 days after infection) (*1*). Accordingly, we assumed that in human hosts, valvular endothelial cells are likely involved. The initial antimicrobial drug therapy, which contained rifampin, may already have contributed to the reduction of the bacterial load but was not completely effective (Figure 1). Thus, following the recommended guidelines for treatment of intracellular rickettsial bacteria with endocardial involvement, we changed to a 6-week course of treatment consisting of rifampin combined with doxycycline (*14*). After the end of the course, we observed a successful response.

We detected *Candidatus* Neoehrlichia mikurensis in 4 of 8 consecutive blood specimens, and repeated analysis showed the disappearance of the pathogen's DNA during the course of treatment (Figure 1). Laboratory diagnosis of ehrlichiosis is severely hampered because the relevant pathogens cannot be cultured on routine media. Serologic tests depend on samples collected during acute phase of illness, obtaining comparative samples in the course of the disease, and demonstrating a >4-fold increase in antibody titers.

Conclusions

We have identified *Candidatus* Neoehrlichia mikurensis in multiple blood samples of a patient who sought treatment for septicemia. Therapeutic success has been shown over time by the fact that the suggested pathogen's DNA was no longer detectable and by a favorable clinical outcome. Surveys of arthropod populations should be conducted to examine the geographic distribution of *Candidatus* Neoehrlichia mikurensis, and species-specific assays could determine the relevance of this organism in human ehrlichial diseases.

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Dr Fehr is an infectious disease specialist and attending physician at the University Hospital of Zurich. His research interests include bacterial pathogens and HIV/AIDS. He has investigated surgical site infections and emerging pathogens in Tanzania.

Dr Bloemberg is head of the Department of Molecular Diagnostics at the Institute of Medical Microbiology, University of Zurich. He is a specialist in molecular genetics and microbial biochemistry. His research interests include bacterial pathogens, biofilms, antibacterial drugs, and antibiotic resistance.

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Address for correspondence: Guido V. Bloemberg, University of Zurich, Institute of Medical Microbiology, Gloriastrasse 32, 8006 Zurich, Switzerland; email: bloemberg@imm.uzh.ch

Evolution of Seventh Cholera Pandemic and Origin of 1991 Epidemic, Latin America

Connie Lam, Sophie Octavia, Peter Reeves, Lei Wang, and Ruiting Lan

Thirty single-nucleotide polymorphisms were used to track the spread of the seventh pandemic caused by *Vibrio cholerae*. Isolates from the 1991 epidemic in Latin America shared a profile with 1970s isolates from Africa, suggesting a possible origin in Africa. Data also showed that the observed genotypes spread easily and widely.

The seventh cholera pandemic began in 1961, and by 1966, it had affected most of Asia. Cholera incidence then decreased slightly until 1971, when an upsurge was observed in Africa and Europe, which had been free of cholera for >100 years (*1*). Cholera rates remained relatively low during the 1980s, with the disease confined to Asia and Africa. However, 2 major cholera outbreaks appeared in the 1990s: first, a resurgence of cholera in Africa, and, second, outbreaks that started in Peru became the first cholera epidemic in Latin America since 1895 (*2*). In addition, a novel serotype caused major outbreaks on the Indian subcontinent in 1992. That strain was referred to as O139 Bengal and was later shown to be a variant of the seventh pandemic clone with its replacement of the O antigen (*1*). Pulsed-field gel electrophoresis (*3*), amplified fragment length polymorphism analysis (*4*), and ribotyping (*1*) have been applied to seventh pandemic isolates but did not fully resolve the relationships of the various outbreaks. In this study, we used genome-wide single-nucleotide polymorphisms (SNPs) to track the evolution and spread of the seventh cholera pandemic, including the O139 Bengal strain.

The Study

The availability of complete genome sequences of a pre–seventh pandemic isolate, M66–2 (*5*), a seventh pandemic isolate, N16961 (*6*), and the partial genome sequence of an O139 Bengal isolate, MO10 (*7*), enabled identification and use of SNPs as evolutionary markers in *Vibrio cholerae*. A set of 18 SNPs was chosen from 125 N16961 SNPs (*5*) and 12 SNPs selected from 59 identified by comparison of the N16961 and MO10 genome sequences. The SNPs selected were mostly from genes with known function and were distributed throughout the 2 chromosomes for the N16961 SNPs and the large chromosome for the MO10 SNPs. We have previously shown that recombinant regions could be identified by the differences in distribution of SNPs in such regions (*5*); for this study, only mutational SNPs were selected.

The 30 SNPs (online Technical Appendix, www.cdc.gov/EID/content/16/7/1130-Techapp.pdf) were used to type a collection of 64 seventh pandemic *V. cholerae* isolates. SNPs were detected by using hairpin primer real-time PCR. SNP data for 3 complete *V. cholerae* genomes (M66–2, N16961, MJ-1236) and 4 partially sequenced genomes (MO10, RC9, B33, CIRS 101) (*7*) were obtained from the National Center for Biotechnology Information (Rockville, MD, USA) and included in the analysis. The 71 isolates were divided into 10 SNP profiles by using the 30 SNPs (online Appendix Table, www.cdc.gov/EID/content/16/7/1130-appT.htm). Three profiles were represented by 1 isolate only, whereas the remaining profiles contained 4–17 isolates. The Simpson index of diversity for all SNPs combined was 0.929.

A maximum-parsimony tree (Figure) was constructed to show the relationships of the SNP profiles. The tree was fully resolved with no reverse or parallel changes in the seventh pandemic isolates. The pre–seventh pandemic strains were used as an outgroup and placed at the base of the tree. Six groups could be distinguished, with each group containing SNP profiles differing by no more than 1 SNP. The ladderized tree shows the stepwise evolution of the SNP profiles and groups. Group I at the bottom of the tree originated in Indonesia in 1961. It contains mostly isolates from Asia from the 1960s but continued to be isolated in Southeast Asia. The other groups evolved sequentially. Group II contains isolates from Africa from the 1970s to the 1990s and all 4 isolates from Latin America; group III contains earlier 1970s isolates from Asia and 1980s isolates from Africa; group IV contains late 1970s and 1980s isolates from Asia only; while group V contains 1990s isolates from Asia and Africa. Group VI contains only O139 isolates with the same SNP profile.

Conclusions

The presence of isolates from Africa in 3 groups can be explained by multiple introductions of cholera into Africa from cholera-endemic regions in Asia. The isolates in the first introduction in the 1970s shared a single origin (group

Author affiliations: University of New South Wales, Sydney, New South Wales, Australia (C. Lam, S. Octavia, R. Lan); University of Sydney, Sydney (P. Reeves); and Nankai University, Tianjin, People's Republic of China (L. Wang)

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II). However, during the late 1980s and early 1990s, cholera outbreaks appeared to be caused by strains from 3 related sources. The first source came from group II, which was already established in Africa, and the second and third sources came from groups III and V in Asia. Because both groups were supported by multiple SNPs, it is less likely that the 1970s isolates from Africa and Asia evolved in parallel to fall into the same groups. Additionally, B33 in Group V carries a classical CTX prophage (*8*), which indicates that this strain likely originated in Asia.

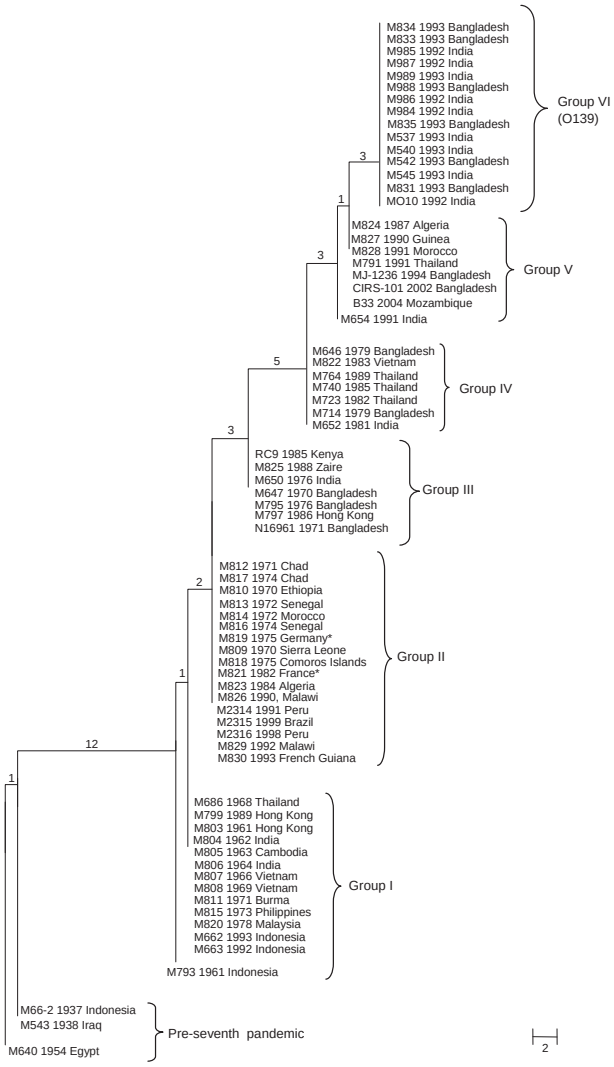

Figure. Maximum parsimony tree of 68 seventh cholera pandemic and 3 pre–seventh cholera pandemic isolates. The tree was based on 18 N16961 seventh pandemic single-nucleotide polymorphisms (SNPs) and 12 MO10 O139 SNPs. The 3 pre–seventh pandemic isolates were used as an outgroup. Each strain name is followed by the year and location of isolation. All 15 O139 isolates had the same SNP profile and are shown as group VI. The numbers on each node represent the number of supporting SNPs. M821 and M819 from France and Germany are likely imported from either Africa or Asia. SNP data for the following isolates were obtained from GenBank: accession nos. RC9, ACHX00000000; MJ-1236, CP001385/CP001486; B33, ACHZ00000000; CIRS 101, ACVA00000000; MO10, AAKF00000000; N16961, AE003852; and M66–2, CP001233. Scale bar indicates number of nucleotide substitutions.

The cholera epidemic in Latin America was originally suspected to have come from Asia and to have been facilitated by the discharge of contaminated ballast water into Peruvian ports by international trade ships (*2*). However, the isolates from Latin America analyzed in this study were closely related to isolates found in Africa in the 1970s and 1990s. Four isolates, 2 from Peru and 1 each from Brazil and French Guiana, had an SNP profile identical to the 12 isolates from Africa that originated during that period. No isolates from Asia fell into this group. This finding suggests that the strain that caused the epidemic in Latin America came from Africa rather than Asia.

The outbreak in Peru occurred in parallel with the upsurge of cholera generally in Africa (*1*) and could have been imported at that time. However, the epidemic strain may have reached Latin America well before it caused the epidemic in 1990s, given the ability of the organism to persist in the marine environment for long periods (*2*). The strain could have been brought into the region during the mass migration from Africa to Latin America in the 1970s (*9*). The isolates from Latin America differ by 1 locus from the other seventh pandemic strains (Asia and Africa) by multilocus enzyme electrophoresis (*10*) and also differ in the *Vibrio* spp. seventh pandemic island-II gene cluster (*11*), which suggests that further evolution occurred after the strain separated from its likely ancestral strain from Africa and supports this latter scenario. The epidemic strain in Latin America could not have originated from the 1990s isolates from Asia in Groups III–V because they arose later than Group II isolates. However, a 1970s lineage in Asia that spread to Africa may have remained in Asia until the 1990s but was not represented in the isolates sampled. Further investigation is needed to resolve this hypothesis. Furthermore, although the SNP profiles of the isolates from Africa and Latin America are identical, they may have diverged substantially because the SNPs used can only determine node positions but not branch length caused by phylogenetic discovery bias (*12*).

Our SNP data clearly show that O139 Bengal was a derivative of the seventh pandemic, as previously suggested (*13*). Nine of the 12 O139 SNPs can now be seen to have arisen in its O1 precursor strain because they were present in seventh pandemic isolates as early as 1979 (online Technical Appendix). These SNPs also resolved the relationships of Groups IV–V. Some studies have suggested that the O139 variant may have multiple origins (*14*). However, our results suggest that these O139 isolates from the then new epidemic have a single origin, which is consistent with earlier ribotyping data (*15*).

Our data show each of the groups/genotypes spread easily and widely to multiple countries or regions. This finding suggests that cholera epidemics or upsurges, which often occurred at the same time in many countries, were caused by the spread of newly arisen genotypes. Additionally, a genotype can also persist for long periods. Thus, in cholera-endemic regions such as Southeast Asia and Africa, cholera can be caused not only by an endemic genotype, but also by new epidemic genotypes. This finding is useful for control of cholera epidemics.

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Ms Lam is a PhD student in medical microbiology at the University of New South Wales. Her research interests include molecular epidemiology and evolution of pathogenic bacteria.

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Address for correspondence: Ruiting Lan, University of New South Wales, School of Biotechnology and Biomolecular Sciences, Sydney, New South Wales 2052, Australia; email: r.lan@unsw.edu.au

Vaccine-associated Paralytic Poliomyelitis in Immunodeficient Children, Iran, 1995–2008

Shohreh Shahmahmoodi, Setareh Mamishi, Asghar Aghamohammadi, Nessa Aghazadeh, Hamideh Tabatabaie, Mohammad Mehdi Gooya, Seyed Mohsen Zahraei, Taha Mousavi, Maryam Yousefi, Kobra Farrokhi, Masoud Mohammadpour, Mahmoud Reza Ashrafi, Rakhshandeh Nategh, and Nima Parvaneh

To determine the prevalence of vaccine-associated paralytic poliomyelitis (VAPP) in immunodeficient infants, we reviewed all documented cases caused by immunodeficiency-associated vaccine-derived polioviruses in Iran from 1995 through 2008. Changing to an inactivated polio vaccine vaccination schedule and introduction of screening of neonates for immunodeficiencies could reduce the risk for VAPP infection.

After establishment of the Global Polio Eradication Initiative in 1988, the incidence of polio worldwide decreased from ≈350,000 cases annually to 1,606 cases in 2009 (*1*). Oral polio vaccine (OPV) has been efficiently used for >40 years and is associated with few adverse events (*2*). Its most commonly recognized adverse event, vaccine-associated paralytic poliomyelitis (VAPP), is estimated by the World Health Organization to cause 1 case per million births and by Minor (*3*) to cause ≈1 case per 6.2 million doses of OPV distributed.

VAPP is clinically indistinguishable from paralytic poliomyelitis caused by wild-type polioviruses (*2*) and occurs among healthy OPV recipients and their contacts, with onset temporally linked (within 60 days) to OPV exposure. Persons with primary immunodeficiencies are at >3,000-fold higher risk for VAPP (*2*,*4*). Isolates from immunodeficient VAPP (iVAPP) patients and some asymptomatic carriers show evidence of prolonged replication as indicated by >1% nucleotide sequence divergence from the corresponding Sabin OPV strain; such vaccine-derived polioviruses (VDPVs) isolated from immunodeficient persons after exposure to OPV are called iVDPVs (*4*,*5*).

Although most mutations involved in reversion of the OPV to a wild-type strain are found in the 5′ untranslated region of the virus genome, mutations have also been found in viral protein (VP) 1, VP2, and VP3 nt sequences (*5*). The >1% demarcation arises from the average rate of VP1 nt divergence of ≈1% per year, suggestive of prolonged replication (*6*,*7*). However, poliovirus evolution rates are variable, especially in the early phases of OPV replication (*2*). Immunodeficient OPV vaccine recipients are potential reservoirs for neurovirulent polio virus reintroduction into the population (*8*). To date, >44 cases in patients with immunodeficiency have been confirmed worldwide that excreted iVDPV for long periods (*9*,*10*). Timely diagnosis and containment of VDPVs needs to be addressed in posteradication strategies in regions where OPV is still used routinely. We present all 6 documented cases of iVAPP caused by iVDPVs diagnosed in Iran during 1995–2008 (Tables 1 and 2).

The Study

Patient 1 was a 17-month-old girl. She had exhibited antibody deficiency and thus received inactivated polio vaccine (IPV). She was a household contact of a healthy OPV-vaccinated sibling. Limited data indicated that paralysis became evident in June 1995. All 3 fecal specimens collected 3–6 days after onset of paralysis yielded VDPV type 2. Recombination with the Sabin 1 strain was detected, with a crossover site at nt 5355 (3A). The girl died 8 days after onset of paralysis with obscured etiology.

Patient 2 was a boy born in January 2005. He received 4 doses of OPV, administered at birth and at 2, 4, and 6 months of age. In August 2005, he was hospitalized with irritability, drowsiness, hypotonia, and right paraparesis. Two collected fecal specimens tested were positive for VDPV type 2. Recombination with the Sabin 1 strain was also found at nt 5358. At baseline, he had mild anemia, hypogammaglobulinemia, and diminished CD4+ T-cell counts. A test result for HIV was negative. The expression of human leukocyte antigen DR on his lymphocytes was low, indicating major histocompatibility complex class II deficiency. His condition deteriorated during the next several months, with involvement of respiratory muscles and 3 episodes of aspiration pneumonia. He died of respiratory failure at 11 months of age. Follow-up fecal cultures during his illness showed persistent VDPV type 2 shedding (*11*).

Author affiliations: Tehran University of Medical Sciences, Tehran, Iran (S. Shahmahmoodi, S. Mamishi, A. Aghamohammadi, N. Aghazadeh, H. Tabatabaie, M. Yousefi, K. Farrokhi, M. Mohammadpour, R. Nategh, N. Parvaneh); Ministry of Health Center for Disease Control and Management, Tehran (M.M. Gooya, S.M. Zahraei, T. Mousavi); and Pediatrics Center of Excellence, Tehran (S. Mamishi, A. Aghamohammadi, M. Mohammadpour, M.R. Ashrafi, N. Parvaneh)

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Table 1. Age at time of paralysis onset, vaccination history, and characterization of isolated polioviruses, for patients with vaccine-associated paralytic poliomyelitis, Iran, 1995–2008*

Patient no.	Age, mo/sex at VAPP onset	OPV, no. doses	Time intervals			Poliovirus type	Viral protein 1 nt divergence,† %
			Last OPV and VAPP onset	Virus shedding from VAPP onset	VAPP onset and death		
1	17/F	0‡	0	1.2 mo	8 d	iVDPV type 2	2.2
2	7/M	4	1.1 mo	3 mo	4 mo	iVDPV type 2	1.1–1.5
3	10/M	4	3.3 mo	2 wk	1 mo	iVDPV type 2	1.7
4	15/M	4	9 mo	5 mo	11 mo	iVDPV type 3	2
5	5/F	2	3.2 mo	5 d	1 mo	iVDPV type 2, iVDPV type 1	Type 2: 1.7–2; type 1: 1.7
6	20/M	4	1.1 mo	3 d	NA§	iVDPV type 2	1.2

*VAPP, vaccine-associated paralytic poliomyelitis; OPV, oral polio vaccine; iVDPV, immunodeficiency-associated vaccine-derived polioviruses.
†From the prototype Sabin strain.
‡Inactivated polio vaccine was administered. Contact case-patient of a healthy OPV-vaccinated sibling.
§Alive to date, has residual paralysis.

Patient 3 was a boy born in January 2006. Beginning at 2 months of age, he had chronic diarrhea, malabsorption, and failure to thrive. Recurrent episodes of pneumonia also developed, beginning when the boy was 4 months of age. OPV was administered at birth and at 2, 4, and 6 months of age. In October 2006, he was referred to hospital showing symptoms of acute paralysis of the left leg of 2 weeks' duration, followed by involvement of his right leg and upper arms, accompanied by drowsiness, fever, and hypotonia. Laboratory results showed lymphopenia; anemia; decreased levels of immunoglobulin (Ig) G, IgA, and IgM; and diminished CD3+, CD4+, and CD8+ T-cell counts (Table 2). VDPV type 2 was isolated from both of his collected fecal specimens. The final diagnosis was severe combined immunodeficiency (SCID) caused by RAG2 mutation (R229W) (N. Parvaneh, unpub. data). The boy died <3 months after onset of paralysis after gram-negative sepsis in January 2007.

Patient 4 was a 15-month-old boy who had fever and weakness of the lower limbs in December 2006. He received 4 doses of OPV, administered at birth and at 2, 4, and 6 months of age. At admission to the hospital, his right leg was completely flaccid, and the left was paretic. VDPV type 3 was isolated from his feces. Recombination with the Sabin 1 strain was detected at the 3Dpol region of the genome. Immunologic workup showed hypogammaglobulinemia and diminished CD19+ B lymphocytes. The final diagnosis was X-linked agammaglobulinemia. The patient was treated with intravenous Ig and physical therapy. Follow-up fecal cultures showed no virus. He died 11 months after onset of paralysis with chronic respiratory insufficiency (*12*,*13*).

Patient 5, a girl born in September 2006, was the third child of healthy parents. She received OPV at birth and in November 2006. In February 2007, she was hospitalized with severe pneumonia and paraparesis. Two fecal specimens collected on days 3 and 5 after onset of paralysis were positive for VDPV types 1 and 2. B cell–negative T cell–negative SCID was diagnosed (Table 2); the girl died of severe sepsis and multiple organ failure in April 2007, 1 month after onset of VAPP.

Patient 6, a boy 2 years of age, had weakness in his right leg. At 7 months of age, progressive paralysis of the extremity developed after a febrile illness. His first fecal specimen was positive for the Sabin 2 strain. He subsequently experienced several episodes of pneumonia and upper respiratory infections necessitating hospitalization. Immunologic workup favored a diagnosis of X-linked agammaglobulinemia (Table 2). Electrodiagnostic studies of the affected limb indicated femoral nerve mononeuropathy. One of 2 additional fecal specimens collected was positive for VDPV type 2. The boy began intravenous Ig

Table 2. Underlying primary immunodeficiency and immunologic findings for patients with vaccine-associated paralytic poliomyelitis, Iran, 1995–2008*

Patient no.	Underlying immunodeficiency	Leukocytes, cells/μL	ALC, cells/μL	CD3,† cells/μL	CD4,† cells/μL	CD8,† cells/μL	CD19,† cells/μL	IgG,‡ mg/dL	IgM,‡ mg/dL	IgA,‡ mg/dL
1	Undefined hypogammaglobulinemia	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	MHC class II deficiency	6,300	3,642	1,216	608	607	1,460	200	<10	<10
3	SCID	1,700	731	138	96	32	10	45	<10	<10
4	XLA	6,500	3,375	2,700	1,404	1,290	35	556	<10	<10
5	SCID	6,800	2,589	336	184	185	160	<10	<10	<10
6	XLA	8,500	4,000	2,760	1,920	835	40	20	58	25

*ALC, absolute lymphocyte count; Ig, immunoglobulin; NA, not available; MHC, major histocompatibility complex; SCID, severe combined immunodeficiency; XLA, X-linked agammaglobulinemia.
†Reference ranges for lymphocyte subpopulations: CD3, 1,900–5,900; CD4, 1,400–4,300; CD8, 500–1,700; CD19, 610–2,600 cells/μL.
‡Reference ranges for immunoglobulins: IgG, 661 ± 219; IgM, 54 ± 23; IgA, 37 ± 18.

substitution (600 mg/kg every 4 weeks, continuing to date). Follow-up fecal samples became negative for polioviruses. His immunodeficiency is under control, and he has only residual paralysis of the right leg.

Conclusions

Although the Sabin 3 strain is associated with the highest rates of VAPP, probably because of low genetic stability, it is rarely associated with formation of VDPV and rarely seen in iVAPP (*2*). Our findings were similar, with iVDPV type 2 being the most common serotype (detected in 5 patients).

In our series, the median interval between administration of the last OPV dose and iVAPP onset was 3.1 months. Khesturiani et al. found a median interval of 2.3 months (*8*). In addition, the median interval between last OPV and onset of VAPP in the 23 iVDPV excretors reported during 1962–2004 was 0.6 years (*2*). Immune deficiency was diagnosed after onset of iVAPP in 5 of our patients. The exception is notable because it illustrates one of the few iVAPP cases in which immunodeficiency was diagnosed before paralytic manifestations (*14*). OPV is routinely administered at birth, when most primary immunodeficiencies are hardly identifiable (*12*). Introduction of neonatal screening programs for some immunodeficiencies such as SCID could help prevent inadvertent exposure of such patients to OPV.

The median interval between last OPV dose and last positive sample among the patients in our series was 3.5 months (range 1.5–14 months). Khesturiani et al. described an interval of 8.8 months–7.8 years (*8*). All the contact samples obtained for our cases were negative, implying an acceptable coverage of OPV in Iran.

Poliovirus accumulates mutations in VP1 region at ≈1% per year (*6*,*7*). However, our experience shows a more rapid evolution (Table 1), assuming that the virus has replicated in the patients' gastrointestinal systems from birth. However, changes in VP1 synonymous third-base codons are more constant during virus evolution and are more reliable indicators of poliovirus replicative age (*4*).

Although the risk for further transmission of iVDPV is relatively low, potential risk for circulation of iVDPV strains always remains. One episode of iVDPV spread (in an Amish population with low coverage of OPV) has been documented (*15*). Because elimination of iVDPV before cessation of OPV use seems impossible, changing to an IPV schedule seems mandatory for global poliovirus eradication.

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Dr Shahmahmoodi is an assistant professor in the Virology Division, School of Public Health, Tehran University of Medical Sciences; and technical supervisor/responsible officer of Iran's National Polio Laboratory. Her research interests include polio viruses and nonpolio enteroviruses.

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Address for correspondence: Nima Parvaneh, Department of Pediatrics, Tehran University of Medical Sciences, No. 62 Gharib St, 14194 Tehran, Iran; email: nparvaneh@tums.ac.ir

Dogs as Sentinels for Human Infection with Japanese Encephalitis Virus

Hiroshi Shimoda, Yoshito Ohno, Masami Mochizuki, Hiroyuki Iwata, Masaru Okuda, and Ken Maeda

Because serosurveys of Japanese encephalitis virus (JEV) among wild animals and pigs may not accurately reflect risk for humans in urban/residential areas, we examined seroprevalence among dogs and cats. We found that JEV-infected mosquitoes have spread throughout Japan and that dogs, but not cats, might be good sentinels for monitoring JEV infection in urban/residential areas.

Japanese encephalitis virus (JEV), a common cause of serious acute encephalitis in humans, is primarily transmitted by *Culex tritaeniorhynchus* mosquitoes and is widely endemic to Southeast Asia and the Western Pacific region (*1*). Annual incidence of Japanese encephalitis (JE) is ≈50,000 cases,with 10,000 deaths (*2*). In Japan during the 1950s, several thousand JE cases occurred each year. However, as a result of a JEV vaccination program, isolation of pig farms, and reduction of mosquitoes, the number of JE cases in Japan has decreased markedly, to <10 cases per year since 1992 (*3*). In 2005, the strong recommendation for JE vaccination was halted because of a severe vaccine-associated side effect in 1 person; however, since 2009, a newly developed JE vaccine has been available and vaccination has been resumed.

Annual serosurveys for JEV antibodies in pigs, the main amplifiers of JEV, tend to show high seropositivity in western Japan (*3*). Our previous study of JEV in wild animals in the Kinki district also showed high seroprevalence: 83% among wild boars and 59% among raccoons (*4*). These data indicate that JEV remains endemic to Japan.

However, serosurveys of wild animals and pigs may not accurately reflect risk for humans because these animals remain separate from human populations and thus may not indicate the prevalence of JEV in urban/residential areas of Japan. Therefore, additional monitoring of the risk for JEV infection in humans in these areas, in addition to annual surveillance of pigs, is needed. To determine seroprevalence in family-owned dogs and cats, which share living space with humans, we conducted serosurveys of JEV in these species.

Author affiliations: Yamaguchi University, Yoshida, Yamaguchi, Japan (H. Shimoda, Y. Ohno, H. Iwata, M. Okuda, K. Maeda); and Kyoritsu Seiyaku Corporation, Kudankita, Chiyoda-ku, Tokyo, Japan (M. Mochizuki)

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The Study

First, to examine whether dogs and cats were infected with JEV, we analyzed serum samples from 100 dogs and 292 cats in Yamaguchi Prefecture, which is in the western part of Honshu, Japan. Dog samples were collected during 2006–2007, and cat samples were collected during 1997–1999 and 2004–2005. An 80% plaque-reduction neutralizing test using virus JEV/sw/Chiba/88/2002 was performed as described (*4*). Virus JEV/sw/Chiba/88/2002 is genetically classified as genotype I (*5*). To analyze the results statistically, we performed χ^2 and Fisher exact probability tests. The significance level was $p<0.05$.

Results showed that 17% of dogs and 1% of cats were seropositive for JEV; thus, seropositivity was ≈10-fold higher among dogs than among cats (Table 1). In addition, outdoor-only dogs (38%) were 3.7-fold more likely to be seropositive than were indoor-only dogs (10%) ($p<0.05$). Antibody prevalence did not differ significantly between male (14%) and female (20%) dogs ($p>0.05$; data not shown).

Next, serum samples from 652 dogs in every district in Japan during 2006–2007 were examined for seroprevalence of JEV. The results showed that 25% of dogs had virus-neutralizing antibodies against JEV. In northern Japan, 0% and 9% of dogs from the Hokkaido and Tohoku districts, respectively, were seropositive; these levels were significantly lower than those for other districts ($p<0.05$). In contrast, in southern Japan, 61% and 47% of dogs in the Shikoku and Kyushu districts, respectively, were seropositive; these levels were significantly higher than those for other districts ($p<0.05$). Seropositivity to JEV in the Kanto (17%), Chubu (18%), Kinki (23%), and Chugoku (26%) districts showed no significant differences ($p>0.05$) (Figure). In addition, 45% of outdoor-only dogs and 8% of indoor-only dogs were seropositive for JEV, thus confirming that outdoor-only dogs were 5.5-fold more likely than indoor-only dogs to be seropositive ($p<0.05$) (Table 2). Regarding the areas of residence, 21% of dogs in urban/residential areas and 43% of dogs in rural areas were seropositive; the results for rural areas were significantly higher than those for urban/residential areas (Table 2). No significant correlation was found between ages of dogs and JEV seropositivity (data not shown).

Discussion and Conclusions

Our findings of significantly higher JEV seropositivity among dogs than cats are similar to those found in a 1954–

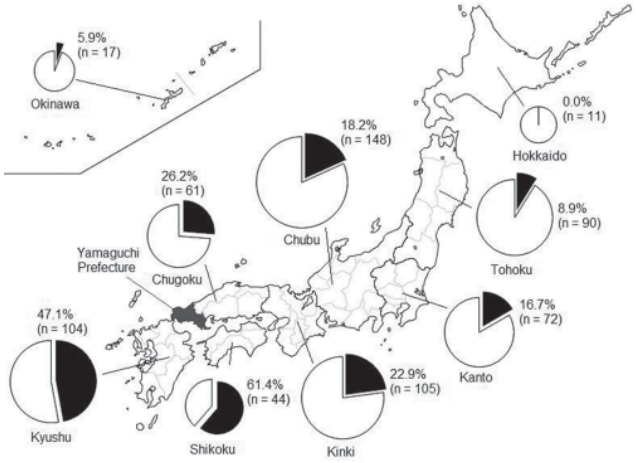

Figure. Seropositivity for Japanese encephalitis virus among dogs in 9 districts of Japan, 2006–2007. Numbers in parenthesis indicate number of dogs tested. The size of each circle indicates the number of samples. Black pie chart segments indicate the proportion of seropositive dogs; white segments indicate proportion of seronegative dogs.

1955 study in which 55% of dogs and 10% of cats were seropositive for JEV (*6*). Studies of another mosquito-vectored virus found 26% of dogs and 9% of cats in Louisiana and 5% of dogs and no cats in New York to be seropositive for West Nile virus (WNV) (*7*,*8*). Previous reports on host feeding patterns of JEV and WNV vectors showed that although *Culex* spp. mosquitoes feed on various mammals, including dogs, cats, and humans, they tend to feed more on dogs than on cats or humans (*9*,*10*). These reports are consistent with our finding that seropositivity was higher among dogs than among cats and humans and indicate that some JEV vectors do occasionally feed on humans.

Our nationwide serosurvey indicated that JEV prevalence was significantly lower in the Hokkaido and Tohoku districts and significantly higher in the Kyushu and Shikoku districts (Figure). Annual serosurveys of pigs have also shown that JEV seropositivity rates are higher for pigs in western than in northern Japan. In addition, during 2005–2007 in Japan, 24 JE cases in humans were reported, most of which occurred in western Japan (*11*). This finding is consistent with our data, suggesting that serosurveys in dogs accurately reflect JEV infection risk for humans in Japan.

Our finding that 45% of outdoor-only dogs were seropositive for JEV is similar to the finding of a previous study, conducted during a WNV epidemic among humans, that 69% of outdoor-only dogs were seropositive for WNV (*7*). A serosurvey in the Kanto district of Tokyo during 1954–1955 showed that 49% of stray dogs were seropositive for JEV (*6*). Results of these studies are similar to our results, indicating that risk for JEV infection remains high

Table 1. Seroprevalence of Japanese encephalitis virus among dogs (2006–2007) and cats (1997–2005), Yamaguchi, Japan*

Animals	No. examined	No. (%) positive
Dogs		
Indoor	58	6 (10)
Outdoor	21	8 (38)
Both or unknown	21	3 (14)
Total	100	17 (17)
Cats	292	3 (1)

*Housing information obtained by questionnaire.

in Japan, particularly in the western part. In addition, confirmation of seropositivity among indoor-only dogs (8%) indicates that JEV-infected mosquitoes may enter houses; thus, infants and elderly persons, who tend to go outside less frequently, might also be at risk for JEV infection.

That seropositivity in rural areas (43%) was significantly higher than that in urban/residential areas (21%) suggests that pig farms and rice paddies in rural areas are associated with JEV. However, the relatively high seropositivity in urban/residential areas suggests that JEV infection risk for humans remains high, even in areas with few pig farms and rice paddies. Because pigs are housed away from humans, serosurveys of pigs in urban/residential areas are limited. Therefore, dogs, which are found in all areas of Japan, may be better sentinels for JEV infection in these areas. However, information such as whether dogs become viremic after JEV infection or how long anti-JEV antibodies last in them remains unclear.

In conclusion, using dogs as sentinels indicated that the risk for human infection with JEV remains high, even in urban/residential areas. Therefore, to assess the continuing risk for JEV infection in humans in urban/residential areas of Japan, we recommend JEV surveillance among pigs every year and surveillance among dogs every several years.

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Table 2. Seroprevalence of Japanese encephalitis virus among dogs throughout Japan, 2006–2007*

Location	No. examined	No. (%) positive
Where dog stays		
Indoors	222	18 (8)
Outdoors	234	105 (45)
Both or unknown	196	41 (21)
Type of area		
Urban/residential	405	86 (21)
Rural	152	65 (43)
Unknown	95	13 (14)

*Housing information obtained by questionnaire.

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Mr Shimoda is a student in the Department of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan. His research interest is zoonoses.

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Address for correspondence: Ken Maeda, Laboratory of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan; email: kmaeda@yamaguchi-u.ac.jp

Rickettsia felis–associated Uneruptive Fever, Senegal

Cristina Socolovschi, Oleg Mediannikov, Cheikh Sokhna, Adama Tall, Georges Diatta, Hubert Bassene, Jean-François Trape, and Didier Raoult

During November 2008–July 2009, we investigated the origin of unknown fever in Senegalese patients with a negative malaria test result, focusing on potential rickettsial infection. Using molecular tools, we found evidence for *Rickettsia felis*–associated illness in the initial days of infection in febrile Senegalese patients without malaria.

Flea-borne spotted fever is widely distributed throughout the world (*1*,*2*). The causative agent is *Rickettsia felis,* an obligate intracellular bacterium (*2*,*3*). Usually *R. felis* infection causes mild to moderate disease characterized by fever, cutaneous rash (sometimes with an inoculation eschar), and neurologic and digestive signs (*1*–*3*). The pathogen has been detected in numerous arthropods, but the main reservoirs are *Ctenocephalides* spp. fleas, which are ectoparasites of domestic cats and dogs (*2*). Mammals that carry fleas around humans contribute to accidental infection of humans with *R. felis* after flea bites (*2*,*4*).

R. felis infection is generally diagnosed on the basis of both serologic assays and bacterial DNA detection by PCR (*2*–*4*). In Africa, human *R. felis* infections have been reported in Tunisia (*5*) but not in Senegal. One case of murine typhus, induced by *R. typhi,* was reported in Spain in an immigrant from Senegal (*6*). Recently, the high incidence of rickettsial diseases was noted in international travelers from sub-Saharan Africa (*7*). However, because rickettsiosis often is misdiagnosed, in the incidence of arthropod-borne spotted fever in humans in Africa may be underestimated (*4*).

The objective of our study was to investigate the origin of unknown fever in Senegalese patients who had a negative test result for malaria. We focused on potential *Rickettsia* spp. infection as a cause of fever.

Author affiliations: Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Marseille, France (C. Socolovschi, O. Mediannikov, D. Raoult); Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Dakar, Senegal (O. Mediannikov, C. Sokhna, G. Diatta, H. Bassene, J.-F. Trape); and Institut Pasteur de Dakar, Dakar (A. Tall)

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The Study

We conducted interviews and sampling during November 2008–July 2009 in 2 rural Senegalese villages in the Sine-Saloum region: Dielmo (13°43′N, 16°24′W) and Ndiop (13°41′N, 16°23′W) (*8*). The villages were included in a longitudinal prospective study, initiated in 1990, for investigations of host–parasite relationships (*8*,*9*). At the beginning of the study, all participants, including parents or legal guardians of all children, gave written individual informed consent. The national ethics committee of Senegal and the local ethics committee of Mediterranean University, Marseille, France, approved this project.

Medical examination and blood sampling were conducted for each person who had a fever >37.5°C. Approximately 200 μL (3–4 drops) of whole blood was collected from each patient by lancet stick of a fingertip for malaria tests and DNA extraction. Our study used only samples negative for *Plasmodium* spp. The first step of DNA extraction was conducted in the village dispensary by using the QIAamp kit (QIAGEN, Hilden, Germany). Binding and washing of samples with QIAGEN columns was performed with an adapted manual pump (Fisher Scientific Inc, Strasbourg, France). Columns were stored at 4°C until final elution was performed in Marseille, France.

We screened 204 samples from 134 patients by quantitative real-time PCR (qPCR) for all spotted fever group rickettsiae with *Rickettsia*-specific *gltA* gene–based RKND03 system. Positive results were confirmed by a newly designed real-time PCR primer and probe combination based on the RC0338 gene (Table 1). Appropriate handling and DNA extraction are controlled by qPCR of the β-actin gene (Table 1). We analyzed data using Epi Info software, version 3.4.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

A total of 103 patients were from Dielmo (391 inhabitants), and 31 were from Ndiop (313 inhabitants). Seventy-two patients were female, and 90 (67%) were >10 years of age. No one died during the study, and all patients with identified rickettsiae infection completely recovered.

We identified 9 samples from 8 patients (6%) positive by both genus-specific qPCR systems (Table 1). The following sequencing of nested PCR *gltA* gene amplicons from all positive samples showed 100% homology with *R. felis* URRWXCal2 (GenBank accession no. CP000053) (*10*). Furthermore, all positive samples were confirmed by *R. felis* species–specific qPCR. One girl 6 years age had 2 *R. felis*–positive blood samples 1.5 months apart. No samples were positive for typhus group rickettsiae, and 1 was was positive for *R. conorii* by sequencing of amplicons (data not shown).

The prevalence of flea-borne spotted fever in all tested samples was 4.4% (9/204). Monthly incidence for positive samples was 4.76% (1/21) in December, 4.76 % (2/42) in

Table 1. Target sequences, primers and probes used in a study of *Rickettsia felis*, rural Senegal, November 2008–July 2009

Quantitative real-time PCR designation and specificity	Target gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Probe
RKND03 system; *Rickettsia* genus-specific	*glt*A	GTG-AAT-GAA-AGA-TTA-CAC-TAT-TTA-T	GTA-TCT-TAG-CAA-TCA-TTC-TAA-TAG-C	6-FAM-CTA-TTA-TGC-TTG-CGG-CTG-TCG-GTT-C-TAMRA
1029 system; *Rickettsia* genus-specific	Hypothetical protein (RC0338 gene)	GAM-AAA-TGA-ATT-ATA-TAC-GCC-GCA-AA	ATT-ATT-KCC-AAA-TAT-TCG-TCC-TGT-AC	6-FAM-CTC-AAG-ATA-AGT-ATG-AGT-TAA-ATG-TAA-A-TAMRA
Rpr 331 system; specific for typhus group rickettsiae	Glycosyltransferase	TGC-TTC-ATG-GGC-AAT-GTC-TG	TTG-AGC-ATA-AAA-CTG-CCC-TGC-T	6-FAM-CGC-TGG-ATT-ATC-AAA-AGA-ATT-AGC-ACG-TAMRA
Specific for *R. felis*	Biotin synthase	ATG-TTC-GGG-CTT-CCG-GTA-TG	CCG-ATT-CAG-CAG-GTT-CTT-CAA	6-FAM- GCT-GCG-GCG-GTA-TTT-TAG-GAA-TGG-G-TAMRA
β-actin; specific for human β-actin gene	Human β-actin	CAT-GCC-ATC-CTG-CGT-CTG-GA	CCG-TGG-CCA-TCT-CTT-GCT-CG	6-FAM-CGG-GAA-ATC-GTG-CGT-GAC-ATT-AAG-TAMRA

January, 16.66% (3/18) in April, 6.89% (2/29) in June, and 2.38% (1/42) in July. Seven patients lived in Dielmo, and 1 lived in in Ndiop. The overall incidence was 1.7% in Dielmo and 0.3% in Ndiop (7/391 vs. 1/313; p = 0.06), and for children <10 years of age 3.5% (5/143) in Dielmo and 0.9% (1/109) in Ndiop. The incidence of flea-borne spotted fever was highest among children <10 years of age (6/252 vs. 2/452; p = 0.02). The average age of infected patients was 15 years (range 2–57 years). Clinical manifestations are detailed in Table 2. No rashes and no eschars were found during examination.

Conclusions

Our study provides molecular evidence for *R. felis* infection in West Africa in the initial days of infection in febrile Senegalese patients who did not have malaria. This infection can be easily misdiagnosed because it lacks specific signs (*2*,*3*). We developed a 2-step DNA extraction protocol from collected whole blood. The first step, directly performed in the rural villages far from standard laboratory facilities, improved sample storage and limited contamination. Accordingly, this eliminated the need to either mount a complete DNA extraction laboratory in the field or to transport fragile samples, such as human blood, from a remote site. This method can be used for research of other infectious diseases in rural area and in other developing countries.

The major clinical signs and symptoms in our study were fever associated with weakness, headache with sleep disorders, and digestive and respiratory signs; we also noted a lack of cutaneous rash or inoculation eschar (*2*–*5*). Another rickettsial study in an indigenous African population reported that cutaneous rash might be imperceptible in patients with pigmented skin (*11*).

Interestingly, a 6-year-old child had 2 positive blood samples for *R. felis* infection; the samples were taken at 1.5 month intervals, which raises the question of potential reinfection, chronic bacteremia, or relapse. Unfortunately, the girl was not treated with antimicrobial drugs between these episodes because the samples arrived together at the laboratory for molecular diagnosis. Relapses have been described for other rickettsial diseases such as epidemic typhus with late relapse (Brill-Zinsser disease) (*12*) and scrub typhus with early relapse (*13*). Our study identified a higher attack rate of flea-borne spotted fever in children <10 years of age with an attack rate of 3.5% in Dielmo during a 9-month period. Some reports of rickettsial diseases in sub-Saharan Africa indicate more infection in

Table 2. Clinical signs and symptoms in and epidemiologic data for patients with flea-borne spotted fever, rural Senegal, November 2008–July 2009*

Village	Sampling date	Age, y/sex	Temperature, °C	Signs and symptoms
Dielmo	2009 Jun	57/F	38.4	Fatigue
	2009 Apr	40/F	39.5	Chills, fatigue, headache, poor appetite, thirst, pharyngitis, sleep disorders, rhinitis, urinary pain
	2009 Jun	2/F	38	Fatigue
	2009 Jan	2/M	38.1	Cough, rhinitis,
	2009 Apr	3/M	38.1	Poor appetite, cough, sleep disorders
	2009 Jul	3/M	38.5	Headache, fatigue, poor appetite, sleep disorders
	2008 Dec	6/F	39.7	Headache
	2009 Jan		38.5	Headache
Ndiop	2009 Apr	10/F	38.7	Fatigue, headache, nausea, vomiting

*Fever was part of the case definition and therefore part of the clinical picture for all patients.

younger persons in whom the disease might be mild or subclinical (*14*).

The incidence of flea-borne spotted fever was higher in Dielmo than in Ndiop. Notably, tick-borne relapsing fever, malaria, and Q fever also are more prevalent in Dielmo than in Ndiop (*8*,*9*). Reasons for the significantly different prevalence of these infectious diseases in the 2 geographically close villages remain unexplained. Our preliminary work over 9 months did not determine a seasonal variation, but we noted more cases in April. Rickettsiae, including *R. felis*, have not been reported as background organisms that may circulate undetected in the blood of humans and thus be detected by chance. Nevertheless, future work on the clarification of the role of *Rickettsia* spp. in public health will include the study of healthy controls from appropriate cohorts.

Finally, we believe that the incidence of *R. felis* infection is largely underestimated and may be responsible in Africa for many cases of uneruptive fevers of unknown origin, including those associated with respiratory, digestive, and neurologic signs. We can speculate that flea-borne spotted fever might be an important neglected public health concern not only in North Africa but also in sub-Saharan Africa. Children are particularly vulnerable to this emerging infection.

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Dr Socolovschi is an assistant professor of infectious diseases and tropical medicine at the Medical School of Marseilles, France. Her research interests focus on vector-borne infectious tropical diseases and medical entomology.

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Address for correspondence: Didier Raoult, Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE), UMR CNRS-IRD 6236-198, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille CEDEX 5, France; email: didier.raoult@gmail.com

Novel Human Parvovirus 4 Genotype 3 in Infants, Ghana

Marcus Panning,[1] Robin Kobbe, Silke Vollbach, Jan Felix Drexler, Samuel Adjei, Ohene Adjei, Christian Drosten, Jürgen May, and Anna Maria Eis-Hübinger

Human parvovirus 4 has been considered to be transmitted only parenterally. However, after novel genotype 3 of parvovirus 4 was found in 2 patients with no parenteral risks, we tested infants in Ghana. A viremia rate of 8.6% over 2 years indicates that this infection is common in children in Africa.

In 2005, a novel human parvovirus, termed parvovirus 4 (PARV4), was identified in a plasma sample from a patient with symptoms resembling those of acute HIV infection (*1*). In 2006, a related virus was discovered in plasma pools for manufacture of plasma-derived medical products; the virus was initially called PARV5 and is now called PARV4 genotype 2 (*2*). Phylogenetic analysis suggested that PARV4 formed a separate novel genus within the subfamily *Parvovirinae*.

Initial PCR analyses of blood and autopsy specimens from adults suggested that PARV4 infection was restricted to persons at risk of parenteral infection with viruses such as hepatitis C virus (HCV) or HIV (e.g., injection drug users) (*3*–*5*). A recent serologic study identified high frequencies of immunoglobulin G against PARV4 in injection drug users who were co-infected with HCV and HIV and in persons with hemophilia who had been exposed to non–virus-inactivated clotting factor concentrates (*6*). Absence of serologic reactivity in adults without parenteral risk factors supported an association between PARV4 and bloodborne transmission.

Only limited genetic diversity has been found among PARV4 sequences, particularly among genotype 1 viruses, suggesting recent emergence and spread among parenterally exposed persons in Europe and the United States (*5*). Because persons with PARV4 genotype 1 DNA in their tissues have been substantially younger than those with genotype 2, a recent shift in prevalence of the 2 genotypes has been suggested; genotype 1 currently predominates (*4*). An even more pronounced shift was found for parvovirus B19 in that nearly complete cessation of genotype 2 and replacement by genotype 1 occurred in the 1960s in western countries (*7*). Furthermore, molecular clock analysis indicated that B19 genotype 3, which is endemic to West Africa and rarely detected outside Africa, may be more ancient than genotypes 1 and 2 (*8*).

Recently, a novel PARV4 variant, termed genotype 3, was identified in tissue samples of 2 adults from Nigeria and the Democratic Republic of the Congo (*9*). Each patient had signs of AIDS but was antibody negative for HCV and had no evidence of parenteral exposure. The absence of parenteral risks raises the possibility of alternative routes of transmission that might affect the general, nonparenterally exposed, population (*9*). Proving nonparenteral transmission would suggest more widespread distribution of PARV4 in humans than previously expected and occurrence of virus in additional population subsets. We therefore studied the occurrence of PARV4 in infants in Ghana.

The Study

We analyzed 279 anonymous blood samples that had been collected during a trial of intermittent preventive malaria treatment for infants from January 2004 through September 2005 (*10*). Samples came from infants from 9 villages in the rural Afigya Sekyere district, Ashanti region, Ghana, where estimated prevalences of HIV-1 and HCV in adults were <3% (*10*) and 2.5% (*11*), respectively. For storage under tropical conditions, an equal volume of whole blood in EDTA was supplemented with buffer AS1 (QIAGEN, Hilden, Germany). DNA isolation was conducted on a BioRobot M48 workstation (QIAGEN), using the MagAttract M48 DNA Mini Kit (QIAGEN) as recommended (sample input volume 200 μL, elution volume 200 μL).

All samples were first tested by a previously described real-time PCR designed to detect established PARV4 genotypes 1 and 2 (*12*). When initial sequencing identified novel PARV4 genotype 3, a specific real-time PCR for this genotype was developed by using primers 5′-ACCAAGGACACCAGACAGTCTT-3′ and 5′-ACGTGTTCAGACCAAAAGGAT-3′ and probe 5′-FAM-CCAGCTCCATACCTTTCAGCAGTTGC-BHQ1-3′. All samples were retested by using a plasmid-based standard derived from the real-time PCR amplicon of sample Ghana19 for absolute quantification of genome copy numbers. The lower limit of detection of this assay was 10 plasmid

Author affiliations: Institute of Virology, Bonn, Germany (M. Panning, S. Vollbach, J.F. Drexler, C. Drosten, A.M. Eis-Hübinger); Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (R. Kobbe, J. May); Ministry of Health/Ghana Health Service, Agona, Ghana (S. Adjei); and Kumasi Centre for Collaborative Research in Tropical Medicine, Kumasi, Ghana (O. Adjei)

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[1]Current affiliation: Institute for Medical Microbiology and Hygiene, Freiburg, Germany.

copies/reaction. Procedures to prevent PCR contamination were strictly adhered to, and negative controls were used throughout.

In total, 24 (8.6%) of 279 samples were positive for PARV4 genotype 3 DNA. At the time of blood collection, no infant had signs of acute infection (fever, rash, myalgia). Positive samples were found for infants in 7 of 9 studied villages, indicating widespread prevalence. To test whether socioeconomic factors might influence prevalence, we conducted a χ^2 test. A significantly lower relative risk of acquiring PARV4 infection was found for children who had access to a kitchen (indicative of higher level of hygiene within household) and who did not live close to a river (Table).

Two age groups were randomly selected from the cohort: 1) 94 infants with a median age of 14.9 months (interquartile range 14.5–15.5 months; 47 male, 47 female) and 2) 185 infants with a median age of 24.0 months (interquartile range 23.5–24.2 months; 92 male, 93 female). Significantly more positive results were seen among infants in the older versus the younger age group (22/185 [11.9%] vs. 2/94 [2.1%], respectively; p<0.006). Viral loads in the whole study group ranged from 420 to 56,000 copies/mL of whole blood (median 3,400 copies/mL). Median viral loads did not differ significantly between the age groups (4,300 vs. 3,200 copies/mL).

A 746-nt fragment of open reading frame (ORF) 1 and a 558-nt fragment of the ORF2 gene and the noncoding region between them were sequenced. Maximal nucleotide distances from previously known PARV4 sequences were 7%–8%. To obtain highly informative sequence datasets for phylogenetic analysis, we concatenated ORF1 and 2 sequences and excluded interfragment recombination by using SimPlot and GARD analysis with the HYPHY package (*13*). We then conducted phylogenetic analysis on the concatenated fragments by using the neighbor-joining method (Figure). The novel viruses from Ghana clustered in a monophyletic clade with the Nigerian PARV4 genotype 3 prototype strain NG-OR. Within this clade, sequences 1, 3, 4, 10, 12, 13, 17, 18, 19, 20, 21, 23, and 24, sampled from the 2 neighboring villages Asamang and Kona, formed a separate monophyletic subclade, suggesting local epidemic transmission of a unique virus lineage.

Conclusions

Although infection with human parvovirus PARV4 has been considered to be restricted to adults and transmitted parenterally, we found high prevalence of PARV4 genotype 3 in blood of infants in Africa. In agreement with findings for the 2 adults with PARV4 genotype 3 infection (*9*), clinical signs in these children were not overt. PARV4 infection might thus be clinically silent, or acute infection might be followed by low-level viremia that is cleared slowly, similar to infection with B19.

Finding PARV4 genotype 3 over a 2-year period in several villages suggests common and ubiquitous prevalence. Parenteral medical treatment could be clearly ruled out as a transmission route for the viremic children >2 months of age because these children were under medical observation. It could not be ruled out for younger children; however, because such treatment is uncommon in the rural Ashanti region, it is unlikely. The possibility of virus transmission by vaccination with inadequately sterilized needles is excluded because vaccination was conducted with single-

Table. Socioeconomic risk factors associated with PARV4 genotype 3 in whole blood from infants, Ghana, January 2004–September 2005*

		PARV4 viremia until month 24		
Factor†	No. tested‡	No. (%) positive	RR (95% CI)	p value§
Kitchen available				
No	137	15 (11.0)	1	
Yes	126	5 (4.0)	0.36 (0.14–0.97)	0.033
Data lacking	16			
River close				
No	197	13 (6.6)	1	
Yes	69	10 (14.5)	2.2 (1.01–4.78)	0.045
Data lacking	13			
Water source				
Pipe	164	10 (6.1)	1	
Pump, well, or borehole	88	10 (11.4)	1.86 (0.81–4.31)	0.140
Other	14	2 (14.3)	2.34 (0.57–9.66)	0.241
Data lacking	13			

*PARV4, parvovirus 4; RR, relative risk; CI, confidence interval.
†Other socioeconomic factors recorded, all without association with PARV4 genotype 3 viremia, were occupation of mother, education of mother, occupation of father, education of father, number of pregnancies, age of mother, age of father, no. children in household, no. adults in household, house type, no. rooms in house, financial situation, knowledge of malaria protection, mosquito protection, electricity in household, availability of radio or television.
‡Socioeconomic interviews could not be conducted for 11 participants, and 5 questionnaires were incomplete.
§χ^2 test.

used syringes. Finding virus in persons without parenteral exposure overlaps with a recently raised suspicion regarding a different epidemiology of PARV4 infection in Africa as opposed to the Northern Hemisphere (*9*).

High prevalence in children in Africa contrasts with the prevalence pattern in the Northern Hemisphere, suggesting different dynamics and routes of transmission. Prenatal or perinatal transient infection can be largely ruled out because the older children had substantially higher rates of viremia. On the contrary, our analysis of socioeconomic factors identified a reciprocal association of transmission risk with access to a kitchen and distance from a river. Foodborne or smear transmission (contact with contaminated objects) are thus suspected. In conclusion, in Africa, novel PARV4 genotype 3 is prevalent among infants who are most likely not at risk for parenteral exposure.

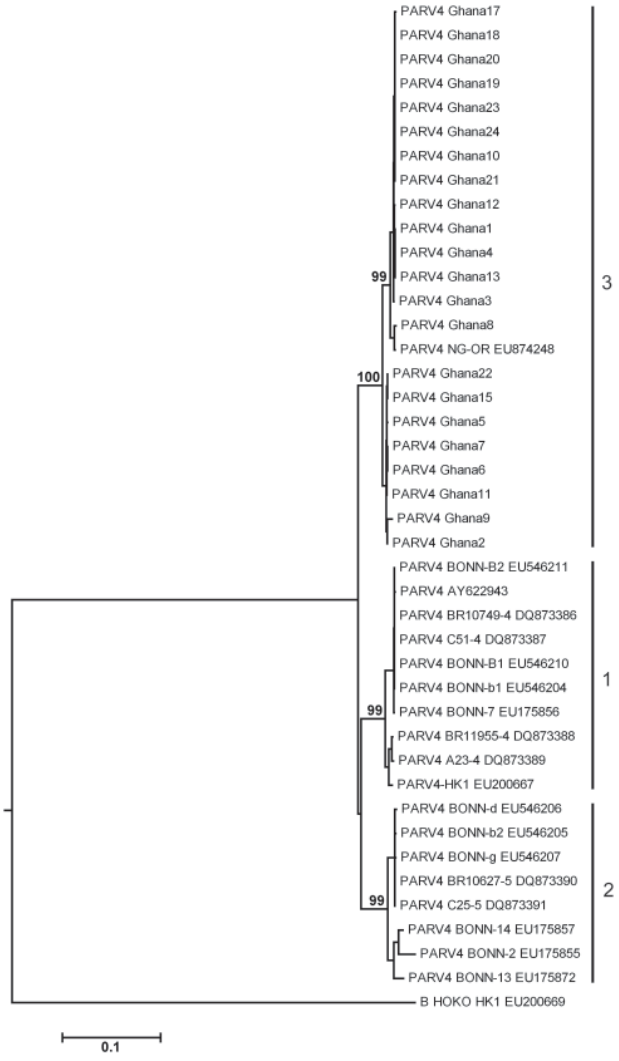

Figure. Phylogenetic analysis of human parvovirus 4 (PARV4) nucleotide sequences. The concatenated dataset of 746 open reading frame (ORF) 1 nt and 558 ORF2 nt (except for strains 14 [550 nt sequenced in ORF1] and 16 [218 nt sequenced in ORF2]) was subjected to neighbor-joining–based phylogenetic analysis with 1,000 bootstrap replicates in MEGA4 by using the Kimura substitution model and the complete deletion option for gaps (*14*). Bovine hokovirus was used as an outgroup because it is the closest relative to human PARV4. Numbers next to branches indicate bootstrap support values in percent (only selected branches are annotated). Numbers next to strain designations indicate PARV4 genotypes 1, 2, and 3. Sequences are deposited in GenBank (accession nos. GU951546–GU951569); sequencing primers available upon request from the authors. Scale bar indicates nucleotide substitutions per site.

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Dr Panning is a physician and medical virologist specializing in emerging viruses. His research interests are development of molecular diagnostic assays for patient care and virus ecology studies.

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Address for correspondence: Anna Maria Eis-Hübinger, Institute of Virology, University of Bonn Medical Centre, Sigmund-Freud-Str. 25 53127 Bonn, Germany; email: eish@virology-bonn.de

Geographic Differences in Genetic Locus Linkages for *Borrelia burgdorferi*

Bridgit Travinsky, Jonas Bunikis, and Alan G. Barbour

Borrelia burdorferi genotype in the northeastern United States is associated with Lyme borreliosis severity. Analysis of DNA sequences of the outer surface protein C gene and *rrs-rrlA* intergenic spacer from extracts of *Ixodes* spp. ticks in 3 US regions showed linkage disequilibrium between the 2 loci within a region but not consistently between regions.

Most bacterial pathogens comprise a variety of strains in various proportions. For *Borrelia burgdorferi*, an agent of Lyme borreliosis, strains differ in their reservoir host preferences (*1*), propensities to disseminate in humans (*2*,*3*), and prevalences in ticks by geographic area (*4*,*5*). Strain identification of *B. burgdorferi* now is predominantly based on DNA sequences of either of 2 genetic loci: 1) the plasmid-borne, highly polymorphic outer surface protein (*ospC*) gene, which encodes outer surface protein C (*6*,*7*), or 2) the intergenic spacer (IGS) between the *rrs* and *rrlA* rDNA, here called IGS1. Other loci for genotyping are the plasmid-borne *ospA* gene (*7*) and the rrfA-rrlB rDNA intergenic spacer, here called IGS2 (*8*). The apparent clonality of *B. burgdorferi* was justification for inferring strain identity from a single locus (*9*,*10*), but the extent of genomewide genetic exchange in this species may have been underestimated (*6*).

Given reports of an association between disease severity and *B. burgdorferi* genotype (*2*,*3*), prediction of a strain's virulence potential from its genotype has clinical, diagnostic, and epidemiologic relevance. But is a single locus sufficient for this assessment?

The Study

To investigate this issue, we determined sequences of *ospC* and IGS1 loci, and in selected cases the *ospA* and IGS2 loci, in 1,522 DNA extracts from *B. burgdorferi*–infected *Ixodes scapularis* nymphs collected from the northeastern, mid-Atlantic, and north-central United States during the summers of 2004, 2005, 2006, and 2007, as described (*4*,*11*). We also included results from 214 infected *I. pacificus* nymphs collected in Mendocino County, California (*5*); 20 infected *I. pacificus* adults from Contra Costa County, California (J. Bunikis and A.G. Barbour, unpub. data); and 10 *B. burgdorferi* genomes (strains B31, ZS7, 156a, 64b, 72a, 118a, WI91-23, 94a, 29805, and CA-11.2a), for which sequences are publicly available (www.ncbi.nlm.nih.gov). Multilocus sequence typing (MLST), based on 8 chromosomal housekeeping genes, had been carried out for several strains represented in the extracts (Table) (*4*,*12*). The corresponding MLST types of the 10 genome sequences were assigned by reference to a *B. burgdorferi* MLST database (http://borrelia.mlst.net) (*12*). For this study, we also determined the MLST type of strain CA8.

The methods for 1) DNA extraction from ticks (*11*), 2) PCR amplification of *ospC*, *ospA*, and IGS1 (*7*), 3) amplification of IGS2 (*8*), and 4) amplification of 8 chromosomal loci for MLST (*12*) have been described. Sequences for both strands were determined from either PCR products or cloned fragments with custom primers (*7*). We followed the basic nomenclature of Wang et al. (*13*) until, after exhausting the alphabet, we assigned both a letter and, arbitrarily, the number 3 (e.g., C3) when a new nucleotide sequence differed by >8% from known *ospC* alleles. We distinguished *ospC* variants with <1% sequence difference by adding a lowercase letter, e.g., Da and Db. Except for *ospC* D3 and Oa, novel polymorphisms were confirmed in at least 1 other sample. To simplify IGS1 nomenclature, we numbered types sequentially, beginning with the original 9 types (*7*); *ospA* alleles (*7*) and IGS2 loci were likewise sequentially numbered. The online Appendix Table (www.cdc.gov/EID/content/16/7/1147-appT.htm) provides accession numbers for all sequences, as well as original and revised names for IGS1 sequences.

For 741 *Ixodes* ticks from northeastern and north-central United States or from northern California, 1 *ospC* allele was identified and sequenced. In the remaining samples, we found a mixture of strains or evidence of ≥2 *ospC* and/or ≥2 IGS sequences (*9*). In 678 (91%) of the 741 samples with a single *ospC*, the allele could be matched with particular IGS1 (Table). We identified 9 unique *ospC* sequences: Fc, Ob, Ub, A3, B3, C3, D3, E3, and F3, all from the north-central United States. Alleles H3 and I3 of California were recently reported by Girard et al. (*5*). Of 32 codon-aligned *ospC* sequences, 6 pairs and 1 trio (Fa, Fb, and Fc) differed in sequence by <1% (Figure, panel A). Nine novel IGS1 sequences, numbered 24–31 and 33, were discovered in samples from which *ospC* alleles were determined.

When we confined analysis to samples from northeastern states, we confirmed linkage disequilibrium between *ospC* and IGS1 loci (*7*,*10*,*14*). However, when results from north-central states and California were included, a different picture emerged (Table, Figure, panel B). Most of the

Author affiliation: University of California, Irvine, California, USA

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ospC alleles showed concordance with the chromosomal loci; monophyletic MLST showed either the same *ospC* allele or a minor variant of it. However, in several instances, the *ospC* alleles were linked to different IGS1 sequences, different *ospA* sequences, and/or different MLST with internal nodes in common. We observed this linkage for *ospC* alleles A, G, Hb, and N. In the case of *ospC* Hb, the shared internal node was deep.

Table. Linkages between *ospC* alleles and other loci in *Borrelia burgdorferi* strains*

ospC	IGS1	Geographic region*	Representative cultured isolate or tick sample†	IGS1-*ospC* associations‡	*ospA*	IGS2	MLST§
A	1	1, 2	**B31**	45/52	1	1	1
A	11	2	*2206617*	4/4	22	1	55
A	10	3	CA4, CA6	14/18	23	1	2
Ba	3	1	**64b**, B373	39/41	3	1	7,58,59
Ba	6	2	51405UT	7/9	14	1	30
Bb	16	4	**ZS7**	–	28	–	20
C	24	1	JD1, BL515	10/10	8	5	11
Da	5	1	*516113*	13/14	5	4	38
Db	5	2	*424404*	13/15	18	7	51
Db	19	3	**CA11.2A**	16/16	27	4	70
E	9	1, 2	N40, B348	17/19	9	1	19
Fa	17	1, 2, 3	B156	61/64	3	4	8
Fb	18	2	MI407	14/19	8	6	–
Fc	18	2	*1469205*	7/8	13	6	56
G	26	1	**72a**, MR616	10/11	9	4	14
G	22	2, 3	*1468503*	9/10	21	4	48,49
Ha/Hb	12	1	B509/**156a**	13/13	2	2	4
Hb	12	2	519014UT	56/65	11	2	32
Hb	13	3	CA92-0953	20/20	23	2	6
Ia	7	1	B500, B331	12/16	7	4	15,16
Ia	7	2	**WI91-23**	5/5	11	4	71
Ib	7	3	CA92-1096	–	30	4	17
J	20	1, 2	**118a**	3/5	8	4	34
K	2	1	297	67/68	2	2	3
K	14	2	*149901*	7/10	31	2	–
L	14	2	47703UT	23/25	8	2	29
M	6	1	**29805**	4/4	2	3	12
M	6	2, 3	CA92-1337	16/16	17	3	13
N	4	1	MR661, *500203*	41/41	4	10	9,36
N	23	2	*51108*	8/10	2	1	43
Oa	27	1	*501427*	1/1	–	–	54
Ob	6	2	*2207807*	6/7	2	–	–
T	28	1	23509	16/16	8	4	37
T	29	2	1476702	10/11	20	4	46
Ua	8	1	**94a**, B485	19/19	8	4	18
Ua	8	2	*48802*	4/4	16	4	47
Ua	17	2	*2207116*	4/4	12	10	–
Ub	30	2	*426905*	3/3	8	9	–
A3	14	2	*2206613*	6/6	19	2	–
B3	23	1, 2	*2250201*	3/3	17	1	57
C3	17	2	*50202*	6/9	15	5	–
D3	31	2	*2150902*	1/1	–	–	–
E3	20	2	*2127701*	4/4	8	8	52
E3	21	3	HRT25	12/12	24	–	–
E3	5	3	LMR28	12/12	25	–	–
F3	5	2	*1456802*	8/12	8	4	–
H3	25	3	CA8	37/40	26	4	(72)
I3	17	3	CA11, CA12	5/5	27	4	–

*Regions: 1, northeastern United States; 2, north-central United States; 3, northern California; 4, western Europe; *osp*, outer surface protein; IGS, intergenic spacer; MLST, multilocus sequence typing; –, MLST not determined.
†Tick samples (*4*) are indicated by *italics*; strains with genome sequences are indicated in **boldface**.
‡Number of tick extracts with the listed IGS1 locus (numerator)/number of extracts with the listed *ospC* allele (denominator).
§MLST from (*4*,*12*) or this study (in parentheses).

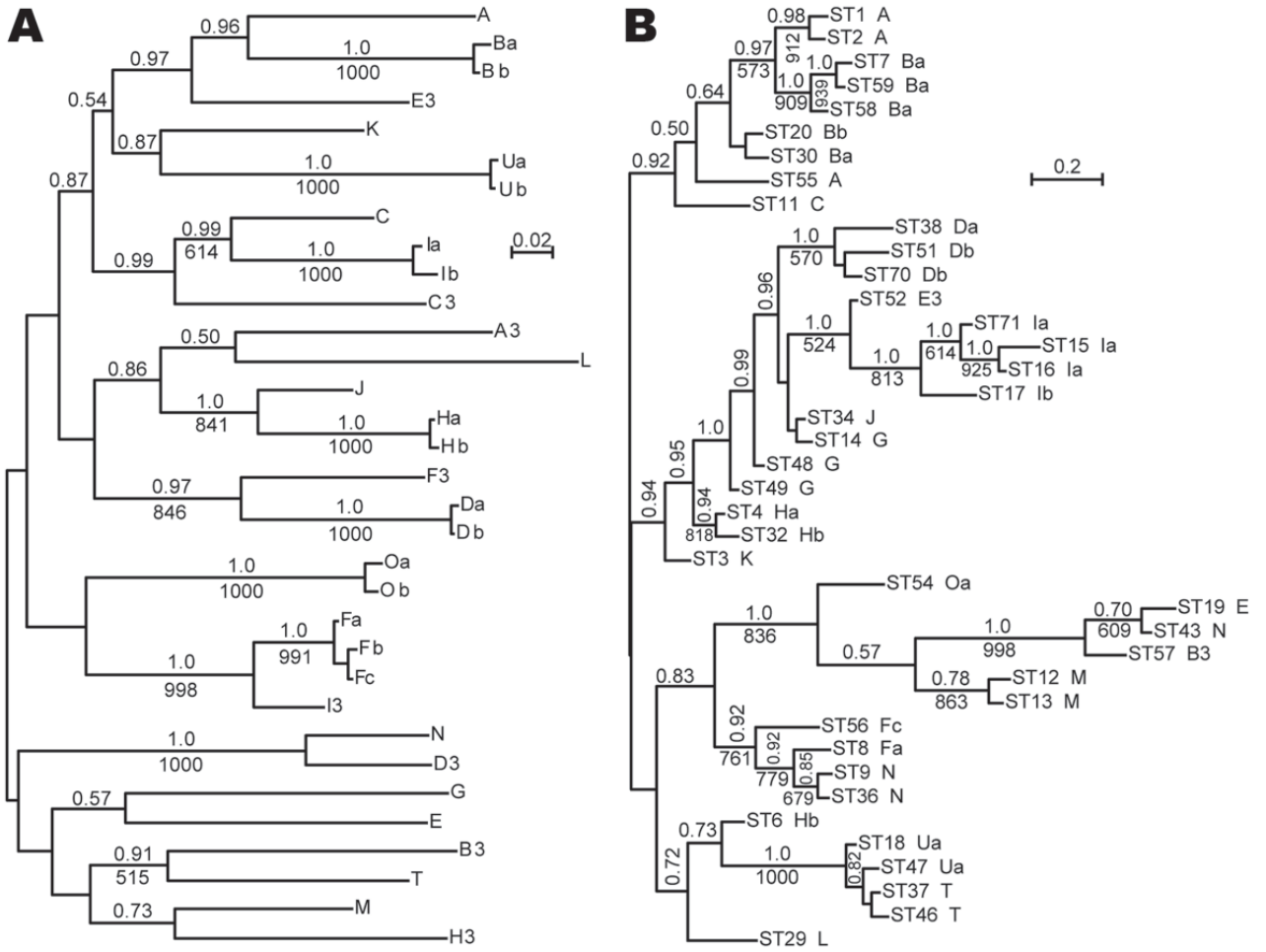

Figure. A) Bayesian and maximum-likelihood phylogenetic inference of outer surface protein C (*ospC*) gene sequences and B) concatenated multilocus sequence typing (MLST) sequences of *Borrelia burgdorferi*. Sequences were aligned by codon. Labels at the tips refer to *ospC* alleles (A) or MLST (ST) and linked *ospC* alleles (B; Table). Consensus phylograms were the output of the MrBayes version 3.1.2 algorithm (http://mrbayes.csit.fsu.edu). There were 500,000 generations with the first 1,000 discarded. Nodes with posterior probabilities of >0.5 are indicated by values above the branches. Below the branches are integer values for nodes with support of >500 of 1,000 bootstrap iterations of the maximum-likelihood method, as carried out with the PhyML 3.0 algorithm (www.atgc-montpellier.fr/phyml). For both data sets and both algorithms, the models were general time reversible with empirical estimations of the proportions of invariant sites and gamma shape parameters. Scale bars indicate genetic distance. GenBank accession numbers for sequences are given in the online Appendix Table (www.cdc.gov/EID/content/16/7/1147-appT.htm).

We applied the Simpson index of diversity, as implemented by Hunter and Gaston (*15*), to the data in the Table to compare the discriminatory power (DP) of genotyping on the basis of a combination of *ospC* and IGS1 sequences with genotyping by 8-locus MLST (*12*). For double-locus typing, there were 43 types were found for 678 strains; DP value was 0.96. For MLST in this data set, 36 types were shown for 554 strains; DP was 0.95. In the study of Hoen et al. in which selection was made for geographic isolation, 37 types were distributed among 78 strains; DP was 0.97 (*4*).

Conclusions

Dependence on a single locus for typing may falsely identify different lineages as the same, especially when the samples come from different regions. Other loci may be as informative as *ospC* or IGS1, but the abundance of extant sequences for these loci justifies their continued use. Uncertainties about the linkage of *ospC* and IGS1 usually can be resolved by sequencing the *ospA* allele (Table). IGS2 provided little additional information in this study.

One interpretation of these findings is that lateral gene transfer of all or nearly all of an *ospC* gene has occurred between different genetic lineages. We previously had not detected recombination at the IGS1 locus on the chromo-

some (*7*), but there may be recombination at other chromosomal loci, as well as plasmid loci (*6*). Besides extending the understanding of the geographic structuring of the *B. burgdorferi* population, the results indicate that the *ospC* allele does not fully represent the complexity of *B. burgdorferi* lineages; thus, inferring phenotypes on the basis of this single locus should be made with caution.

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Ms Travinsky is a senior research associate in the Department of Microbiology and Molecular Genetics, University of California, Irvine. Her research interests include the genetic diversity and phylogeography of *Borrelia* species.

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Address for correspondence: Alan G. Barbour, University of California, Irvine, 3012 Hewitt, Irvine, CA 92697-4028, USA; email: abarbour@uci.edu

Accumulation of L-type Bovine Prions in Peripheral Nerve Tissues

Yoshifumi Iwamaru, Morikazu Imamura, Yuichi Matsuura, Kentaro Masujin, Yoshihisa Shimizu, Yujing Shu, Megumi Kurachi, Kazuo Kasai, Yuichi Murayama, Shigeo Fukuda, Sadao Onoe, Ken'ichi Hagiwara, Yoshio Yamakawa, Tetsutaro Sata, Shirou Mohri, Hiroyuki Okada, and Takashi Yokoyama

We recently reported the intraspecies transmission of L-type atypical bovine spongiform encephalopathy (BSE). To clarify the peripheral pathogenesis of L-type BSE, we studied prion distribution in nerve and lymphoid tissues obtained from experimentally challenged cattle. As with classical BSE prions, L-type BSE prions accumulated in central and peripheral nerve tissues.

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disorder of cattle characterized by accumulation of a protease-resistant form of a normal cellular prion protein (PrPres) in the central nervous system. The scientific literature in general has assumed that BSE in cattle is caused by a uniform strain (classical BSE). However, different neuropathologic and molecular phenotypes of BSE (atypical BSEs) have recently been reported from various countries (*1*). Recent data from Western blot analyses of field cases of atypical BSEs are characterized by a higher (H-type BSE) or lower (L-type BSE) molecular mass of the unglycosylated form of PrPres than is classical BSE (*2*). The origins of atypical BSEs remain obscure; unlike classical BSE, atypical BSE has been detected mainly in aged cattle and suggested as a possible sporadic form of BSE (*3*).

Several lines of evidence demonstrate that classical BSE and a variant form of Creutzfeldt-Jacob disease are most likely caused by the same agent (*4*,*5*). Transmission of classical BSE to humans has been proposed to result from ingestion of contaminated food. Whether atypical BSEs are transmissible to humans remains uncertain; however, human susceptibility to L-type BSEs is suggested by recent experimental transmission in primates (*6*) and mice transgenic for human prion protein (PrP) (*7*) by using the most effective route of intracerebral inoculations of prions. The L-type BSE prion is much more virulent in primates and in humanized mice than is the classical BSE prion, which suggests the possibility of zoonotic risk associated with the L-type BSE prion. These findings emphasize the critical importance of understanding tissue distribution of L-type BSE prions in cattle because, among the current administrative measures for BSE controls, the specified risk materials removal policy plays a crucial role in consumer protection.

In Japan, atypical BSE was detected in an aged Japanese Black cow (BSE/JP24) (*8*). We recently reported the successful transmission of BSE/JP24 prions to cattle and showed that the characteristics of these prions closely resemble those of L-type BSE prions found in Italy (*9*). In this study, we report the peripheral distribution of L-type BSE prions in experimentally challenged cattle.

The Study

The Animal Ethics Committee and Animal Care and Use Committee of the National Institute of Animal Health approved the study. Five Holstein calves 2–3 months of age were intracerebrally injected with 1 mL of 10% (w/v) brain homogenates prepared from the medulla oblongata of BSE/JP24. In our earlier report, experimentally challenged cattle appeared to display clinical signs indicative of BSE at 11 months postinoculation (mpi) (*9*). Animals were sequentially euthanized before and after the onset of clinical signs (cattle identification codes 8515 and 496 at 10 and 12 mpi, respectively) and at the terminal stage of the disease (cattle identification codes 528, 1061, and 5566 at 16 mpi). A wide range of tissues was sampled at subsequent necropsy. We provisionally categorized the adrenal gland as nerve tissue because of the presence of chromaffin cells in the medulla of the gland.

Western blot analysis for PrPres was performed on obex tissue samples as described previously by using anti-PrP monoclonal antibody T2 (*9*). PrPres was detectable in all obex samples obtained 10, 12, and 16 mpi, suggesting that transmission of L-type BSE prions to these animals was successful. Dilution of the protease-treated brain sample and analysis of Western blot results showed that the detection threshold for PrPres was 1.25 μg of brain tissue equivalent (data not shown).

A variety of nerve and lymphoid tissue samples were investigated for accumulation of PrPres by Western blot analysis by using phosphotungstic acid precipitation, as described previously (*10*); examples of cattle tissue samples obtained 10 and 16 months mpi (codes 8515 and

Author affiliations: National Institute of Animal Health, Tsukuba, Ibaraki, Japan (Y. Iwamaru, M. Imamura, Y. Matsuura, K. Masujin, Y. Shimizu, Y. Shu, M. Kurachi, K. Kasai, Y. Murayama S. Mohri, H. Okada, T. Yokoyama); Hokkaido Animal Research Center, Hokkaido, Japan (S. Fukuda, S. Onoe); and National Institute of Infectious Diseases, Tokyo, Japan (K. Hagiwara, Y. Yamakawa, T. Sata)

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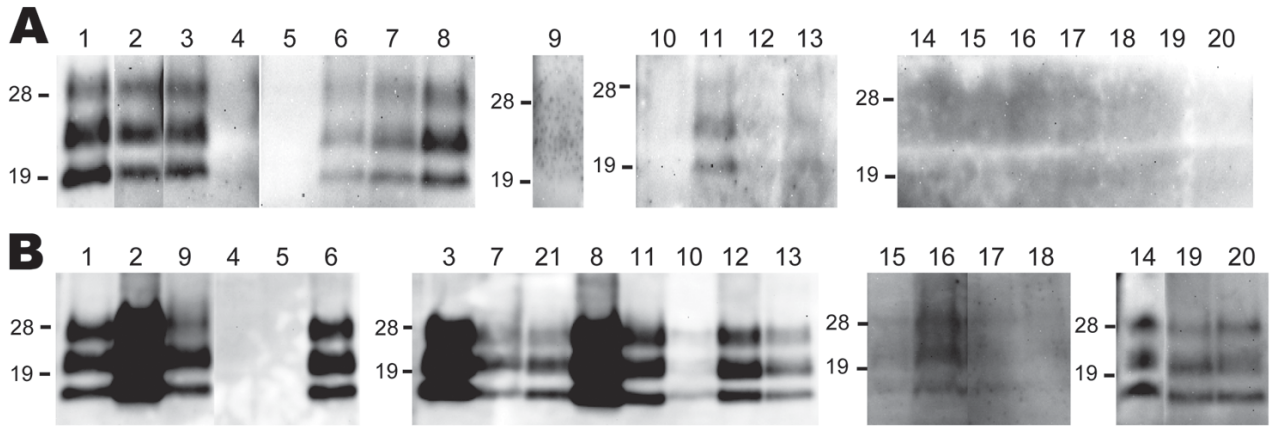

Figure 1. Western blot analysis of a protease-resistant form (PrPres) of a normal cellular prion protein in nerve tissue samples obtained from cattle 10 (A) and 16 (B) months postinoculation (cattle identification codes 8515 and 1061, respectively). The nerve tissues tested are shown above the lanes: 1, trigeminal ganglia; 2, pituitary gland; 3, anterior cervical ganglion; 4, facial nerve; 5, hypoglossal nerve; 6, cranial mesenteric ganglia; 7, vagus nerve (cervical part); 8, stellate ganglia; 9, adrenal gland; 10, phrenic nerve; 11, vagus nerve (pectoral part); 12, vagosympathic trunk (pectoral part); 13, vagosympathetic trunk (lumbar part); 14, accessory nerve; 15, suprascapular nerve; 16, brachial nerve plexus; 17, median nerve; 18, radial nerve; 19, sciatic nerve; 20, tibial nerve, 21, middle cervical ganglion. The equivalent of 100 mg of tissue was loaded. Western blots were probed with monoclonal antibody T2 to detect PrPres. Molecular mass standards (kDa) are indicated on the left of each panel.

1061, respectively) are shown in Figure 1. In cattle at the preclinical stage, PrPres was detectable in all tested ganglia and barely detectable in the vagus nerve and vagosympathic trunk. In cattle at the terminal stage, PrPres was barely detectable in the forelimb nerves (suprascapular nerve, brachial nerve plexus, median nerve, and radial nerve), whereas substantial amounts of PrPres were present in other nerve tissues except for facial and hypoglossal nerves (Table). A broader nerve tissue distribution of PrPres was observed in cattle at 16 mpi than at 10 and 12 mpi. Contrary to what we found in nerve tissues, we detected no PrPres from tests performed on lymphoid tissues obtained from any of the 5 cattle studied.

Infectivity of selected nerve tissues (including the obex, sciatic nerve, adrenal gland, brachial nerve plexus, and vagus nerve) obtained from cattle euthanized at 10, 12, and 16 mpi (codes 8515, 498, and 5566, respectively) was analyzed by intracerebral injection into mice transgenic for bovine prion protein, as described previously (*11*). As a negative control, mice were injected with cells from the brainstem of a normal cow. The presence of PrPres in the brains of all mice used in the experiment was determined by Western blot analysis. Infectivity was detected in all nerve tissues tested, regardless of the presence of detectable PrPres (Figure 2). Control mice showed no apparent abnormality >500 days postinoculation.

Conclusions

We report accumulation of L-type atypical BSE prions in peripheral nerve tissues sampled from intracerebrally challenged cattle. Our study demonstrated that almost all of the peripheral nerve tissues tested became PrPres positive in a time-dependent manner, whereas no PrPres was detectable in lymphoid tissues, even in cattle with fatal atypical BSE. Our results suggest the possibility that, like classical BSE prions, L-type BSE prions propagated in the central nervous system and were spread centrifugally by nerve pathways (*11*,*12*). In

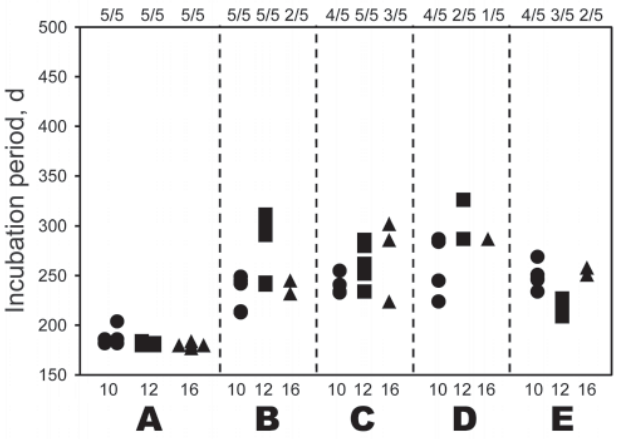

Figure 2. Bioassay using nerve tissues obtained from bovine spongiform encephalopathy JP24 prion-inoculated cattle. Inocula from selected tissues—obex (A), sciatic nerve (B), adrenal gland (C), branchial nerve plexus (D), and vagus nerve cervical part (E)—were prepared from cattle euthanized at 10 (code 8515, circle), 12 (code 498, square), and 16 (code 5566, triangle) months postinoculation were and inoculated intracerebrally into mice transgenic for bovine prion protein. Ratios above graph indicate number of prion-diseased mice/number of inoculated mice at 500 d postinoculation.

Italy, L-type BSE prions have been characterized in detail by using cattle challenged intracerebrally. However, PrPres was not detected in their peripheral tissues, including the peripheral nerves (*13*). The reason for the discrepancy in PrPres detection is unclear. In view of the similarities between the L-type and BSE/JP24 prion characteristics (*9*), this discrepancy may result from differences in the methods used for PrPres detection.

We detected infectivity in the nerve tissue samples (including samples from the obex, sciatic nerve, adrenal gland, brachial nerve plexus, and vagus nerve) obtained 10, 12, and 16 mpi. On the basis of the incubation time of 223 ± 25 (mean ± SD) days in mice injected with a 1,000-fold dilution of the obex homogenate, infectious titers in peripheral nerve tissues appeared to be 1,000 × lower than those estimated in the obex during endpoint titration of infectivity.

Our results demonstrate that L-type atypical BSE prions can be distributed in the peripheral nerve tissues of intracerebrally challenged cattle. These findings are useful for understanding L-type BSE pathogenesis and accurately assessing the risks associated with this disease. Investigations of prion distribution in cattle that have been orally challenged with L-type BSE prions are critical.

Table. Western blot detection of PrPres in tissue samples obtained from cattle intracerebrally challenged with BSE/JP24 prion*

	Cattle identification codes				
Tissue samples	8515 (10 mpi)	498 (12 mpi)	528 (16 mpi)	1061 (16 mpi)	5566 (16 mpi)
Nerve tissues					
Obex	+	+	+	+	+
Spinal cord	+	+	+	+	+
Cauda equina	+	+	+	+	+
Optic nerve	+	+	+	+	+
Pituitary gland	+	+	+	+	+
Trigeminal ganglia	+	+	+	+	+
Cranial cervical ganglia	+	+	+	+	+
Stellate ganglia	+	+	+	+	+
Vagosympathic trunk	+	+	+	+	+
Cranial mesenteric ganglia	+	+	+	+	+
Vagus nerve	+	+	+	+	+
Facial nerve	–	–	–	–	–
Hypoglossal nerve	–	–	–	–	–
Phrenic nerve	–	+	+	+	+
Accessory nerve	–	+	+	+	+
Suprascapular nerve	–	–	+	+	+
Brachial nerve plexus	–	–	+	+	+
Median nerve	–	–	+	+	–
Radial nerve	–	–	+	+	–
Sciatic nerve	–	+	+	+	+
Tibial nerve	–	+	+	+	+
Adrenal gland	–	+	+	+	+
Lymphoid tissues					
Spleen	–	–	–	–	–
Tonsil	–	–	–	–	–
Parotid lymph nodes	–	–	–	–	–
Lateral retropharyngeal lymph nodes	–	–	–	–	–
Mandibular lymph nodes	–	–	–	–	–
Brachiocephalic lymph node	–	–	–	–	–
Anterior cervical lymph node	–	–	–	–	–
Axillary lymph nodes	–	–	–	–	–
Superficial inguinal lymph nodes	–	–	–	–	–
Subiliac lymph nodes	–	–	–	–	–
Popliteal lymph nodes	–	–	–	–	–
Splenic lymph nodes	–	–	–	–	–
Hepatic lymph nodes	–	–	–	–	–
Internal iliac lymph nodes	–	–	–	–	–
External iliac lymph nodes	–	–	–	–	–
Mesenteric lymph nodes	–	–	–	–	–

*PrPres, prion protein resistant; BSE, bovine spongiform encephalopathy; mpi, months postinoculation; +, positive for PrPres; –, negative for PrPres.

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Dr Iwamaru is a veterinarian and senior researcher with the Prion Disease Research Center, National Institute of Animal Health. His research focuses on the molecular mechanisms underlying neurodegenaration in prion diseases.

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Address for correspondence: Yoshifumi Iwamaru, Prion Disease Research Center, National Institute of Animal Health, Kannondai 3-1-5, Tsukuba, Ibaraki 305-0856, Japan; email: gan@affrc.go.jp

Cryptococcus gattii Genotype VGIIa Infection in Man, Japan, 2007

Koh Okamoto, Shuji Hatakeyama, Satoru Itoyama, Yoko Nukui, Yusuke Yoshino, Takatoshi Kitazawa, Hiroshi Yotsuyanagi, Reiko Ikeda, Takashi Sugita, and Kazuhiko Koike

We report a patient in Japan infected with *Cryptococcus gattii* genotype VGIIa who had no recent history of travel to disease-endemic areas. This strain was identical to the Vancouver Island outbreak strain R265. Our results suggest that this virulent strain has spread to regions outside North America.

Cryptococcus neoformans and *C. gattii* are closely related species of yeast; *C. gattii* was previously classified as *C. neoformans* var. *gattii* (*1*). Although both species cause pulmonary or central nervous system infections, they differ in their ecology, epidemiology, and pathobiology. *C. neoformans* is the most common *Cryptococcus* spp. worldwide and mainly affects immunocompromised hosts. In contrast, *C. gattii* mainly affects immunocompetent hosts and often forms mass-like lesions (cryptococcomas).

Multilocus sequence typing can be used to divide this species into 4 molecular genotypes, VGI–VGIV, which differ in epidemiology and virulence (*1*,*2*). *C. gattii* was believed to be restricted to tropical and subtropical areas such as Australia, Southeast Asia, and South America (*1*). However, in 1999, a *C. gattii* infection outbreak occurred on Vancouver Island, British Columbia, Canada (*3*), which has a temperate climate.

During the Vancouver Island outbreak, most human, animal, and environmental isolates obtained belonged to VGIIa (major genotype, 90%–95% of isolates) and VGIIb (minor genotype, 5%–10% of isolates) (*2*,*3*). These strains have now spread to mainland British Columbia and the Pacific Northwest region of the United States (*1*,*4*,*5*). The potential for further spread of this strain, particularly the VGIIa genotype, is a serious concern because it is highly virulent in mammals and can infect immunocompetent persons (*2*).

Author affiliations: University of Tokyo Hospital, Tokyo, Japan (K. Okamoto, S. Hatakeyama, S. Itoyama, Y. Nukui, Y. Yoshino, T. Kitazawa, H. Yotsuyanagi, K. Koike); and Meiji Pharmaceutical University, Tokyo (R. Ikeda, T. Sugita)

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We report a case of cerebral cryptococcoma caused by *C. gattii* VGIIa (a strain identical to the Vancouver Island outbreak major genotype strain R265) in a patient from Japan who had no recent travel history to known disease-endemic areas.

The Patient

In 2007, a 44-year-old man (sign painter) from Japan who was not infected with HIV came to the University of Tokyo Hospital with a 2-month history of headache and loss of right-sided vision in both eyes (right homonymous hemianopsia). He had had a diagnosis of hyperglycemia 3 years before admission but had declined to seek medical treatment. His medical history was otherwise unremarkable. He was an ex-smoker who rarely consumed alcohol and was not taking any prescription medications (including corticosteroids or other immunosuppressive drugs). He had traveled to Guam in 1990 and Saipan in 1999 and had no other history of overseas travel. He reported exposure to his dog, and he did not spend time in wilderness areas. The patient often worked near construction sites in urban locations.

When hospitalized, the patient was afebrile and had stable vital signs. Physical examination showed agraphia (inability to write), anarithmia (inability to count numbers), right homonymous hemianopsia, and Romberg sign. Laboratory evaluations showed increased glucose (367 mg/dL) and hemoglobin A1c (10.5%) levels. Results of a complete blood cell count and hepatic and renal function tests were within normal limits. Levels of electrolytes and C-reactive protein were also within normal limits. Results of a test for antibodies to HIV-1/2 were negative.

An enhanced brain magnetic resonance imaging scan showed a 4.4 × 4.1 × 3.3–cm lobulated mass in the left occipital lobe with surrounding edema. The lesion had low signal intensity on T1-weighted images, moderate signal intensity on T2-weighted images, and rim enhancement on T1-weighted images after administration of gadolinium (Figure). A chest computed tomography scan showed a 1.8 × 1.2–cm nodule in the left lower lung (S8 segment).

The brain mass was completely resected because it was suspected to be a tumor. Pathologic evaluation of the resected specimen showed that the mass was a large abscess containing encapsulated yeast. The yeast could be seen after staining with periodic acid–Schiff and Grocott methenamine silver stains. Specimen cultures were positive for *Cryptococcus* spp. and led to a diagnosis of cerebral cryptococcosis.

The patient was treated with liposomal amphotericin B (4 mg/kg/d for 3 weeks) and flucytosine (100 mg/kg/d for 3 weeks). A cerebrospinal fluid (CSF) sample obtained by lumbar puncture soon after mass resection showed a slightly increased protein level but no leukocytosis or a low

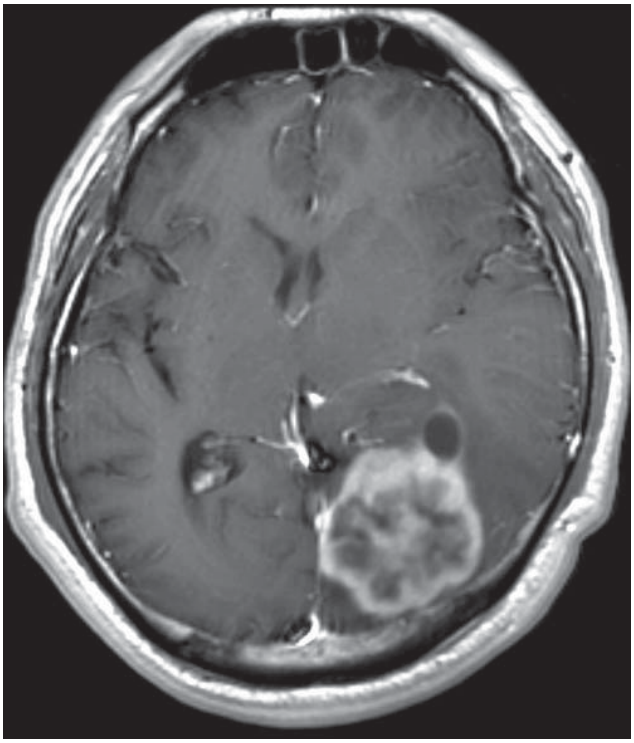

Figure. Postcontrast T1-weighted magnetic resononance image of the brain of a 44-year-old man with cerebral cryptococomma in Japan, 2007, showing a rim-enhancing lobulated mass (lower right) with surrounding edema in the left occipital lobe.

glucose concentration. Fungal cultures of the CSF showed negative results. Cryptococcal antigen titers were >512 in serum and 64 in CSF.

After 3 weeks of induction therapy, the patient received consolidation and maintenance therapy (oral fluconazole, 400 mg/d) for 1 year. The pulmonary nodule decreased in size after antifungal treatment, suggesting that this lesion was a pulmonary cryptococcoma. At the 1-year follow-up visit, the cerebral cryptococcoma had not recurred, and the pulmonary nodule had disappeared.

The cryptococcal strain isolated from the brain was identified as serotype B by slide agglutination test (*6*) and identified as *C. gattii* by rRNA gene sequence analysis (*7*). Eleven unlinked loci (*SXI1, IGS, TEF1, GPD1, LAC1, CAP10, PLB1, MPD1, HOG1, BWC1*, and *TOR1*) analyzed by multilocus sequence typing, according to the method of Fraser et al. (*2*), were identical to those of the Vancouver Island major genotype strain R265 (genotype VGIIa) (Table) (*2*,*8*,*9*). MICs of this isolate were 0.125, 0.5, 1.0, and 0.011 μg/mL for amphotericin B, flucytosine, fluconazole, and itraconazole, respectively.

Conclusions

Japan has not been considered a *C. gattii*–endemic area. There have been only 2 reports of *C. gattii* infections in Japan; both infections apparently originated in Australia (*10*,*11*). Retrospective surveillance studies found no evidence of *C. gattii* infection or colonization in Japan (*12*; Ministry of Health, Labor and Welfare of Japan, 2003, unpub. data). Although the source of the infection in the patient reported here has not been identified, it appears to have originated in Japan. Infections with *C. gattii* have not been reported in the places the patient had visited (Guam and Saipan) (*13*,*14*). Analysis of persons who traveled to Vancouver Island indicated that the median incubation period of *C. gattii* infection is 6–7 months (range 2–11 months) (*13*), although an incubation period of 13 months has been reported for 1 patient (*14*).

C. gattii genotype VGIIa may have been present in the Pacific Northwest region of the United States long before the Vancouver Island outbreak (*2*,*4*). However, no human cases were identified in the United States until January 2006 (*4*,*5*). Genotype VGIIa has now spread from Vancouver Island to mainland British Columbia and the Pacific Northwest region of the United States, possibly because of human activity or animal migration (*1*). However, this genotype has not been reported in any other region, although similar isolates have been obtained from South America (*1*) and from patients who had traveled to affected areas (*1*,*8*). Thus, our findings indicate possible global dispersal of this strain.

Our results suggest that *C. gattii* VGIIa genotype may be spreading. In North America, this genotype has been isolated from various environmental specimens such as tree surfaces, soil, water, and air (*1*,*4*). Soil is believed to be a major potential reservoir of this organism (*1*,*15*). Thus, ecologic and environmental studies on *C. gattii* in Japan are needed to determine likely reservoirs and to improve understanding of *C. gattii* epidemiology. Although many clinical laboratories in Japan currently do not differentiate between *C. neoformans* and *C. gattii* infections, identifica-

Table. Multilocus sequence typing profiles of 2 *Cryptococcus gattii* strains, Japan, 2007*

Strain	Multilocus sequence typing profile†										
	SXI1α	*IGS*	*TEF1*	*GPD1*	*LAC1*	*CAP10*	*PLB1*	*MPD1*	*HOG1*	*BWC1*	*TOR1*
JP01	18	4	7	1	4	1	1	5	1	1	1
R265	18	4	7	1	4	1	1	5	1	1	1

*JP01, strain from Japan (patient in this study); R265, Vancouver Island genotype VGIIa strain.
†GenBank accession nos. for multilocus sequencing typing alleles: *SXI1α*_18, DQ096308; *IGS*_4, DQ096314; *TEF1*_7, DQ096364; *GPD1*_1, DQ096377; *LAC1*_4, DQ096400; *CAP10*_1, DQ096416; *PLB1*_1, DQ096343; *MPD1*_5, DQ096334; *HOG1*_1, DQ096456; *BWC1*_1, DQ096428; *TOR1*_1, DQ096470.

tion of cryptococcal isolates to the species level, especially in apparently immunocompetent patients, is needed.

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Dr Okamoto is a chief resident in the Department of Infectious Diseases at the University of Tokyo Hospital. His research interests are infections in immunocompromised patients and fungal infections.

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Address for correspondence: Shuji Hatakeyama, Department of Infectious Diseases, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; email: shatake-tky@umin.ac.jp

Saffold Cardioviruses of 3 Lineages in Children with Respiratory Tract Infections, Beijing, China

Lili Ren, Richard Gonzalez, Zhengde Xie, Yan Xiao, Yongjun Li, Chunyan Liu, Lan Chen, Qingqing Yang, Guy Vernet, Gláucia Paranhos-Baccalà, Qi Jin, Kunling Shen, and Jianwei Wang

To clarify the potential for respiratory transmission of Saffold cardiovirus (SAFV) and characterize the pathogen, we analyzed respiratory specimens from 1,558 pediatric patients in Beijing. We detected SAFV in 7 (0.5%) patients and identified lineages 1–3. However, because 3 patients had co-infections, we could not definitively say SAFV caused disease.

Saffold cardiovirus (SAFV) is a new piconavirus, originally identified from fecal samples of a female infant with fever of unknown origin (*1*). SAFV has since been reported worldwide, and 8 lineages have been identified (*1*–*6*). Although serologic surveys have shown that SAFV-3 infection occurs early in life (*7*), the pathogenicity of SAFV is still unclear.

Because SAFVs are mainly detected in fecal samples, virus transmission is thought to occur by the fecal–oral route (*1*,*3*–*7*). However, 2 research groups also found SAFV-2 lineage in respiratory secretions (*2*,*4*). Thus, we investigated whether the respiratory tract route could be an additional transmission route and whether SAFV lineages other than SAFV-2 may also infect the respiratory tract. We identified and characterized 7 SAFV strains, which belonged to 3 distinct lineages, from respiratory samples of children with lower and upper respiratory tract infections (LRTIs and URTIs, respectively).

Author affiliations: State Key Laboratory for Molecular Virology and Genetic Engineering, Beijing, People's Republic of China (L. Ren, Q. Jin, J. Wang); Institute of Pathogen Biology, Beijing (L. Ren, R. Gonzalez, Y. Xiao, L. Chen, Q. Yang, Q. Jin, J. Wang); Fondation Mérieux, Lyon, France (R. Gonzalez, Y. Li, G. Vernet, G. Paranhos-Baccalà); and Beijing Children's Hospital, Beijing (Z. Xie, C. Liu, K. Shen)

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The Study

We assessed 2 cohorts. Cohort 1 comprised 1,032 children (617 boys and 415 girls) with acute LRTIs, hospitalized in Beijing Children's Hospital (BCH), from whom nasopharyngeal aspirates were collected from May 2007 through March 2009. The patients ranged in age from 2 weeks to 16 years (mean age 31.3 months, median 9 months). Cohort 2 comprised 506 BCH outpatient children (277 boys and 229 girls) with acute URTIs, from whom throat swabs were collected from May through August 2009. These patients ranged in age from 4 months to 16 years (mean age 46.7 months, median 37 months).

Virus nucleic acids in clinical samples were extracted by using the NucliSens easyMAG system (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. SAFV RNA was detected by nested reverse transcription–PCR (RT-PCR) by using primers selective for the 5′ untranslated region (UTR) (*3*). The viral protein (VP) 1 gene was amplified by using 3 pairs of primers as previously described (*3*,*5*,*6*). The full genomic sequences were obtained by a genome walking method (*7*). The 5′ and 3′ UTR sequences were determined by using the RACE System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After being cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), all PCR products were verified by sequencing. In addition, all screened specimens were tested for known respiratory viruses as previously described (*8*,*9*). *Mycoplasma pneumoniae* was detected by using the gelatin particle agglutination test kit (SERODIA-MYCO II, Fujirebio, Japan).

For phylogenetic analysis, we constructed neighbor-joining trees based on the distances of SAFV nucleotide or amino acid sequences by using MEGA 4.0 (*10*). We used SimPlot (version 3.5.1) to analyze possible recombination between viral genome sequences (*11*).

LLC-MK2 cells were used to isolate SAFV as previously described (*12*). Cells were collected either when cytopathic effects were observed or after 12 days postinoculation, and then they were tested for SAFV by RT-PCR.

We detected SAFV RNA in 4 (0.4%) of the 1,032 nasopharyngeal aspirates from patients with LRTIs and in 3 (0.6%) of the 506 throat swab specimens from outpatients with URTIs. The SAFV-positive patients (4 girls and 3 boys) were 5 months to 9 years of age (Table). SAFV infection did not appear to have a predominant time for occurrence: cases were detected in a range of months for the periods covered (August and December 2007, October and November 2008, and June 2009). All SAFV-positive patients exhibited symptoms of respiratory tract infection, such as coughing, gasping, sneezing, or fever. Of the 4

Table. Characteristics of patients with Saffold cardiovirus, Beijing, China, 2007–2009*

Patient no.	Age/sex	Sampling date	Diagnosis	Clinical signs	Other underlying diseases	Co-infecting organisms	SAFV lineage
BCH133	9 y/F	2007 Aug 7	Pneumonia	Cough	Tuberculosis, hepatic dysfunction	*Mycoplasma pneumoniae*	1
BCH350	5 mo/M	2007 Dec 7	Broncho-pneumonia	Cough, gasping	Respiratory failure	RSV	3
BCH895	2 y, 8 mo/M	2008 Oct 6	Pneumonia	Fever, cough, spitting, gasping, chills	No	EV	1
BCH1031	4 y/M	2008 Nov 16	Peribronchitis	Fever, cough, spitting, gasping, chills, sneezing	No	None	3
BCHU79	1 y, 6 mo/F	2009 Jun 5	URTI	Fever, cough	Unknown	None	2
BCHU115	1 y, 10 mo/F	2009 Jun 6	URTI	Fever, cough	Unknown	None	3
BCHU353	4 y, 6 mo/F	2009 Jun 17	URTI	Fever, cough	Unknown	None	2

*SAFV, Saffold cardiovirus; RSV, respiratory syncytial virus; EV, enterovirus; URTI, upper respiratory tract infection.

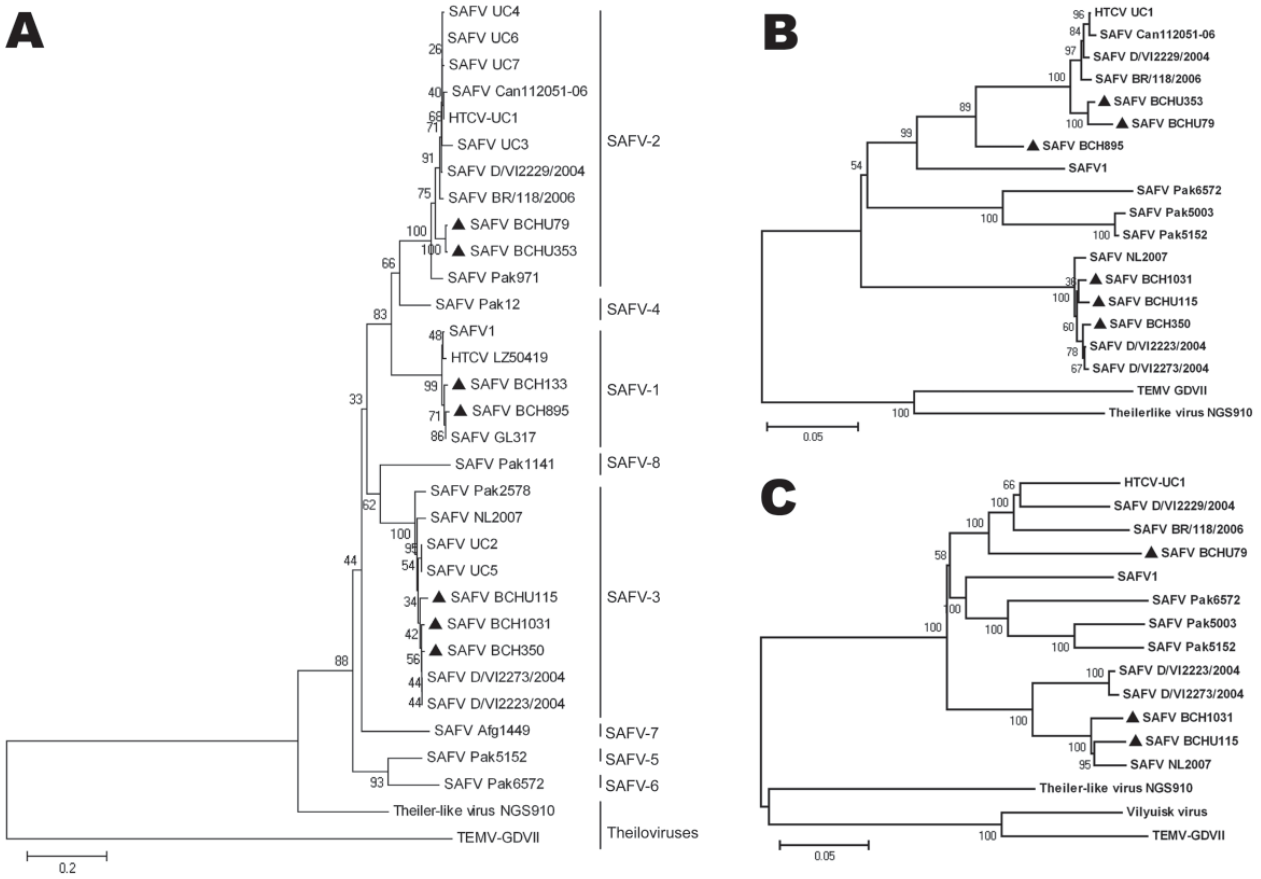

Figure 1. Phylogenetic analysis of Saffold cardiovirus (SAFV) strains obtained in Beijing, China, 2007–2009, based on viral protein (VP) 1 (A), P1 capsid proteins (B), and full-length genomes (C). The trees, with 500 bootstrap replicates, were generated by using the neighbor-joining algorithm in MEGA 4.0 (*10*). Strains identified in this study are indicated by a specific identification code (BCH or BCHU), followed by the patient number and labeled with dark triangles (BCH133, BCH350, BCH895, BCH1031, BCHU79, BCHU115, and BCHU353). GenBank accession nos. of the complete genome sequences are BCH1031 GU943513, BCHU115 GU943514, and BCHU79 GU943518; of the P1 gene, BCH350 GU943515, BCH895 GU943516, and BCHU353 GU943517; and of the VP1 gene of BCH133, GU126461. SAFV-1 (prototype), UC1-UC7, Can112051-06, D/VI2229/2004, BR/118/2006, D/VI2273/2004, D/VI2223/2004, LZ50419, GL317, Pak12, Pak971, Afg1449, Pak1141, Pak2578, Pak5003, Pak5152, Pak6572, and NL2007, TEMV-GDVII, Theiler-like virus NGS910, and Vilyuisk virus were used as reference sequences (GenBank accession nos. NC009448, NC010810, EU604745-EU604750, AM922293, EU681176-EU681179, FJ586240, FJ464767, FJ463600-FJ463602, FJ463604, FJ463605, FJ463615-FJ463617, FM207487, X56019, AB090161, and EU723237). Scale bars incdicate nucleotide substitutions per site.

SAFV-positive patients with LRTIs, 2 had underlying illnesses, i.e., tuberculosis, hepatic dysfunction, or respiratory failure (Table). All SAFV-positive patients recovered within 7–11 days. No major differences were found in disease duration between patients who had underlying diseases and those who did not.

Co-infections with additional respiratory pathogens were detected for 3 of 7 SAFV-positive patients, all in the first cohort. These pathogens were respiratory syncytial virus (1 patient), enterovirus (1 patient), and *M. pneumoniae* (1 patient) (Table). A SAFV-3 strain was isolated from sample BCH1031. Starting at 5 days postinoculation, cytopathic effects were observed. Virus was not isolated from other SAFV-positive samples (data not shown).

We identified 3 genetic lineages, SAFV-1, -2 and -3, on the basis of phylogenetic analysis of VP1, the most diverse protein of picornaviruses (*6*) (Figure 1). Multiple-alignment analysis, based on reference sequences of each lineage available in GenBank, showed that the identity of the VP1 amino acid sequences among the strains in the same lineage was 92.6%–100% and among strains belonging to different lineages, 63.3%–100% (online Appendix Table, www.cdc.gov/EID/content/16/7/1158-appT.htm). We found no obvious differences in amino acid sequences, nor any new motifs in these sequences, between strains detected in respiratory samples and those in fecal samples within the same lineage.

To further characterize the variation of SAFVs, we amplified the P1 region sequences of 6 strains identified in this study. The identity of all available nucleotide sequences of the P1 region among SAFVs was 68.5%–97.2%, whereas that of amino acid sequences was 74.9%–99.4%. The VP1 CD and VP2 EF loop structures, which display the greatest amino acid divergence among different SAFVs and are associated with tropism and virulence (*6*,*7*), were analyzed by amino acid alignment. Similar to previously reported findings (*6*,*7*), we found that the sequences in both loops among SAFVs were highly diverse among SAFVs (Figure 2). The amino acid identities among all known SAFVs were 53.1%–100% in CD loops and 40.3%–100% in EF loops; amino acid identities of SAFVs versus animal cardioviruses were 29.1%–41.1% in CD loops and 19.2%–32.6% in EF loops. We did not find any major differences among the available amino acid sequences of CD loops (91.1%–100.0%) or EF loops (97.9%–100.0%) of SAFVs within the same lineage for samples collected from either the gastrointestinal or respiratory tracts.

The complete genome sequence of BCH1031 (SAFV-3) was obtained, as were nearly complete genome sequences of BCHU115 (SAFV-3) and BCHU79 (SAFV-2), which

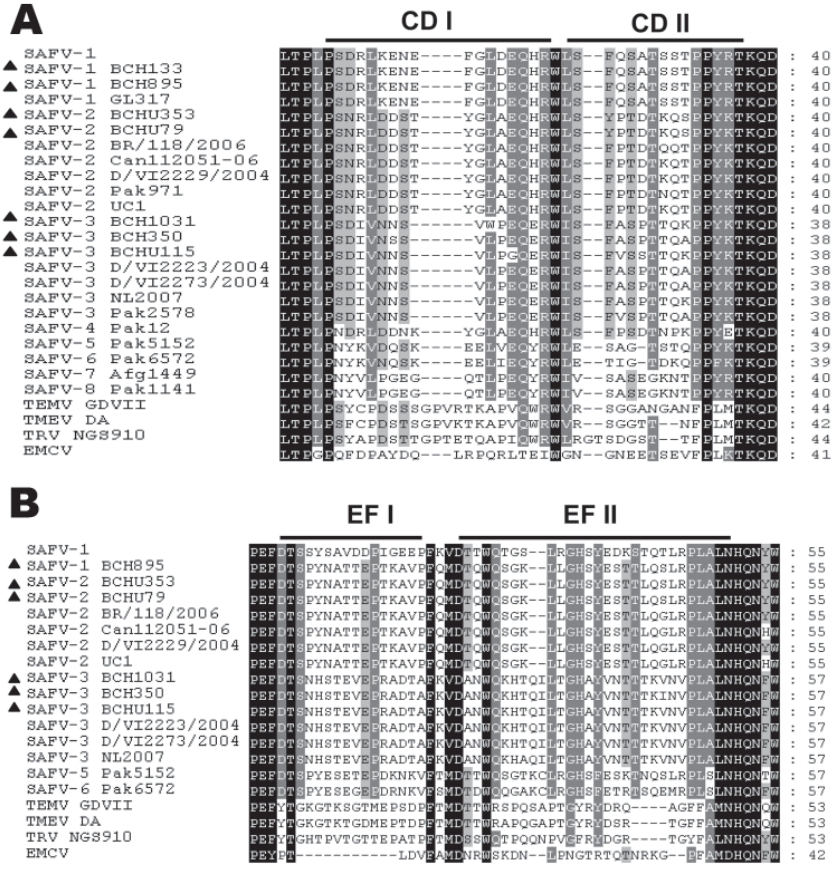

Figure 2. Alignment of Saffold cardiovirus (SAFV) viral protein (VP) 1 CD (A) and VP2 EF (B) loop sequences from strains isolated in Beijing, China, 2007–2009. Columns highlighted in black show absolute amino acid conservation; those highlighted in gray show amino acids with highly similar properties. Strains identified in this study are labeled with dark triangles (BCH133, BCH1031, BCHU115, BCH350, BCH895, BCHU353 and BCHU79) (GenBank accession nos. GU126461, GU943513–GU943518), SAFV-1 (prototype), SAFV-1 GL317, SAFV-2 Can112051-06, SAFV-2 BR/118/2006, SAFV-2 D/VI/2229/2004, SAFV-2 Pak971, SAFV-2 UC1, SAFV-3 Pak2578, SAFV-3 D/VI/2223/2004, SAFV-3 D/VI/2273/2004, SAFV-3 NL2007, SAFV-4 Pak12, SAFV-5 Pak5152, SAFV-6 Pak 6572, SAFV-7 Afg1449, SAFV-8 Pak1141, Theiler-like virus NGS910, TMEV GDVII, and EMCV were used as reference sequences (GenBank accession nos. NC009448, FJ464767, AM922293, EU681177, EU681176, FJ463601, NC010810, FJ463605, EU681179, EU681178, FM207487, FJ463600, FJ463616, FJ463617, FJ463602, FJ463604, AB090161, X56019, X87335).

covered full-length coding sequences as well as the 3′ UTR and a partial 5′ UTR (Figure 1). SimPlot analysis showed relatively high (>86%) identity between strains within the same lineage. No clear evidence of genetic recombination between the SAFV strains was found (online Appendix Figure, www.cdc.gov/EID/content/16/7/1158-appF.htm).

Conclusions

Although 8 lineages of SAFV have been detected in fecal samples worldwide, only SAFV-2 has been detected in respiratory samples (*2*,*4*). In addition, only SAFV-1 had been reported in China (*5*,*13*). In this study, we found that SAFV lineages 1, 2, and 3 co-circulated in patients with respiratory infections in Beijing.

Although SAFV is known to be transmitted by the fecal–oral route (*1*,*3*–*7*), as are other picornaviruses (*14*), our detection of SAFV in respiratory samples suggests that various SAFV lineages may also be transmitted through the respiratory tract and may be associated with disease. However, other respiratory viruses and *M. pneumoniae* were co-detected in 3 of the 7 SAFV-positive patients. Given that we did not conduct assays for common respiratory bacteria (and the number of SAFV-positive cases was limited), whether SAFV actually caused the observed symptoms in the patients cannot be definitively determined.

The genetic diversity of SAFVs in respiratory samples can complicate the relationship between SAFVs and disease because different genotypes of the same picornavirus species may cause different clinical signs and symptoms (*6*,*14*). Further investigations are needed to clarify any possible link between the pathogenicity and genetic diversity of SAFV.

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Dr Ren works as a scientist at the Dr Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences–Fondation Mérieux and State Key Laboratory of Molecular Virology and Genetic Engineering. Her research is focused on the etiology of infection and pathogenesis of respiratory viruses.

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Address for correspondence: Jianwei Wang, 9# Dong Dan San Tiao, Dongcheng District, Beijing 100730, People's Republic of China; email: wangjw28@163.com

Novel Swine Influenza Virus Reassortants in Pigs, China

Yuhai Bi,[1] Guanghua Fu,[1] Jing Chen,[1] Jinshan Peng, Yipeng Sun, Jingjing Wang, Juan Pu, Yi Zhang, Huijie Gao, Guangpeng Ma, Fulin Tian, Ian H. Brown, and Jinhua Liu

During swine influenza virus surveillance in pigs in China during 2006–2009, we isolated subtypes H1N1, H1N2, and H3N2 and found novel reassortment between contemporary swine and avian panzootic viruses. These reassortment events raise concern about generation of novel viruses in pigs, which could have pandemic potential.

Genetic characterization of pandemic (H1N1) 2009 virus has indicated that it may have derived from swine (*1*,*2*). However, because of the lack of systematic swine influenza surveillance, the generation pathway of the novel virus is uncertain. Therefore, we attempted to obtain more information about swine influenza viruses isolated from pigs.

The Study

During December 2006–February 2009 in the People's Republic of China, 3,546 samples from 3 main swine industry provinces—Fujian (765 samples), Guangdong (1,276 samples), and Shandong (1,505 samples)—were collected for influenza surveillance. Nasal and tracheal swab samples were collected from apparently healthy domestic pigs at abattoirs. Virus isolation and identification were performed as described (*3*). Of 29 strains of influenza A virus obtained, 19 were subtype H1N1, 1 subtype H1N2, and 9 subtype H3N2. Subtype H1N2 was isolated from diseased pigs in Guangdong Province in 2006; the others were isolated from healthy pigs. Isolation rates for subtypes H1N1 and H3N2 were 0.54% and 0.25%, respectively, indicating that subtype H1N1 viruses were predominant in the sampled pig population.

Author affiliations: China Agricultural University, Beijing, People's Republic of China (Y. Bi, G. Fu, J. Chen, J. Peng, Y. Sun, J. Wang, J. Pu, Y. Zhang, H. Gao, J. Liu); Shandong Animal Disease Control Center, Jinan, People's Republic of China (J. Chen, F. Tian, J. Liu); Veterinary Laboratories Agency–Weybridge, Addlestone, Surrey, UK (I.H. Brown); China Rural Technology Development Center, Beijing (G. Ma); and Chongwen Animal Health Inspection Institute, Beijing (J. Peng)

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To determine genetic and antigenic characteristics, we conducted phylogenetic and antigenic analysis of all isolates. Cross–hemagglutination-inhibition showed that the subtype H3N2 viruses could be divided into 2 distinct antigenic groups (online Appendix Table 1, www.cdc.gov/EID/content/16/7/1162-appT1.htm). Viruses of subtype H1 (swine/Shandong/101/2008, swine/Shandong/327/2009, and swine/Shandong/275/2009) reacted well with antiserum to the European avian-like swine virus, swine/FJ/204/2007, but not with antiserum to classical swine (H1N1) virus, swine/Guangdong/1/2005. The other 6 subtype H1N1 isolates reacted strongly with antiserum to swine/Guangdong/1/2005 (online Appendix Table 1; online Appendix Table 2, www.cdc.gov/EID/content/16/7/1162-appT2.htm), indicating that the antigenicity of the subtype H1N1 viruses could also be divided into 2 distinct antigenic groups. Subtype H1N2 virus (swine/Guangdong/1222/2006) had low reactivity with swine/Guangdong/1/2005 and swine/Fujian/204/2007 (online Appendix Table 2), indicating that the antigenicity of subtype H1N2 isolate differed from that of classical and European avian-like swine viruses.

Phylogenetic analysis showed that the H3 hemagglutinin (HA) tree separated into avian and human lineages (online Technical Appendix, panel A, www.cdc.gov/EID/content/16/6/1162-Techapp.pdf), implying that at least 2 independent H3 sublineages of virus prevail in pigs in China. Neuraminidase (NA) genes of the 9 subtype H3N2 and 1 subtype H1N2 isolates were located in distinct lineages (online Technical Appendix, panel B). A cluster was formed by 4 strains of H3N2—swine/Fujian/43/2007, swine/Guangdong/811/2006, swine/Shandong/106/2007, and swine/Shandong/133/2007—and the cluster grouped with Eurasian avian (H9N2) viruses. Four H3N2 strains—swine/Guangdong/211/2006, swine/Guangdong/423/2006, swine/Guangdong/223/2006, and swine/Guangdong/968/2006—were located in the intermediate human sublineage represented by A/Beijing/39/75 (H3N2). One subtype H3N2 isolate, swine/Guangdong/7/2006, grouped closely with A/Moscow/10/99 (H3N2), and the subtype H1N2 isolate swine/Guangdong/1222/2006 shared close similarities with North American swine triple reassortant viruses (online Technical Appendix, panel B). These findings showed that viruses of avian, intermediate human, and recent human N2 sublineages were prevalent in pigs in China.

Phylogenic analysis of subtype H1 HA showed that the 9 subtype H1N1 isolates were located in either the classical or European avian-like swine lineages (online Technical Appendix, panel C). Swine/Guangdong/1222/2006, together with subtype H1N2 isolates from Hong Kong and subtype H1N2 strains from Guangxi, have a sister-like relationship with those of pandemic (H1N1) 2009 virus (online Technical Appendix, panel C). Consistent with character-

[1]These authors contributed equally to this article.

istics of HA genes, NA genes of the 6 influenza (H1N1) strains isolated belong to classical swine lineages (online Technical Appendix, panel D). The other 3 isolates—swine/Shandong/101/2008, swine/Shandong/275/2009, and swine/Shandong/327/2009—together with pandemic (H1N1) 2009 virus, were located in the European avian-like swine group.

The polymerase acidic protein (PA) gene of swine/Guangdong/7/2006 (H3N2) was closely related to that of duck/Guangdong/12/2000 (H5N1) (online Technical Appendix, panel G), and other internal genes of swine/Guangdong/7/2006 were located in the recent human subtype H3N2 lineages (online Technical Appendix, panels E, F, and H–J). The matrix (M) gene of the 3 isolates—swine/Guangdong/211/2006(H3N2), swine/Guangdong/223/2006 (H3N2), and swine/Guangdong/423/2006 (H3N2)—grouped in classical swine lineage (online Technical Appendix, panel I), and other internal genes were located in intermediate human subtype H3N2 lineage (online Technical Appendix, panels E–H and J). Except for the fact that the PA gene of swine/Guangdong/968/2006 and NA gene of swine/Guangdong/811/2006 are of the Eurasian H9N2 avian virus lineage (online Technical Appendix, panels B and G), the other internal genes are located in the same lineages with the 3 viruses swine/Guangdong/211/2006, swine/Guangdong/223/2006, and swine/Guangdong/423/2006 (online Technical Appendix, panels E–J). The polymerase basic protein 1 (PB1), PA, NP, and nonstructural (NS) genes of swine/Shandong/106/2007 (H3N2) and swine/Shandong/133/2007 (H3N2) belong to the Eurasian avian lineage grouping with the H9N2 viruses (online Technical Appendix, panels F–H and J). The PB2 and M genes of the 2 isolates group in human subtype H1N1 lineage (online Technical Appendix, panels E and I). The PA and M genes of swine/Fujian/43/2007 (H3N2) belong to recent human-like H3N2 virus lineages (online Technical Appendix, panels G and I); the NS gene originates from European avian-like virus (online Technical Appendix, panel J), and the PB2, PB1, and NP genes were located in the Eurasian avian lineages with subtype H9N2 viruses (online Technical Appendix, panels E, F, and H). Except for the M gene, all other internal genes of swine/Guangdong/1222/2006 have a sister-like relationship with those of pandemic (H1N1) 2009 virus (online Technical Appendix, panels E–J). The PB1 gene of swine/Shandong/275/2009 was an exception, grouping with Eurasian avian subtype H9N2 virus (online Technical Appendix, panel F). All 6 internal genes of the 3 Shandong isolates were located in the European avian-like lineages (online Technical Appendix, panel E–J). All 8 genes of the 6 subtype H1N1 Guangdong isolates formed 1 cluster and belonged to classical swine lineages (online Technical Appendix, panels C–J), indicating that none of these viruses were recent reassortants (Table).

Conclusions

Influenza A subtypes H1N1, H1N2, and H3N2 viruses co-circulate in China. Genetic analysis showed that the single subtype H1N2 virus and all subtype H3N2 viruses

Table. Genetic origin of swine influenza viruses*

Isolate	Gene							
	PB2	PB1	PA	HA	NP	NA	M	NS
Swine/GD/7/2006 (H3N2)	R-H	R-H	A-H5	R-H	R-H	R-H	R-H	R-H
Swine/SD/106/2007 (H3N2)	H-H1	A-H9	A-H9	R-H	A-H9	A-H9	H-H1	A-H9
Swine/SD/133/2007 (H3N2)	H-H1	A-H9	A-H9	R-H	A-H9	A-H9	H-H1	A-H9
Swine/FJ/43/2007 (H3N2)	A-H9	A-H9	R-H	R-H	A-H9	A-H9	R-H	S-A
Swine/GD/211/2006 (H3N2)	H-75	H-75	H-75	H-75	H-75	H-75	C	H-75
Swine/GD/223/2006 (H3N2)	H-75	H-75	H-75	H-75	H-75	H-75	C	H-75
Swine/GD/423/2006 (H3N2)	H-75	H-75	H-75	H-75	H-75	H-75	C	H-75
Swine/GD/811/2006 (H3N2)	H-75	H-75	H-75	H-75	H-75	A-H9	C	H-75
Swine/GD/968/2006 (H3N2)	H-75	H-75	A-H9	H-75	H-75	H-75	C	H-75
Swine/GD/1222/2006 (H1N2)	A-A	R-H	A-A	C	C	R-H	C	C
Swine/GD/611/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/GD/322/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/GD/33/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/GD/446/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/GD/109/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/GD/628/2006 (H1N1)	C	C	C	C	C	C	C	C
Swine/SD/327/2009 (H1N1)	S-A	S-A	S-A	S-A	S-A	S-A	S-A	S-A
Swine/SD/101/2008 (H1N1)	S-A	S-A	S-A	S-A	S-A	S-A	S-A	S-A
Swine/SD/275/2009 (H1N1)	S-A	A-H9	S-A	S-A	S-A	S-A	S-A	S-A

*PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleocapsid protein; NA, neuraminidase; M, matrix; NS, nonstructural; GD, Guangdong; R-H, recent human-like (H3N2); A-H5, Eurasian avian (H5N1); SD, Shandong; H-H1, human-like (H1N1); A-H9, Eurasian avian (H9N2); FJ, Fujian; S-A, European avian-like swine; H-75, intermediate human-like (H3N2) (A/Victoria/75-like); C, classical swine; A-A, American avian.

examined were either double- or triple-reassortant viruses, which have been rarely documented in China. Finding a gene fragment ostensibly of highly pathogenic avian influenza (H5N1) virus in a subtype H3N2 virus implies that subtype H5N1 viruses may be able to contribute genes to virus pathogenic processes in pigs. Moreover, European avian-like swine (H1N1) virus undergoes reassortment with avian (H9N2) viruses.

Some researchers have hypothesized that pigs may serve as hosts for genetic reassortment between human and avian influenza viruses (*4*). Our results show that subtypes H3N2 and H1N2 and 1 European avian-like swine (H1N1) virus were all derived from relatively recent reassortment events. The gene fragments of the subtype H3N2 viruses comprised those of human subtype H3N2 (A/Victoria/75-like and A/Moscow/99-like) and the strains H1N1 classical swine, Eurasian H5N1, and H9N2 avian. Infection of pigs with avian H5N1 and H9N2 viruses in China has been reported, and swine H1 and H3 viruses appear widely established in the pig population in China and elsewhere in Southeast Asia (*5*–*9*). These findings raise more questions about the generation of novel viruses, which may have zoonotic potential, in pigs.

Pandemic (H1N1) 2009 virus probably resulted from reassortment of recent North American influenza subtypes H3N2 and/or H1N2 swine viruses with Eurasian avian-like swine viruses (*2*). The current situation, therefore, presents continued risk for further reassortment of swine influenza virus in pig populations and continued spread of pandemic (H1N1) 2009 virus to pigs worldwide. Systematic influenza virus surveillance in pigs is needed in China.

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Dr Bi is a PhD student at the College of Veterinary Medicine, China Agricultural University, and has research interests in the epidemiology and pathogenic mechanism of influenza virus and paramyxovirus.

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Address for correspondence: Jinhua Liu, Key Laboratory of Zoonosis of Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing 100193, People's Republic of China; email: ljh@cau.edu.cn

Long-term Shedding of Influenza A Virus in Stool of Immunocompromised Child

Benjamin A. Pinsky, Samantha Mix, Judy Rowe, Sheryl Ikemoto, and Ellen J. Baron

In immunocompromised patients, influenza infection may progress to prolonged viral shedding from the respiratory tract despite antiviral therapy. We describe chronic influenza A virus infection in an immunocompromised child who had prolonged shedding of culturable influenza virus in stool.

Human influenza A virus infections are typically localized to the respiratory tract, and viral presence in the gastrointestinal (GI) tract is rarely observed. Isolation of influenza virus from the stool is most commonly documented in human infections of avian influenza subtype H5N1. In these infections, virus in the stool may be related to a disseminated infection with exceptionally high viral titers, atypical of seasonal, circulating influenza infections (*1*). Interestingly, infections with the influenza A pandemic (H1N1) 2009 virus can be associated with a high rate of GI symptoms (*2*). Although seasonal influenza RNA in stool has been described, we report here the culturing of H1 virus from stool.

Case Report

The patient was a 4.5-year-old boy who had received a bone marrow transplant for Wiskott-Aldrich syndrome 4 years earlier; he also had chronic graft versus host disease of the GI tract. In January 2008, fever and respiratory symptoms developed in the patient, and direct fluorescent antibody (DFA) testing of a nasopharyngeal swab specimen showed influenza A infection. Over the next several months, he had additional influenza A–positive DFAs and was given oral and aerosolized ribavirin for chronic infection. Despite this aggressive antiviral treatment, >24 respiratory specimens were positive for influenza A by DFA or culture over the next year.

When the patient was ≈3 months into an extended hospitalization at the Lucile Packard Children's Hospital (complicated by pseudomonal and enterococcal bacteremia, as well as disseminated aspergillosis), he experienced multiple daily episodes of nonbilious, nonbloody emesis (≈2–3/day) and loose stools without blood or mucous (up to 12/day). The clinical team suspected a graft versus host disease flare but also conducted a work up of the patient to identify an infectious process. Results of blood cultures, stool culture, *Clostridium difficile*–toxin B cytotoxicity assay, and stool examination for ova and parasites were all negative. Results of a urine culture were positive for enterococcus. Culture of stool samples was enterovirus positive and over the subsequent 2 weeks, enterovirus was isolated from 2 additional viral stool cultures. These cultures were not evaluated for the presence of influenza virus. Three weeks after the initial stool testing, results of viral stool culture and repeat stool studies were negative. However, the diarrhea and emesis persisted, and ≈8 weeks after the onset of symptoms, another stool specimen was sent for viral culture.

After 3 days, the culture demonstrated cytopathic effect on primary RhMK cells but not on human foreskin fibroblasts, MRC-5 fibroblasts, or A549 lung carcinoma cells, which is consistent with enterovirus infection. However, results of immunofluorescent staining of the RhMK cells (by using a panenterovirus blend of monoclonal antibodies) were negative. Staining results were also negative with serotype group–specific reagents, including the coxsackie virus B blend, echovirus blend, enterovirus 70 and 71, poliovirus blend, coxsackie virus A9, and coxsackie virus A24 (all enterovirus reagents from Millipore/Light Diagnostics, Billerica, MA, USA). An astute technologist associated the pattern of cells showing a cytopathic effect with the patient's concurrent influenza A–positive respiratory specimen and long history of influenza infection. She then set up a standard respiratory virus DFA panel (Millipore/Light Diagnostics), which included fluorescein-conjugated antibodies for the detection of influenza A and B; respiratory syncytial virus; parainfluenza 1, 2, and 3; and adenovirus. Strikingly, the specimen was strongly positive for influenza A virus and showed obvious hemadsorption with guinea pig erythrocytes. Two months later, influenza A was again isolated from the patient's stool, which suggested persistent infection of the GI tract with influenza A virus. Subsequent nucleic acid testing revealed that this chronic influenza A infection was caused by the seasonal, circulating subtype H1N1 virus. Overall, the patient shed influenza A from respiratory secretions for >1.5 years and from stool for >2 months.

Conclusions

Because viral stool cultures from patients with respiratory infections are infrequently ordered, the true occurrence of influenza virus in stool is unknown. The few studies to date have considered viral RNA in fecal specimens as a marker of GI influenza infection, which may not ac-

Author affiliations: Stanford University, Stanford, California, USA (B. Pinsky, J. Rowe, S. Ikemoto, E.J. Baron); and Willamette University, Salem, Oregon, USA (S. Mix)

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curately reflect the shedding of intact virus or the capacity for transmission. One study of 4 children with respiratory symptoms and confirmed influenza infection showed that half had influenza RNA in stool (*3*). In contrast, 6 (<1%) of 627 patients with GI symptoms had detectable influenza RNA in fecal samples (*4*). Similarly, influenza RNA was detected in 21 (2.9%) stool samples from 733 children in Indonesia who had concurrent diarrhea and influenzalike illness (*5*). Notably, in this study, culturable influenza B virus was isolated from the stool of 1 patient. Future studies will be required to ascertain the incidence of influenza virus in the stool of children and adults with influenzalike illness and respiratory influenza infection. Furthermore, given the importance of this issue for infection control and the limited number of laboratories that perform stool viral culture, additional work will be necessary to correlate influenza RNA in feces with the presence of infectious virus.

The lack of a fully intact immune system likely predisposed our patient to chronic influenza infection and spread of the virus to the GI tract. This patient had received a bone marrow transplant for primary immunodeficiency as well as immunosuppressive therapy for graft versus host disease with methylprednisolone, tacrolimus, sirolimus, and daclizumab. Prolonged viral shedding from the respiratory tract and the development of antiviral resistance is well documented in immunocompromised patients, including patients who have received bone marrow transplants (*6*). Although bone marrow transplant patients appear more susceptible to lower respiratory tract disease, in particular, during influenza outbreaks (*7*), influenza virus in stool samples from this patient population has not been well studied. Notably, our patient was not treated with either of the common classes of anti-influenza drugs, the neuraminidase inhibitors or adamantanes, but rather received a long-term course of ribavirin. Although clinical cases of ribavirin-resistant influenza virus infection have not yet been reported, genotypic and phenotypic analysis of this patient's isolate may show resistance or other virus-specific factors associated with chronic influenza and the presence of virus in stool. Although culturable influenza A virus was isolated from the stool of our patient, whether it played a causative role in the patient's gastroenteritis could not be determined.

While influenza virus likely spread to the patient's GI tract after a primary respiratory infection, the route of dissemination remains unknown. One possibility is direct GI inoculation by swallowing respiratory secretions. Because influenza viruses enter the cell through acid-activated fusion with the endosomal membrane (*8*), a low pH environment, for example in the human stomach, is thought to render most influenza viruses noninfectious by prematurely inducing an irreversible conformational change in the viral hemagglutinin (*9*). However, the sensitivity of influenza virus to low pH inactivation appears dependent on strain and subtype (*10*). Our patient was on a proton pump inhibitor, which would reduce gastric acidity. Another possibility is that the virus reached the GI tract hematogenously, as is suspected in human cases of avian influenza (*1*).

Whether the influenza subtype that infected this patient is capable of local GI replication in humans is also unclear. In the GI tract, the virus likely encountered the proteases necessary for hemagglutinin cleavage and activation (*11*). However, the H1 hemagglutinin has relative specificity for α 2,6-linked sialic acid, a cell-surface glyco-conjugate not normally found on mucosa of the colon or small intestine (*12*,*13*). Nevertheless, this binding specificity is not absolute (*14*), and 2,3-linked sialic acids are abundantly expressed on colorectal epithelial cells (*13*). Future studies should assess the ability of influenza viruses to replicate in the human intestinal epithelium.

Early epidemiologic study of the pandemic (H1N1) 2009 virus suggested that it produced diarrhea, vomiting, or both, in ≈25% of case-patients, more often than the previous seasonal, circulating influenza viruses (*2*). Consistent with the GI symptoms of human infection, experimental respiratory inoculation of ferrets with human isolates of the pandemic strain results in high influenza virus titers in the intestinal tract of infected animals (*15*). Because knowledge of transmission of this novel virus is limited, the Centers for Disease Control and Prevention recommends that all bodily fluids, including the diarrheal stool of infected persons, be assumed to be infectious and handled with precautions. With the emergence of this pandemic (H1N1) 2009 strain known to produce GI symptoms, further research addressing the presence of influenza virus in stool could have major consequences for both infection control and disease management.

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Dr Pinsky is a molecular genetic pathology fellow at Stanford University. His primary research interest is the development of novel diagnostics for infectious diseases.

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Address for correspondence: Benjamin A. Pinsky, Stanford University School of Medicine, Department of Pathology, 300 Pasteur Dr, Lane 235, Stanford, CA 94305-5324, USA; email: bpinsky@stanford.edu

Human Parechovirus Infections in Monkeys with Diarrhea, China

T.L. Shan,[1] C.M. Wang,[1] L. Cui, Eric Delwart, C.L. Yuan, W. Zhao, W. Guo, X.Q. Dai, Y. Yu, and X.G. Hua

Information about human parechovirus (HPeV) infection in animals is scant. Using 5′ untranslated region reverse transcription–PCR, we detected HPeV in feces of monkeys with diarrhea and sequenced the complete genome of 1 isolate (SH6). Monkeys may serve as reservoirs for zoonotic HPeV transmissions and as models for studies of HPeV pathogenesis.

Members of the *human parechovirus* (HPeV) species are small, nonenveloped RNA viruses that are members of the family *Picornaviridae,* genus *Parechovirus*. HPeV can be classified into at least 8 genotypes on the basis of sequence similarity of their capsid protein (HPeV-1–HPeV-8). HPeV-1 and HPeV-2, formerly known as echovirus 22 and echovirus 23, were originally considered to belong to the genus *Enterovirus* (*1*,*2*) but after genome sequencing were reclassified as members of a new genus in the family *Picornaviridae* (*3*). Recently, 6 other genotypes of parechovirus were isolated from young children with gastrointestinal, respiratory, and severe neurologic signs (*4*–*11*). Other HPeV genotypes continue to be characterized (www.picornaviridae.com/parechovirus/hpev/hpev.htm).

Despite the frequent infections and numerous HPeV genotypes detected in humans, information about HPeV infection in animals is scant. In this study, we detected HPeV in feces of monkeys with diarrhea and sequenced the complete genome of 1 isolate (SH6).

The Study

In April 2008, fecal specimens were collected from 116 macaques (3–6 years of age) with diarrhea on a monkey farm in People's Republic of China. Feces were suspended to 10% (wt/vol) in phosphate-buffered saline (0.01 M, pH 7.4), and total RNA was extracted from 200 μL by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Viral RNA was dissolved in 30 μL RNase-free water and stored at –80°C.

Primers (outside-L 5′-CTAGAGAGCTTGGCCGTCGG-3′, outside-R 5′-GTACCTTCTGGGCATCCTTC-3′, inside-L 5′-GGCCTTATACCCCGACTTGC-3′, and inside-R 5′-GGCCTTACAACTAGTGTTTG-3′) (*12*) were used for reverse transcription nested PCR to identify diverse HPeV genotypes by amplification of a 518-bp fragment located in the 5′ untranslated region (UTR). The expected-size DNA bands were excised from an agarose gel, purified with the AxyPrep DNA gel extraction kit (Axygen, Union City, CA, USA), cloned into pMD-18T vector (TaKaRa, Dalian, China), and sequenced (Applied Biosystems 3730 DNA Analyzer; Invitrogen). Feces from 6 of 116 monkeys were positive for HPeV. The HPeV sequences were compared with those of the HPeV genotype reference strains by using BLAST (www.ncbi.nlm.nih.gov/BLAST). Five of the 6 sequences showed closest identity to the 5′ UTR of HPeV-1 (90%–94%). The viral protein (VP)3/VP1 region of these 5 viruses was then PCR amplified and sequenced to confirm type 1 identity (*13*). The last monkey feces–derived sequence showed 97%–98% nucleotide identity to HPeV-6 strains. This strain was fully sequenced.

A 674-nt region of the 5′ UTR, an open reading frame (ORF) encoding a polyprotein precursor of 2,182 aa, and a partial 3′ UTR of 88 nt (7,311 bp) were sequenced. The near full genome showed 96% nucleotide identity with the genotype 6 reference genome (AB252582). The polyprotein encoded capsid proteins VP0 (312 aa), VP3 (229 aa), and VP1 (234 aa) and nonstructural proteins 2A (150 aa), 2B (122 aa), 2C (329 aa), 3A (117 aa), 3B (20 aa), 3C (200 aa), and 3D (469 aa). The integrin binding motif arginine–glycine–aspartic acid was identified close to the C terminus of VP1 (*8*,*14*).

We performed phylogenetic analysis using the nearly full genome of SH6 and 17 representative HPeV and related viruses (Figure). Results confirmed that SH6 belonged to genotype 6 and clustered closely with the reference genome from Japan and a strain from the Netherlands (EU077518), forming an HPeV-6 subgroup (Figure).

Conclusions

Previous studies have documented frequent human infections with different HPeV genotypes (www.picornaviridae.com/parechovirus/hpev/hpev.htm). We detected HPeV genotypes 1 and 6 in the feces of 6 of 116 monkeys with diarrhea. A similar analysis of a healthy monkey control

Author affiliations: Shanghai Jiao Tong University, Shanghai, People's Republic of China (T.L. Shan, L. Cui, C.L. Yuan, W. Zhao, W. Guo, X.Q. Dai, Y. Yu, X.G. Hua); Huazhong Agricultural University, Wuhan, People's Republic of China (C.M. Wang); Blood Systems Research Institute, San Francisco, California, USA (E. Delwart); and University of California, San Francisco (E. Delwart)

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[1]These authors contributed equally to this article.

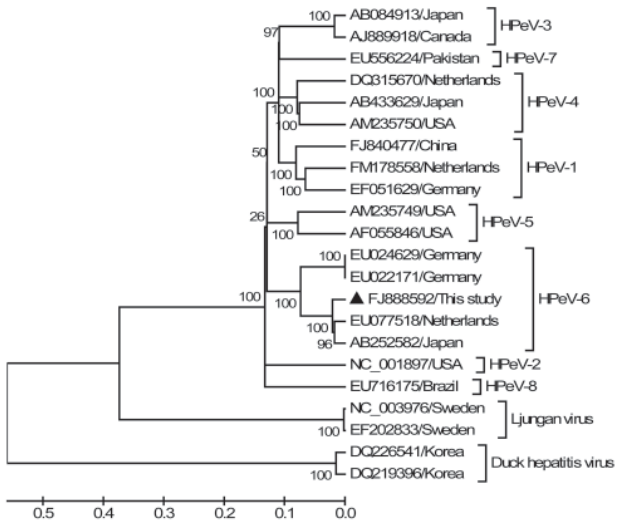

Figure. Phylogenetic analysis of the complete genomes. Phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates using MEGA4.0 software (www.megasoftware.net) with an alignment of the nearly full genome isolated in this study and 17 human parechovirus (HPeV) and related genomes. Bootstrap values are indicated at each branching point. GenBank accession numbers and countries of origins are indicated. The isolate of genotype 6 is marked with a triangle. Scale bar indicates estimated phylogenetic divergence.

group is needed to determine whether an association exists between HPeV infections of monkeys and diarrhea. On the basis of the close similarities between virus feces–derived sequences and HPeV, these viruses might have been transmitted by the fecal–oral route from humans to monkeys. The multiple HPeV genotypes detected indicated that viral transmission occurred on multiple occasions. Monkeys may therefore serve as an animal model for HPeV infection and possibly pathogenesis.

Mr Shan is a PhD candidate at Shanghai Jiao Tong University in Shanghai, China. His interests focus on discovery of novel viruses from biological samples using metagenomic methods.

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Address for correspondence: X.G. Hua, Building of Agriculture & Biology, Shanghai Jiao Tong University, 800 Dongchuan Rd, Shanghai, 200240, People's Republic of China; email: hxg@sjtu.edu.cn

ACC-1 β-Lactamase–producing *Salmonella enterica* Serovar Typhi, India

To the Editor: Typhoid fever, caused by *Salmonella enterica* serovar Typhi, is a serious form of enteric fever. In 2000, the worldwide number of typhoid cases was estimated to be >21,000,000, and there were >200,000 deaths from this disease (*1*).

Ciprofloxacin is the first-line drug of choice for treatment of patients with typhoid fever, but there has been an increase in strains resistant to ciprofloxacin (*2*) and resistance to third-generation cephalosporins has emerged (*3*). There are sporadic reports of high resistance to ceftriaxone in typhoidal salmonellae (*3*,*4*) in which CTX-M-15 and SHV-12 extended spectrum β-lactamases (ESBLs) have been reported. To date, there are no reports of *AmpC* β-lactamases in typhoidal salmonellae. *AmpC* β-lactamases confer resistance to a broad spectrum of β-lactams, which greatly limits therapeutic options. We investigated an isolate of *S.* Typhi by using serotyping, antimicrobial drug susceptibility testing, PCR screening for β–lactamase genes, and sequence analysis to confirm the identity of the isolate and the β–lactamase gene involved in conferring resistance to this isolate.

The isolate was obtained in Bangalore, India, in August 2009, from the blood of a female patient (14 years of age) who was hospitalized because of signs and symptoms of enteric fever. She had no history of having received antimicrobial drugs. After a blood sample was cultured, the patient was empirically treated with ceftriaxone but did not clinically improve.

Culture yielded gram-negative bacteria after 48 hours. The isolate was identified by standard biochemical methods as *S.* Typhi. Identification was confirmed by using *Salmonella* spp. polyvalent O, O9, and H:d antisera (Murex Biotech, Dartford, UK). Susceptibility to antimicrobial drugs was assessed by using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute guidelines (www.clsi.org). The isolate was resistant to ampicillin, piperacillin, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, aztreonam, amoxicillin/clavulanate, and cefepime. It was susceptible to chloramphenicol, trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin, and meropenem.

Treatment was changed to ciprofloxacin (500 mg every 12 h for 7 d). The patient recovered within 72 hours and was discharged. MICs were determined for ciprofloxacin, gatifloxacin, ofloxacin, ceftazidime, ceftriaxone, and amoxicillin/clavulanate by using the Etest (AB Biodisk, Solna, Sweden) (Table). MIC for ceftriaxone was confirmed by an agar dilution method (www.clsi.org). The isolate was tested for ESBLs by using a method with disks containing ceftazidime (30 μg) and ceftazidime/clavulanate (30 μg/10 μg). The *AmpC* disk test for detection of plasmid-mediated *AmpC* β-lactamase was conducted according to standard methods (*5*).

PCR screening and sequencing was performed to identify β-lactamase resistance genes bla_{TEM}, bla_{SHV}, bla_{OXA-1} group, bla_{CTX-M}, and *AmpC* as described (*6*,*7*). Sequencing of β-lactamase gene amplicons was conducted at the Vector Control Research Centre in Pondicherry, India. The BLASTN program (www.ncbi.nlm.nih.gov/BLAST) was used for database searching. We also used a nested PCR specific for the flagellin gene of *S.* Typhi to confirm identity of the isolate (*8*). The nested PCR amplicon was sequenced to confirm identity of the flagellin (*fliC*) gene of *S.* Typhi. Sequencing of the flagellin gene product was conducted by Cistron Bioscience (Chennai, India).

The isolate was negative for ESBL production. PCR amplification and sequencing showed that the isolate harbored bla_{TEM-1} and $bla_{ACC-1.}$ The isolate was negative by PCR for other β-lactamases tested. TEM-1 is one of the most commonly encountered β-lactamases in the family *Enterobacteriaceae* and can hydrolyze narrow-spectrum penicillins and cephalosporins.

We report ACC-1 *AmpC* β-lactamase in typhoidal salmonellae. *S.* Typhi could have acquired the *AmpC* β- lactamase from drug-resistant bowel flora. After the isolate was found to be highly resistant to ceftriaxone, the change in therapy to ciprofloxacin helped in recovery of the patient without any sequelae.

ACC-1 *AmpC* β-lactamases originated in *Hafnia alvei* and are now found in various members of the family *Enterobacteriaceae* (*9*). The ACC-1 *AmpC* β-lactamases are exceptional in that they do not confer resistance to cephamycins (*10*). Our isolate contained bla_{TEM-1} and bla_{ACC-1} and was resistant to cefoxitin and cefepime but susceptible to meropenem. Bidet et al. (*9*) reported isolating *Klebsiella pneumoniae* resistant to cefoxitin and cefepime and intermediate resistance to imipenem. Atypical resistance was attributed to ACC-1 β-lactamase production and loss of a 36-kDa major outer membrane protein (*9*). We did

Table. MICs for isolate of *Salmonella enterica* serovar Typhi, Bangalore, India, 2009

Drug	MIC
Amoxicillin/clavulanic acid	>256
Piperacillin/tazobactam	12
Ceftazidime	>256
Cefotaxime	>256
Ceftriaxone	>256
Ciprofloxacin	0.094

not analyze changes in the outer membrane proteins responsible for alteration of permeability.

Continual monitoring of drug resistance patterns is imperative. Antimicrobial drug susceptibility testing should be conducted for clinical isolates, and empirical antimicrobial drug therapy should be changed accordingly. *AmpC* β-lactamase genes will eventually be transferred to typhoidal salmonellae, which may pose a threat to public health. Spread of broad-spectrum β-lactamases would greatly limit therapeutic options and leave only carbapenems and tigecycline as secondary antimicrobial drugs.

Bindiganavile N. Gokul, Godfred A. Menezes, and Belgode N. Harish

Author affiliations: Sri Devaraj Urs Medical College, Kolar, India (B.N. Gokul, G.A. Menezes); Fortis Hospitals, Bangalore, India (B.N. Gokul); and Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India (G.A. Menezes, B.N. Harish)

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Address for correspondence: Belgode N. Harish, Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry 605006, India; email: drbnharish@yahoo.com

Endocarditis Caused by *Actinobaculum schaalii*, Austria

To the Editor: In May 2009, a 52-year-old man was hospitalized with middle cerebral artery stroke and fever of unknown origin. He had a complicated medical history of middle cerebral artery stroke and mechanical valve replacement of the aortic valve 2 years earlier and gastric-duodenal angiodysplasia. Two months before the most recent hospitalization, he had been hospitalized because of fever and anemia; blood cultures were positive; Gram stain identified coryneform rods that did not grow in culture. Antimicrobial drug therapy with levofloxacin (400 mg 1×/d) was initiated, and the patient was discharged.

At the most recent admission, laboratory testing showed a leukocyte count of 5.92×10^3 cells/μL, with 81% neutrophils, 7% lymphocytes, and 9% monocytes; thrombocyte count was 338×10^3 cells/μL. C-reactive protein level was 62.6 mg/L (reference value <8 mg/L). Basic serum and urine chemical profiles and urine culture were unremarkable. Empiric antimicrobial drug therapy with piperacillin–tazobactam (4.5 g 3×/d) was initiated and discontinued after 5 days because of clinical improvement. The next day, the patient's condition deteriorated, C-reactive protein level increased from 15 mg/L to 32 mg/L, and blood was collected for culture on the day after piperacillin-tazobactam discontinuation and the next 2 days. After 4 days of incubation, bacterial growth was detected in 1 aerobic and 3 anaerobic samples. Gram stain showed positive coryneform rods. Within 48–72 hours, the isolate yielded growth on blood, chocolate, and Schaedler agar; colonies were 1–2 mm in diameter and gray. The specificity of the organism was unsatisfactory with the system we used (API Coryne sys-

tem; bioMérieux, Craponne, France) (Table).

A 16S rRNA gene analysis was performed by using eubacterial universal primers. Subsequently, a BLAST search (www.ncbi.nlm.nih.gov/BLAST) of the partial 16S rRNA gene sequence (730 bp) was performed by using the taxonomy browser of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Homology of 99.7% (728/730 bp) was detected for *Actinobaculum schaalii*. The isolate was deposited in GenBank under accession no. GQ355962. MICs were obtained for various antimicrobial drugs, including amoxicillin–clavulanic acid (0.25 mg/L), piperacillin–tazobactam (0.125 mg/L), and levofloxacin (1 mg/L).

Infectious disease specialists were consulted. On physical examination, the patient exhibited Janeway lesions on hands and feet and a temperature of 38.4°C. Transesophageal echocardiogram showed filiform vegetation on the aortic valve, which was not consistent with echocardiographic major criteria. According to the modified Duke criteria (*5*), the patient's condition fulfilled 1 major clinical criterion (at least 2 positive cultures of blood samples collected 12 hours apart) and 3 minor clinical criteria (prosthetic aortic valve, temperature >38°C, Janeway lesions). Accordingly, definite infective prosthetic valve endocarditis was diagnosed. Intravenous antimicrobial drug therapy with piperacillin-tazobactam (4.5 g 3×/d) was initiated, followed by oral therapy with amoxicillin–clavulanate acid (1 g 3×/d) for 8 weeks. Because a repeated transesophageal echocardiogram 10 days after initiation of antimicrobial drug therapy showed no infective endocarditis, heart surgeons declined to replace the prosthetic valve. The patient's condition improved, and 2 weeks later he was discharged in good clinical condition.

Four species within the genus *Actinobaculum* have been described: *A. massiliae* (causing urinary tract infection [UTI] and superficial skin infection), *A. urinale* (isolated from human urine), *A. suis*, and *A. schaalii* (*1*,*3*,*4*,*6*). *A. schaalii*, which is difficult to identify by culture, has been reported to cause UTI in elderly patients with underlying urologic conditions; a few studies have reported subsequent urosepsis, abscess formations, and osteomyelitis (*1*,*3*,*6*–9). Recently, Bank et al. (*7*) reported development of a TaqMan real-time quantitative PCR for *A. schaalii* and consecutive detection of the organism in 22% of 252 routine urine samples of patients

Table. Comparison of isolated *Actinobaculum schaalii* with related human pathogens reported in the literature

	Isolate that caused endocarditis	Reaction of (reference)*				
Characteristic	*A. schaalii*†	*A. schaalii* (*1*,*2*)	*Actinobaculum massiliae* (*3*)	*Actinobaculum urinale* (*4*)	*Arcanobacterium pyogenes*	*Actinomyces turicensis* (*1*,*2*)
Catalase reaction	–	–	–	–	–	–
β-hemolysis on sheep blood agar	–	–/w‡	–	w	+	w
Nitrate reduction	–	–	–	–	–	–
Pyrazinamidase	–	V	+	–	–	–
Pyrrolidonyl arylamidase	+	+	–	–	+	–
Alkaline phosphatase	+	–§	–	–	V	–
β-glucuroniadase	–	–	–	+	+	–
β-galactosidase	–	–	–	–	+	–
α-glucosidase	+	+	+	–	+	+
N-acetyl-β-glucosaminidase	–	–	–	–	V	–
Esculin hydrolysis	–	–§	–	–	–	–
Urease activity	–	–	–	+	–	–
Gelatin hydrolysis	–	–	–	–	+	–
Acid from						
Glucose	–	V	+	+	+	+
Ribose	+	+	+	+	+	+
Xylose	+	V	+	–	+	+
Mannitol	–	–	–	–	–	–
Maltose	+	+	+	+	+	V
Lactose	–	–	–	–	+	–
Sucrose	+	V	–	+	V	+
Glycogen	–	–	+	–	V	–

*API Coryne system (bioMérieux, Craponne, France) profile for our isolate was compared with those described in the references (*Actinobaculum schaalii* [14 strains], *A. massiliae* [1 strain], *A. urinale* [1 strain], *A. turicensis* [43 strains]) and those in the manufacturer´s database for *Arcanobacterium pyogenes*. +, ≥90% of strains positive; –, ≥90% strains negative; V, variable; w, weak.
†In API Coryne, the strain gave the profile number 4110621 (unacceptable profile because of lack of specificity).
‡*A. schaalii* was described as nonhemolytic for 5 patients (*1*) and as showing weak β-hemolysis only after 2–5 d in 9 cases (*2*).
§Reported as positive for 1 of 14 strains.

>60 years of age (*8*). Those findings suggest that *A. schaalii* is a common undetected pathogen, especially in elderly patients with unexplained chronic UTI.

We report infective endocarditis caused by *A. schaalii*. To our knowledge, infective endocarditis caused by *Actinobaculum* spp. has not been reported. However, several reports have documented endocarditis caused by *Arcanobacterium* spp. and *Actinomyces* spp., which are phylogenetically related to *Actinobaculum* spp. (*10*).

Characteristics of the patient reported here differed from those of patients in previous reports. He had no underlying urologic condition and could not recall any symptoms usually associated with UTI during the year before hospital admission. Urine culture remained negative for *Actinobaculum* spp. despite prolonged incubation for 5 days on chocolate agar in an atmosphere of 5% CO_2 and on Schaedler agar under anaerobic conditions.

This report highlights the usefulness of the recent development of a specific real-time PCR by Bank et al. (*7*), which may prove effective not only for patients typically at risk for *A. schaalii* but also for patients with a wider spectrum of infection. More studies are needed to identify the real prevalence of disease caused by this difficult-to-cultivate organism because it may occur in many other groups of patients.

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Martin Hoenigl, Eva Leitner, Thomas Valentin, Gernot Zarfel, Helmut J.F. Salzer, Robert Krause, and Andrea J. Grisold

Author affiliation: Medical University of Graz, Graz, Austria

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Address for correspondence: Andrea J. Grisold, Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Universitaetsplatz 4, A-8010 Graz, Austria; email: andrea.grisold@medunigraz.at

Mycobacterium chelonae Wound Infection after Liposuction

To the Editor: We recently investigated a case of *Mycobacterium chelonae* abdominal wound infection after liposuction performed under local anesthesia at an outpatient medical office. Our aim was to determine whether other cases of atypical mycobaterial infections had previously occurred after liposuction. *M. chelonae* is widely distributed in soil and water, including tap water. Atypical mycobacterial infections have been associated with skin and soft tissue infections, including infections after cosmetic surgeries, and outbreaks have been documented (*1*–*4*). Previously reported potential sources of liposuction equipment contamination have been inadequate disinfection or sterilization after rinsing of liposuction equipment with tap water, tap water used in cleaning liposuction cannulae, or the quaternary ammonium solution used to disinfect liposuction equipment (*2*,*4*). Increased numbers of procedures performed in freestanding medical centers (not connected with hospitals) that are not routinely monitored by infection control committees or equivalent oversight bodies may contribute to atypical mycobacterial infection (*1*).

Our investigation showed that proper cleaning, disinfection, and sterilization of liposuction equipment and other infection control issues at this medical office were concerns.

Except for the physician, only unlicensed medical assistants worked at this office. This staff had been trained to clean and sterilize liposuction equipment, but no written procedures existed for processing reusable liposuction equipment, no logs were kept of autoclave use for sterilization, and preventive maintenance checks and verification of sterility on the autoclave by using biological indicators as recommended by the manufacturer were not performed. The office did not have any general written infection control policies. Office staff mixed leftover solutions from open small bottles of povidone iodine and placed this mixture into larger containers. Staff stored wet alcohol-soaked cotton balls in multiuse containers for wiping tops of multidose vials instead of using individual alcohol prep pads; and 70% isopropyl alcohol solution from an open nonsterile bottle was used instead of sterile irrigation solutions to flush the liposuction suction cannula to dislodge tissue from the ports during the procedure.

Case finding and surveillance of acid-fast bacilli results routinely reported to our public health tuberculosis program did not indicate any other cases of postliposuction wound infections caused by atypical mycobacteria associated with this office. Laboratory testing of environmental samples, including tap water and faucet aerator samples, also did not indicate a source for *M. chelonae* in this outpatient office. This case was likely an isolated occurrence in which the case-patient acquired infection through an environmental source unrelated to this office. However, because of the infection control concerns observed in this office and because the incubation period for *M. chelonae* can be as long as 5 months (*2*), the physician was advised to develop infection control policies and procedures; develop protocols for cleaning, disinfecting, and sterilizing liposuction equipment in accordance with the manufacturer's recommendations; ensure autoclave sterility by using biological indicators; educate office staff about basic infection control practices and use of aseptic techniques; and notify public health officials of any further infections postliposuction.

Risk factors that cause or contribute to infectious disease outbreaks in outpatient settings include inadequate cleaning, disinfection, sterilization, and storage of instruments and equipment; inappropriate use of barrier equipment, such as gloves, by healthcare personnel; inadequate handwashing practices by healthcare workers; failure to use aseptic techniques; and lack of familiarity with established infection control practices by ambulatory care personnel (*5*). Also, in the outpatient setting, responsibility for implementing an infection control program usually is not assigned to a specific person (*5*), and outpatient medical offices are not routinely monitored by oversight bodies or infection control committees as are hospitals and outpatient surgical centers (*6*).

The California Business and Professions Code requires that outpatient surgery settings using anesthesia, other than local anesthesia or peripheral nerve blocks, be accredited by an oversight body. However, because this facility used only local anesthesia, it did not fall under this code of regulations for facility accreditation, oversight, certification standards, and quality assurance for general public health safety and welfare.

Lack of adherence to basic infection control principles, specifically in outpatient settings, has resulted in outbreaks (*1*,*7*–*10*). Our findings at this medical office further highlight the unaddressed infection control problems in outpatient settings. Because of insufficient oversight for the outpatient setting, professional organizations, state medical boards, and federal and state authorities should consider the need to systematically address infection control standards and monitoring tailored for this setting. As more healthcare procedures move to the outpatient setting, ensuring appropriate infection control practices can prevent outbreaks.

Moon J. Kim and Laurene Mascola

Author affiliation: Department of Public Health, Los Angeles, California, USA

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Address for correspondence: Moon J. Kim, Acute Communicable Disease Control Program–County of Los Angeles, Department of Public Health, 313 N Figueroa St, Rm 222, Los Angeles, CA 90012, USA; email: mokim@ph.lacounty.gov

Influenza A Pandemic (H1N1) 2009 Virus and HIV

To the Editor: The effects of influenza A pandemic (H1N1) 2009 virus infection in HIV-infected patients are unknown. We describe an HIV-infected patient with severe pandemic (H1N1) 2009 virus infection.

The patient was a 37-year-old, HIV-positive, former intravenous drug user in a methadone-substitution program. She had a history of smoking, hepatitis C, and mild chronic obstructive pulmonary disease not requiring treatment. Since 2007 her viral load had been <50 HIV RNA copies/mL, for which she received tenofovir, emtricitabine, and lopinavir/ritonavir. Her CD4 count in March 2009 was 542 cells/μL (25%).

On June 24, 2009, the patient entered the Hospital Universitario La Paz after 3 days of dyspnea and fever, without cough or sputum. Temperature was 39°C, blood pressure 118/74 mm Hg, pulse rate 110 beats per minute, respiratory rate 30 breaths per minute, and oxygen saturation 85% on room air (fraction of inspired oxygen [FiO_2] 21%). Lung wheezes were audible. Laboratory testing showed leukocytosis with neutrophilia and oxygen partial pressure (pO_2) 70.9 mm Hg (FiO_2 21%). Chest radiograph findings were consistent with bacterial pneumonia (Figure, panel A). Empirical treatment with clarithromycin and ceftriaxone was started. After full clinical recovery, the patient was discharged on June 30 and prescribed oral clarithromycin and cefixime.

At a routine follow-up visit on July 2, the woman was asymptomatic and had fewer leukocytes and neutrophils, creatine kinase 800 U/L (reference <145), and lactate dehydrogenase 373 U/L (reference <247). On July 3, she returned to the hospital because of dyspnea and high fever (38.5°C). Oxygen saturation was 75%, pO_2 33.8 mm Hg (FiO_2 21%), and blood pressure 90/54 mm Hg. Chest radiographs showed alveolar infiltrates in the right lower lobe (Figure, panel B). Because her deterioration was attributed to nosocomial infection, she was given meropenem, linezolid, and levofloxacine and was hospitalized. Within the next 96 hours, her condition deteriorated further to drowsiness, hypotension, oxygen saturation 92%, pO_2 60.5 mm Hg (FiO_2 50%), and new radiographic bilateral alveolo-interstitial infiltrates appeared (Figure, panel C).

On July 7, real-time reverse transcription–PCR of a nasopharyngeal swab confirmed influenza A pandemic (H1N1) 2009 virus (*1*). Other infectious causes for bacterial and viral pneumonia were excluded. The patient received mechanical ventilation for 7 days and oseltamivir. She was discharged after 21 days. She still had dyspnea after exertion and radiologic sequelae on chest radiograph (Figure, panel D).

No patients or healthcare workers who had had contact with the patient had confirmed pandemic (H1N1) 2009

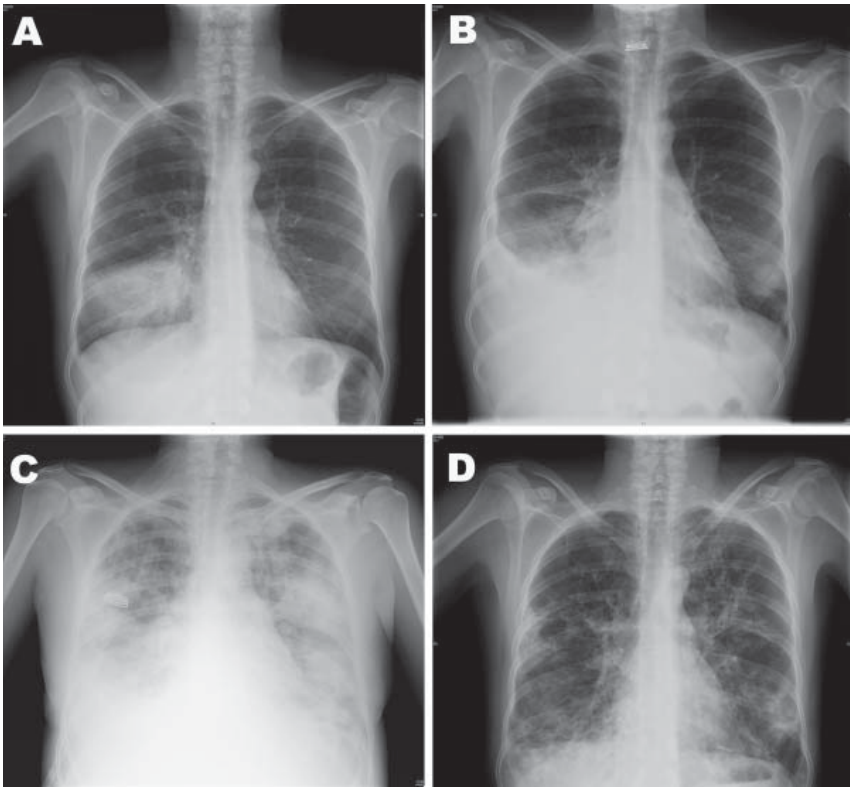

Figure. Chest radiographs of 37-year-old, HIV-positive woman with severe pandemic (H1N1) 2009 virus infection, 2009. A) June 24, alveolar infiltrate in the right lower lobe. B) July 3, minimal pleural effusion, alveolar infiltrate on right lower lobe, and possibly left lower lobe infiltrate. C) July 6, bilateral alveolo-intertitial infiltrates. D) July 29, bilateral peribroncovascular thickening with fibro-cicatricial changes; conserved lung volumes.

virus infection. The patient shared a room with a highly immunocompromised HIV patient, who was negative for pandemic (H1N1) 2009 virus but received oseltamivir as prophylaxis.

Few data are available on pandemic (H1N1) 2009 virus infection in immunocompromised patients. Of 30 patients with pandemic (H1N1) 2009 in California (*2*), 6 had underlying conditions involving immunosuppression but none was infected with HIV. The clinical course of pandemic (H1N1) 2009 virus infection in immunocompromised patients was similar to that in nonimmunocompromised patients, although not all received oseltamivir. The patient reported here was not severely inmunosuppresed; her CD4 count was stable at >300 cells/μL.

Severe pandemic (H1N1) 2009 virus infection most commonly produces fever, dyspnea, respiratory distress, and bilateral patchy pneumonia (*3*), which can initially be interpreted as bacterial pneumonia and consequently treated with antimicrobial drugs (*2*–*7*). We believe that the patient reported here first had a community-acquired pneumonia with a rapid response to treatment and that she secondarily had respiratory distress caused by pandemic (H1N1) 2009 virus pneumonia. We cannot confirm whether the pandemic (H1N1) 2009 virus infection was nosocomial or community acquired.

The increased creatine kinase and lactate dehydrogenase and lymphopenia in this patient resemble that reported in Mexico by Perez-Padilla et al. (*3*). Although these laboratory parameters are unspecific, they could serve as an alert to pneumonia caused by pandemic (H1N1) 2009 virus instead of bacteria. The Centers for Disease Control and Prevention (CDC) recommends testing all HIV-infected patients suspected of having pandemic (H1N1) 2009 virus infection (*8*). The Spanish Ministry of Health and Madrid Department of Health recommends this testing for patients with an erratic outcome from common pneumonia.

For HIV-infected patients who meet case definitions for confirmed, probable, or suspected pandemic (H1N1) 2009 virus infection, CDC recommends empiric antiviral drug treatment (*8*). There are no known contraindications for co-administration of oseltamivir, a neuraminidase inhibitor, with antiretroviral medications; no interactions have been demonstrated (*8*,*9*).

Although clinical signs and treatment of pandemic (H1N1) 2009 are similar for patients with and without HIV infection, HIV-infected patients with suspected pandemic (H1N1) 2009 virus symptoms should be treated as soon as possible. CDC recommends the use of influenza antiviral drugs, but this recommendation might change as additional data on this therapy for HIV-infected patients become available. At this time, however, we strongly recommend use of antiviral drugs for HIV-infected patients with suspected pandemic (H1N1) 2009 virus.

Marta Mora, Elena Rodriguez-Castellano, José Ramón Pano-Pardo, Juan González-García, Carolina Navarro, Juan Carlos Figueira, and José Ramón Arribas

Author affiliation: Hospital Universitario La Paz, Madrid, Spain

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Address for correspondence: Elena Rodriquez-Castellano, Hospital Universitario La Paz, Internal Medicine, Paseo de la Castellana 261 Madrid, Madrid 28670, Spain; email: elena.rodriguez.c@gmail.com

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.

Roseomonas sp. Isolated from Ticks, China

To the Editor: *Roseomonas*, which produces pink colonies, is a newly described genus of gram-negative bacteria (*1*). Human infections with *Roseomonas* spp. have been reported in the past decade, mostly in immunocompromised persons with underlying diseases such as acute leukemia, cancer, and rheumatoid arthritis (*2*–*5*). A healthy woman was reported to be infected by *R. gilardii* after being bitten by a spider (*6*), which indicated possible transmission by an arthropod.

As a part of an investigation of tick-borne diseases, we collected actively questing and feeding ticks in Xinjiang Autonomous Region, People's Republic of China, in the summers of 2007 and 2008 (*7*). Ticks were washed in 75% ethanol, 30% hydrogen peroxide, and sterile distilled water. Five ticks of the same species, sex, and developmental stage were pooled and ground in 1 mL of saline. A 0.1-mL suspension was placed on cysteine heart agar plates containing chocolate and 9% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and supplemented with colistin, amphotericin, lincomycin, trimethoprim, and ampicillin. Eggs laid by engorged female ticks were collected and kept at room temperature.

Fourteen days after hatching, larval ticks were processed as a batch by using the same methods described above. After 2–3 days of incubation at 37°C, pink colonies were observed in 9 cultures, 8 of which originated from engorged female *Dermacentor nuttalli* ticks. The other culture originated from larval ticks, the progeny of an engorged female *D. nuttalli* tick. Colonies were pinpoint, pale pink, shiny, raised, and mucoid. The pink color of the colonies became pronounced when the bacteria were transferred onto plates containing Luria-Bertani agar. Bacteria were gram-negative, plump, coccoid rods, in pairs or short chains. Electron microscopy showed that each organism was ≈0.7 × 1.1 μm.

The 9 isolates showed identical phenotypic and biochemical characteristics, which were similar to those of previously reported *Roseomonas* spp. (*1*). However, the isolates required a lower salt concentration (≤4% NaCl) and a higher temperature (37°C instead of ≤35°C) for optimal growth than other *Roseomonas* spp. Antimicrobial drug susceptibility tests showed that the isolates were susceptible to aminoglycosides (amikacin, gentamicin, and tobramycin), tetracycline, and a β-lactam (imipenem) and resistant to cephalosporins (similar to *R. cervicalis*) (*1*) and sulfamethoxazole.

To further characterize the *Roseomonas* sp. isolated in this study, we amplified and sequenced the 16S rRNA gene. Sequences of the 9 isolates were identical to each other and showed 98%–99.1% similarity with reported species within the genus *Roseomonas*. A phylogenetic tree based on 16S r RNA genes (Figure) showed that the *Roseomonas* sp. identified in this study (representative strain XTD 510, GenBank accession no. EU742165) was in the same branch as *R. cervicalis* ATCC 49957 (GenBank accession no. AY150047). The new isolate was not genetically related to *R. fauriae* and *R. genespecies* 6, which have been reported as not belonging to the genus *Roseomonas* (*8*). The new isolate was also distinct from 2 other species from China, *R. lacus* TH-G33 (GenBank accession no. AJ 78600), which was isolated from freshwater lake sediment (*9*), and *Roseomonas* sp. JS018 (GenBank accession no. DQ 010108), which was isolated from soil (*10*).

We isolated a novel *Roseomonas* sp. from adult *D. nuttalli* ticks and their larval progeny and obtained evidence of transovarial transmission. Although we cannot conclude that ticks are vectors or reservoirs of *Roseomonas* spp., their roles in transmitting the bacteria deserve further study. *D. nuttalli* ticks

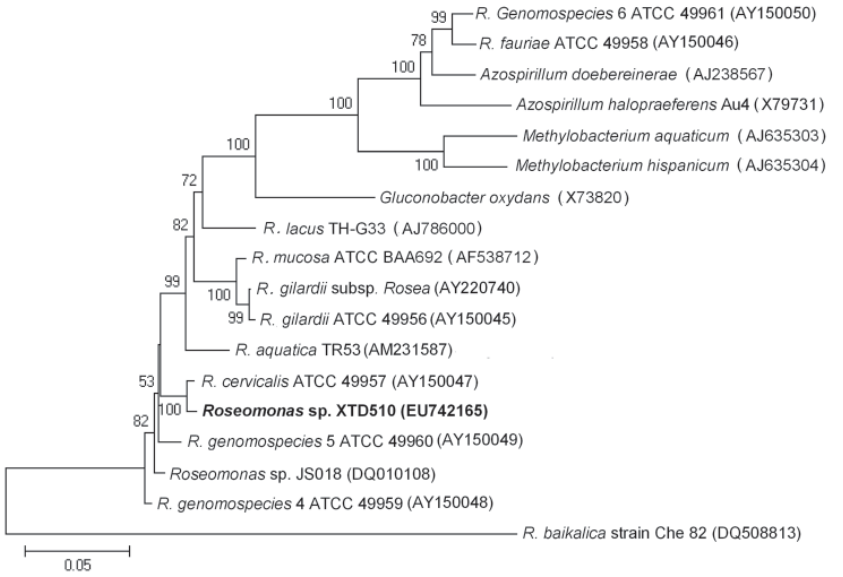

Figure. Unrooted phylogenetic tree based on 16S rRNA gene sequences of *Roseomonas* spp. Tree was constructed by using MEGA 4.0 software (www.megasoftware.net) and the neighbor-joining method with 1,000 bootstrap replicates. Genetic distances were calculated by using the Kimura 2-parameter correction at the nucleotide level. Bootstrap values >50% are shown. The isolate obtained in this study is shown in **boldface**. GenBank accession numbers of reference strains are marked after each strain name. Scale bar indicates nucleotide substitutions per site.

are a dominant species in the study area and usually parasitize a variety of wild and domestic animals. These ticks often feed on humans as alternative hosts. Because this *Roseomonas* sp. is not a common pathogen, its role in public health and veterinary medicine is unkown.

Phenotypic characterization of the isolates indicated similarities with previously reported *Roseomonas* spp. Phylogenetic analysis showed that the novel *Roseomonas* sp. is closely related to *R. cervicalis*, which was isolated from a cancer patient. Our isolates also differed from 2 reported strains isolated from freshwater lake sediment in Jiangsu Province, China (*9*) and from soil in Fujian Province, China (*10*). This result indicated the species diversity of the genus *Roseomonas*, which might be related to different bacterial origins. Because of the unique biochemical characteristics, antimicrobial drug susceptibilities, and novel isolation source of our isolates, the pathogenesis of this organism should be investigated.

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Wei Liu,[1] Fang Zhang,[1] Er-Chen Qiu, Jun Yang, Zhong-Tao Xin, Xiao-Ming Wu, Fang Tang, Hong Yang, and Wu-Chun Cao

Author affiliations: Beijing Institute of Microbiology and Epidemiology, Beijing, People's Republic of China (W. Liu, F. Zhang, E.-C. Qiu, X.-M. Wu, H. Yang, W.-C. Cao); Chinese People's Armed Police Force Center for Disease Control and Prevention, Beijing (J. Yang, F. Tang); and Chinese National Human Genome Center, Beijing (Z.-T. Xin)

[1]These authors contributed equally to this article.

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Address for correspondence: Wu-Chun Cao, Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, 20 Dong-Da St, Fengtai District, Beijing 100071, People's Republic of China; email: caowc@nic.bmi.ac.cn

Misidentification of *Mycobacterium kumamotonense* as *M. tuberculosis*

To the Editor: Because of slow growth of mycobacteria, use of rapid tests to identify them is strongly recommended; rapid tests are widely used as an advanced diagnostic tool in clinical laboratories (*1*,*2*). These tests are particularly useful for diagnosing extrapulmonary mycobacterioses and identifying unusual mycobacteria as etiologic agents (*3*). Commercial probes are frequently used for rapid and specific identification of mycobacteria, especially *Mycobacterium tuberculosis* complex. However, cross-reactivity of DNA probes between mycobacterial species could result in incorrect diagnosis and treatment of patients (*4*,*5*). Misidentification could be a problem if a newly described species, such as *M. kumamotonense* (*6*), were an etiologic agent of a disease.

In July 2006, we obtained a fine-needle, puncture aspiration biopsy specimen from a cervical lymph node of a 30-year-old man at Doce de Octubre Hospital (Madrid, Spain). The patient was a recent immigrant from Paraguay and was HIV positive (C2 stage of infection). A biopsy specimen from a cervical lymph node showed necrotizing granulomatous lymphadenopathy. A computed tomographic scan showed cervico-thoraco-abdominal, multiple cervical,

supraclavicular, axillar, paratracheal, and mediastinal lymphadenopathies. The patient had a CD4 cell count of 219 cells/mm^3 and an HIV viral load of 197,181 copies/mL.

The aspiration sample was positive for acid-fast bacilli by fluorescent staining. The clinical isolate (designated 1369) obtained from the aspirate sample was grown in liquid media (MGIT Diagnostic Kit; Becton Dickinson Diagnostics, Sparks, MD, USA) and identified as *M. tuberculosis* complex by using the AccuProbe System (bioMérieux, Marcy l'Etoile, France).

A diagnosis of lymphoid tuberculosis was made, and the patient was treated with isoniazid, rifampin, ethambutol, and pyrazinamide. After 1 month, rifampin was withdrawn because of a cutaneous exanthem. Three months later, the clinical status of the patient had improved, fever had disappeared, and sizes of cervical and axillary lymph nodes had decreased. Treatment with tenofovir, emtricitabine, and lopinavir/ritonavir was started. Two weeks later, an immune reconstitution syndrome and adenopathies developed, but these resolved in 1 month.

Five months after treatment was started, susceptibility testing in a reference laboratory showed that isolate 1369 was *M. kumamotonense*. The isolate showed 100% identity with the 16S rRNA gene sequence of *M. kumamotonense* (GenBank accession no. AB239925). Results of PCR restriction analysis of heat shock protein 65 gene (*7*) (http://app.chuv.ch/prasite/index.html) were consistent with those for *M. kumamotonense*. The isolate was susceptible to ethambutol, rifampin, cycloserine, and ethionamide and resistant to isoniazid, streptomycin, pyrazinamide, and kanamycin.

Because of the improvement in the clinical status of the patient, treatment continued without modification for 18 months. At this time, his CD4 cell count was 488 cells/mm^3 and his HIV viral load was ≤50 copies/mL. In July 2009, the patient was asymptomatic and had a CD4 cell count of 631 cells/mm^3 and an HIV viral load ≤50 copies/mL.

To confirm misidentification of *M. kumamotonense* as a member of the *M. tuberculosis* complex, other commercial probes were tested. Isolate 1369 was also misidentified as *M. tuberculosis* complex by Inno-LIPA v2 (Innogenetics, Ghent, Belgium). The isolate was identified as *Mycobacterium* sp. by Geno-Type (Hain Lifescience, Nehren, Germany). The 3 commercial probes we used had different genome region specificities, all in the mycobacterial ribosomal operon. The AccuProbe System was specific for 16S rDNA, Inno-LIPA v2 was specific for internal transcribed spacer 1, and Geno-Type was specific for 23S rDNA. Only Geno-Type did not show cross-reactivity between *M. tuberculosis* complex and *M. kumamotonense*. The clinical isolate was identified as *M. kumamotonense*, a new, slow-growing mycobacterium that was first isolated from an immunocompetent patient in Japan (*6*). We showed that this species caused extrapulmonary disease in an HIV-positive patient.

Misidentification of *M. kumamotonense* as *M. tuberculosis* complex by commercial DNA probes has serious clinical implications. Once a patient is given a diagnosis of tuberculosis, he or she will be treated with specific drugs for a long period and be prone to adverse side effects. Furthermore, *M. kumamotonense* is resistant to many drugs used during typical treatment. After a diagnosis of tuberculosis, patient contacts need to be investigated to identify new cases. Emerging mycobacterial pathogens, such as *M. kumamotonense*, may also cause pulmonary and extrapulmonary infections that are also caused by other members of this genus and could be misidentified as *M. tuberculosis*.

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Almudena Rodríguez-Aranda, María S. Jiménez, Jesús Yubero, Fernando Chaves, Rafael Rubio-García, Elia Palenque, María J. García, and M. Carmen Menéndez

Author affiliations: Hospital Universitario Doce de Octubre, Madrid, Spain (A. Rodríguez-Aranda, F. Chaves, R. Rubio Garcia, E. Palenque); Instituto de Salud Carlos III, Madrid (M.S. Jiménez); and Universidad Autónoma de Madrid, Madrid (J. Yubero, M.J. García, M.C. Menéndez)

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Address for correspondence: M. Carmen Menéndez, Department of Preventive Medicine, Public Health and Microbiology, School of Medicine, Autonoma University of Madrid, St/ Arzobispo Morcillo s/n 28029, Madrid, Spain; email: carmen.menendez@uam.es

Mycobacterium conceptionense Infection after Breast Implant Surgery, France

To the Editor: *Mycobacterium fortuitum* complex members are rapidly growing mycobacteria found in water and soil (*1*). These opportunistic pathogens are responsible for posttraumatic skin and soft tissue infections. They also account for 60%–80% of postsurgical wound infections caused by rapidly growing mycobacteria (*2*), particularly after breast surgery (with or without prosthetic implants) (*3*). *M. conceptionense*, an emerging member of the *M. fortuitum* complex, was initially described in a case of osteomyelitis that occurred after an open fracture of the tibia (*4*). We report a case of *M. conceptionense* infection that occurred after breast surgery.

A woman 58 years of age had a left mastectomy with lymph node dissection and chemotherapy for breast carcinoma in March 2004. Three years later, she underwent breast reconstruction that used a cutaneomuscular latissimus dorsi flap with a prosthetic implant. Immediately after surgery, a fever (39°C) developed, but 3 blood cultures remained sterile. No treatment was administered, and she became afebrile within 3 days.

At day 15 after surgery, a serous discharge appeared in the tip of the skin flap. By day 21, the patient was again febrile, and the wound discharge was swabbed for analysis. On day 27, she underwent surgical revision with ablation of the breast implant, drainage, and sample collection. The leukocyte count was normal. However, the C-reactive protein level was 99 mg/L, and the erythrocyte sedimentation rate was 111 mm (first hour). Treatment with intravenous amoxicillin/clavulanic acid was started. Although the biologic parameters normalized, the serous discharge continued. Microscopic examination of specimens from days 21 and 27 yielded no bacteria in Gram- and Ziehl-Nielsen–stained pus specimens, and standard bacteriologic cultures remained sterile. *M. conceptionense*, identified by partial *rpo*B gene sequencing (100% identity with GenBank accession no. AY859695.1) (*4*), grew in both specimens after 8 days of incubation at 37°C under a 5% CO_2 atmosphere in Coletsos medium (bioMérieux, La Balme-les-Grottes, France). By the Etest method (*4*), both isolates were susceptible to several antimicrobial drugs, including clarithromycin, amikacin, ciprofloxacin, and doxycycline. The patient was treated with ciprofloxacin, azythromycin, and amikacin for 3 weeks, followed by ciprofloxacin and azythromycin for 4 weeks.

At patient's relapse 3 months later, *M. conceptionense* exhibiting identical antimicrobial drug susceptibility pattern was again isolated from the wound fluid. The patient was then treated with ciprofloxacin, azythromycin, and doxycycline for 6 months; subsequently, doxycycline alone was given for a total of 18 months. Results from the 2-month follow-up examination were unremarkable.

M. conceptionense was unambiguously identified by partial *rpoB* gene sequencing, a first-line tool for accurate identification of nontuberculous mycobacteria (*5*). A pathogenic role for *M. conceptionense* was supported by 1) its repetitive isolation from the wound;

Table. Three cases of *Mycobacterium conceptionense* infection in female patients*

Patient age, y	Clinical situation	Identification	Treatment		Reference
			Nature	Duration, mo	
31	Posttraumatic osteitis	16S rRNA, *soda, hsp65*, *recA*, *rpoB*†	Antimicrobial drug therapy: AMC	3	(*4*)
43	Subcutaneous abscess without trauma	partial 1,464-bp 16S rRNA gene‡	Surgery and antimicrobial drug therapy: COT and CLA; then DOX and CLA; then LIN and CLA	5	(*10*)
58	Breast implant infection	*rpo*B§	Surgery and antimicrobial drug therapy: CIP and AZY; then CIP, AZY, and DOX; then DOX	18	This report

*AMC, amoxicillin/clavulanic acid; COT, cotrimoxazole; CLA, clarithromycin; DOX, doxycycline; LIN, linezolid; CIP, ciprofloxacin, AZY, azythromycin. The outcome for all 3 patients was favorable.
†GenBank accession nos.: 16S rRNA, AY859684; *rpoB*, AY859695; *hsp65*, AY859678; *sodA*, AY859708; *recA*, AY859690.
‡GenBank accession no. AM884289.1.
§GenBank accession no. AY859695.1.

2) the absence of any other pathogen; and 3) wound healing during appropriate, long-term antimicrobial drug treatment. However, the source of infection remained unknown. The patient had a tattoo on the skin flap used for the breast reconstruction, and ink has been shown to be a source for rapidly growing mycobacteria other than *M. conceptionense* (*6*). However, the tattoo was 5 years old and clinically safe.

Recent reports have identified 12 *M. conceptionense* isolates from water collected in a cooling tower (*7*) and 9 isolates from various freshwater fish species (*8*). The type strain of *M. conceptionense* had been isolated after prolonged exposure of the patient to fresh water (*4*). These observations suggest that water is a potential source for *M. conceptionense*. In this patient, use of contaminated aqueous solutions or inadequately rinsed surgical equipment (*9*) was unlikely the source of infection because further investigations indicated proper use of sterilized, nonreusable surgical equipment. At home, the patient used well water, but results of tests used to detect *M. conceptionense* by culture and the presence of the *rpoB* gene in well water remained negative.

Because *M. conceptionense* is an emerging pathogen with only 2 reported cases of infection (*4*,*10*), the optimal treatment is unknown (Table). Current recommendations for breast implant infection from *M. fortuitum* include 6 months of appropriate antimicrobial drug treatment in addition to implant removal because surgery alone yields relapse within 4–6 weeks (*2*,*3*). Increased worldwide use of breast implants is likely to increase the number of *M. conceptionense* infections and will raise questions about the optimal management of these infections.

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Sandrine Thibeaut, Pierre-Yves Levy, Marie-Laure Pelletier, and Michel Drancourt

Author affiliations: Université de la Méditerranée, Marseille, France (S. Thibeaut, P.-Y. Levy, M. Drancourt); and Clinique la Casamance, Aubagne, France (P.-Y. Levy, M.-L. Pelletier)

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Address for correspondence: Michel Drancourt, Unité des Rickettsies, Faculté de Médecine, Université de la Méditerranée, 27 Blvd Jean Moulin, 13385 Marseille CEDEX 5, France; email: michel.drancourt@univmed.fr

Rapid Diagnostic Tests and Severity of Illness in Pandemic (H1N1) 2009, Taiwan

To the Editor: The recent pandemic (H1N1) 2009 (*1*) demonstrates the need for more sensitive rapid diagnostic tests (RDTs) to distinguish between influenza and other respiratory viruses, enhance influenza surveillance, and institute early antiviral therapy for patients who are severely ill or at high risk (*2*). In anticipation of the global spread of pandemic (H1N1) 2009, on August 15, 2009, the government of Taiwan began performing RDTs at clinics and hospitals for patients with influenza-like illness. This initiative was based in part on the notion that patients with higher viral loads would be more likely to have a positive RDT result and more severe disease. We report that RDTs may have paradoxically lower sensitivity for pandemic (H1N1) 2009 virus for patients with respiratory failure requiring mechanical ventilation, extracorporeal membrane oxygenation (ECMO) or both than for those without respiratory failure.

National Taiwan University Hospital is a 2,200-bed teaching hospital in Taiwan. This hospital provides primary and tertiary care and ECMO. All patients admitted with presumed severe influenza were immediately treated with oseltamivir during the 2009 pandemic. From July 25 through

December 28, 2009, we studied 20 patients with confirmed disease and 3 adult patients with suspected disease who met the US Centers for Disease Control and Prevention case definitions for pandemic (H1N1) 2009 (*3*).

An RDT (QuickVue A+B; Quidel, San Diego, CA, USA) was performed by using nasopharyngeal swab specimens. Genetic material specific for pandemic (H1N1) 2009 viruses was detected in nasopharygeal or throat swab specimens by real-time reverse transcription–PCR at the Centers for Disease Control and Prevention, Taiwan (*4*). Demographic and clinical characteristics of the 23 patients are shown in the Table.

Severity of illness was assessed within 24 hours of admission by determining the Acute Physiology and Chronic Health Evaluation II score (*5*). The highest Sequential Organ Failure Assessment score was calculated to predict outcome of critically ill patients during their stay in the intensive care unit (*6*). The Student *t* test was used to assess continuous variables, and χ^2 or Fisher exact tests were used to assess discrete variables. A p value <0.05 was considered significant. Statistical analyses were performed by using SAS software version 9.1 (SAS Institute, Cary, NC, USA).

There were no differences in age, sex, body mass index, underlying diseases, or occurrence of secondary bacterial infection between patients who received mechanical ventilation (n = 15) and those who did not (n = 8). There were no significant differences between the 2 groups in the median number of days from onset of illness to access to medical care. Patients receiving mechanical ventilation had higher severity-of-illness scores and longer times in the intensive care unit and the hospital. Sensitivity of the RDT was 100% for patients who did not receive mechanical ventilation and 26.7% for those who did (p<0.0001).

Median age of the 8 patients who received ECMO was 31 years. Only 3 patients had underlying diseases. RDT results were positive for only 1 of these patients. Five patients were tested by RDT more than one time before transfer or hospitalization. The interval from onset of illness to the first RDT was 1 d for 1 patient, 2 d for 4 patients, 3 d for 2 patients, and 6 d for 1 patient. Failure of the RDT to detect influenza was associated with a delay of ≥5 d in instituting antiviral therapy for 6 of 8 patients who received ECMO. However, ECMO was stopped for 7 patients who were discharged from hospital after a median duration of 23 d (interquartile range 11.5–54 d) of ECMO.

Table. Characteristics of 23 hospitalized patients with confirmed (n = 20) and suspected (n = 3) pandemic (H1N1) 2009, Taiwan*

Characteristic	No mechanical ventilation (n = 8)	Mechanical ventilation	
		Non-ECMO (n = 7)	ECMO (n = 8)
Age, y			
Mean ± SD	39.9 ± 12.8	44.4 ± 19.5	34.6 ± 15.0
≥65, no. (%)	0	1 (14.3)	0
Male sex, no. (%)	4 (50)	2 (28.6)	4 (50)
BMI, median (IQR)	24.2 (21.2–31.3)	19.4 (17.3–22.6)	27.6(21.9–33.6)
No. (%) with other diseases	4 (50)	7 (100)	3 (37.5)†
Highest SOFA score, mean ± SD‡	0.89 ± 0.93§	9.7 ± 5.6	10.5 ± 4.0
SOFA score ≥4, no. (%)	0§	7 (100)	8 (100)
APACHE II score, mean ± SD¶	1. 6 ± 2.5§	17 ± 6.9	19.6 ± 3.8
APACHE II score ≥15, no. (%)	0§	5 (71.4)	8 (100)
Secondary bacterial infection			
Streptococcus pneumoniae	1	0	1
β-Streptococci, non-A, B, D	0	0	1
Klebsiella pneumoniae	0	1	0
Duration from illness onset to first medical access, d, median (IQR)	2 (1–2.2)	3 (2.5–3)	2 (2–3)
RDT sensitivity, n/N (%)	8/8 (100) §	3/7 (42.9)	1/8 (12.5)
Duration from illness onset to antiviral therapy, d, median (IQR)	4 (3.8–5.8)	6 (4.5–6)	6.5 (5.5–7.2)
Duration of ICU stay, d, median (IQR)	0 (0–2)§	15 (11.5–27.5)	49.5 (22.8–56.2)
Length of hospital stay, d, median (IQR)	5.5 (4–9.2)§	27 (21.5–54.2)	55.5 (30.2–71.2)
28-day mortality rate, no. (%)	0	1 (14.3)	0
Complications			
Ventilator dependent	0	3	3
Hemodialysis	0	0	1

*ECMO, extracorporeal membrane oxygenation; BMI, body mass index; IQR, interquartile range; SOFA; Sequential Organ Failure Assessment; APACHE, Acute Physiology and Chronic Health Evaluation; RDT, rapid diagnostic test; ICU, intensive care unit.
†A 49-year-old man who had a renal transplant, a 17-year-old woman with congenital heart disease, and a 64-year-old man with diabetes and hypertension.
‡Range 0–24. Higher values indicate more severe disease.
§p<0.05 for 8 patients without mechanical ventilation vs. 15 patients with mechanical ventilation with and without ECMO support.
¶Range 0–71. Higher values indicate more severe disease.

This report demonstrates an apparently paradoxical inverse relationship between a positive RDT result and severity of illness among patients with pandemic (H1N1) 2009. This observation cannot be explained by differences in the time to access to medical care, performance of RDT (*7*), or prior antiviral therapy. Variants of pandemic (H1N1) 2009 virus may preferentially infect the lower respiratory tract in certain hosts (*8*). Invasive properties of pandemic (H1N1) 2009 virus and severity of illness may be more closely related to heterogeneity in host immunity than to viral load (*9*). US Centers for Disease Control and Prevention guidance advises that "hospitalized patients with suspected influenza should receive immediate empiric antiviral treatment…, a negative RIDT or DFA test result does not exclude influenza virus infection…" (*10*). Moreover, this guidance also recommends that collection of lower respiratory tract specimens may be useful for reverse transcription–PCR testing to improve diagnosis for patients suspected of having severe lower respiratory tract disease caused by pandemic (H1N1) 2009 virus. The current findings strongly support this recommendation, particularly for severely ill patients.

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Tsui-Mai Kao, Un-In Wu, and Yee-Chun Chen

Author affiliations: National Taiwan University Hospital, Taipei, Taiwan; and National Taiwan University College of Medicine, Taipei

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Address for correspondence: Yee-Chun Chen, Department of Internal Medicine, National Taiwan University Hospital, No. 7 Chung-Shan South Rd, Taipei 100, Taiwan; email: yeechunchen@gmail.com

Human *Brucella canis* Infections Diagnosed by Blood Culture

To the Editor: Brucellosis is a worldwide zoonosis caused by *Brucella* spp. The 4 species known to infect humans are *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* (*1*). Since 1999, 11 cases in Japan have been reported. Although no bacteria were isolated, serum antibody detection indicated that 4 were caused by *B. melitensis* or *B. abortus* acquired abroad and the other 7 by *B. canis* (*2*). Of these 7 patients, 2 were presumed to have received their infection from dogs, and the sources of infection for the other 5 are unclear. We report 2 cases of *B. canis* infection diagnosed by blood culture.

Patient 1 was a 71-year-old male pet shop manager with hypertension. He came to Chubu Rosai Hospital, Nagoya, Japan, on August 9, 2008, after having fever and fatigue for 3 weeks, which were nonresponsive to third-generation cephalosporins. At the time of admission, his temperature was 37.8°C, but physical examination findings were unremarkable. On day 2, gram-negative coccobacilli were detected in a culture of blood collected at the time of admission. Ceftriaxone (1 g 1×/d) was administered, but fever persisted. On day 5, coccobacilli were growing poorly on culture media. Because the patient's history indicated the possibility of a zoonotic disease, doxycycline (100 mg 2×/d) was administered. Thereafter, the patient's fever and generalized symptoms resolved. The blood specimen and isolated bacteria were sent to the National Institute of Infectious Disease, *B. canis* was identified by combinatorial PCR (*3*). Serum tube agglutination test indicated an antibody titer against *B. canis* of 1,280 (Table). On day 10, streptomycin (1 g 1×/d) was added to the treatment regimen. On day 33, the patient was discharged; his laboratory

values were almost within reference limits, and he continued taking doxycycline for 6 weeks and streptomycin for 2 weeks.

Patient 2, a previously healthy 44-year-old co-worker of patient 1, exhibited similar signs and symptoms—fever and general fatigue—that started around the same time as for patient 1 (3 weeks before August 9, 2008). Physical examination findings at that time were unremarkable. Blood tests indicated moderate liver dysfunction. Treatment with fosfomycin was not effective. On August 19, the day after the diagnosis of brucellosis was made for patient 1, patient 2 came to Chubu Rosai Hospital, where *B. canis* was identified from blood culture. Serum antibody titer was 320 (Table). This patient was treated with doxycycline (100 mg 2×/d) plus rifampin (600 mg 1×/d) for 6 weeks. All signs, symptoms, and liver dysfunction resolved.

Neither patient had an immune disorder. About 2 months before illness onset they had each handled, without protection, the placenta of an aborted dog fetus. Negative antibody results were obtained for other persons at risk for infection: laboratory workers who were exposed to the patients' specimens, the patients' families, and a veterinarian who had been stuck by a needle when collecting blood from pet shop dogs to examine for antibody against *B. canis.* We prescribed doxycycline plus rifampin for 3 laboratory workers because brucellosis is among the most commonly reported laboratory-acquired bacterial infections and because postexposure prophylaxis is recommended for persons at high risk for exposure (*4*).

Several days after identification of *B. canis* for patient 1, the dogs in the pet shop (37 dogs, 23 adults and their 14 puppies) were examined for antibody against *B. canis* by using the microplate agglutination test (*5*) and for the *B. canis*–specific gene by combinatorial PCR (*3*). A total of 6 dogs were positive for antibody (titers 320–5,120) and the specific gene; 5 were positive for antibody only (titers 320–5,120), and 4 were positive for the specific gene only. Only adult dogs had positive results. Blood cultures were positive for 6 dogs that were antibody positive. Dogs that were determined by any method to be infected and their puppies (with negative test results) were euthanized. Since January 2008, a total of 8 puppies from the infected dogs had been sold; they were located, tested, and found to not have antibody against *B. canis*. The local government reported this information to the Ministry of Health, Labour and Welfare, Japan, and the ministry shared the information with related organizations.

Caution is necessary when basing diagnosis on serum tube agglutination test because *B. canis* has rough surface antigen and does not cross-react with *B. abortus* antigen (smooth *Brucella* spp.), which is usually used to diagnose brucellosis (*1*). Furthermore, because brucellosis is relatively rare and signs and symptoms are nonspecific, the number of cases reported is thought to be underestimated (*6*–*8*). A recent report showed that 2.5% of dogs in Japan have antibody against *B. canis,* but adult dogs are rarely seriously ill despite this generalized systemic infection (*5*,*9*). Thus, if a febrile person has signs and symptoms of unknown cause and a history of close contact with dogs, brucellosis should be considered and appropriate action to prevent spread of infection should be taken.

Table. Laboratory data for 2 patients infected with *Brucella canis*, Japan, 2008

Patient no.	Isolation of *B. canis* by blood culture	*B. canis* titer (date of sample collection)*	*B. abortus* titer (date of sample collection)*
1	+	1,280 (Aug 11) 1,280 (Sep 30) 320(Nov 4)	<40 (Aug 11)
2	+	320 (Aug 19) 320 (Oct 7) 160 (Nov 11)	<40 (Aug 19)

*Titers determined by serum tube agglutination test.

Atsushi Nomura, Koichi Imaoka, Hajime Imanishi, Hideaki Shimizu, Fumiko Nagura, Kayaho Maeda, Tatsuhito Tomino, Yoshiro Fujita, Masanobu Kimura, and Gerald H. Stein

Affiliations: Chubu Rosai Hospital, Nagoya, Japan (A. Nomura, H. Imanishi, H. Shimizu, F. Nagura, K. Maeda, T. Tomino); National institute of Infectious Diseases, Tokyo, Japan (K. Imaoka, M. Kimura); Toyota Memorial Hospital, Toyota, Japan (Y. Fujita); and University of Florida, Gainesville, Florida, USA (G. Stein)

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Address for correspondence: Atsushi Nomura, Department of Nephrology, Chubu Rosai Hospital, 1-10-6 Komei, Minato-Ku, Nagoya 455-8530, Japan; email: naotmuusria@yahoo.co.jp

Oseltamivir-Resistant Pandemic (H1N1) 2009 in Patient with Impaired Immune System

To the Editor: We detail the development of oseltamivir-resistant pandemic (H1N1) 2009 in a chronically immunocompromised patient and the pitfalls encountered when treating such patients with neuraminidase inhibitors. On August 6, 2009, a 56-year-old man was seen in the emergency room of a local hospital with a 24-hour history of fever, myalgia, coryzal symptoms, and cough. He was on day 3 of a postexposure course of oseltamivir (75 mg 1×/d); influenza A had been presumptively diagnosed for his wife after she had similar symptoms.

The patient's medical history showed grade IVB nodular sclerosing Hodgkin lymphoma, which had been diagnosed in 2001. Lymphoma was initially treated with chemotherapy, but relapse required autologous peripheral stem cell transplantation in July 2005. Further relapses in 2006 and 2007 were treated with radiotherapy and chemotherapy, respectively, before the patient underwent an allogeneic peripheral stem cell transplantation in July 2008. This treatment was complicated by graft-versus-host disease, and the patient required ongoing immunosuppression.

When hospitalized, the patient was being treated with cyclosporine A (50 mg/d) and prednisolone (20 mg/d). Physical examination showed a temperature of 39°C and wheezing from the left lung. Initial tests showed a neutrophil count of 2.02×10^9/L, a lymphocyte count of 0.87×10^9/L, and a C-reactive protein level of 33 mg/L. He was started on piperacillin–tazobactam and gentamicin, and oseltamivir was increased to the treatment dose of 75 mg 2×/d. A nasopharyngeal aspirate collected on August 7 contained pandemic (H1N1) 2009 viral RNA by real-time PCR for generic influenza A (*1*) and capillary sequencing for subtype H1N1 (testing by Micropathology Ltd, Coventry, UK). By August 9, the patient was still febrile, and zanamivir (10 mg 2×/d) was started. Oseltamivir was given for a total of 7 d and zanamivir for 3 d.

Nose and throat swabs taken on August 21 still contained pandemic (H1N1) 2009 viral RNA. Real-time PCR and pyrosequencing demonstrated a histidine-to-tyrosine substitution (H275Y) in the neuraminidase gene associated with oseltamivir resistance (Respiratory Virus Unit, Centre for Infections, Health Protection Agency; methods not in public domain). A mixture of wild-type and resistant virus was present (A. Lackenby, pers. comm.). The sample from August 7 did not contain this mutation, suggesting a de novo H275Y substitution secondary to oseltamivir use.

The patient improved and was discharged on August 23 but returned for treatment on September 7 with worsening fever and cough. Nose and throat swabs obtained on September 11 were PCR negative, but follow-up samples on September 25 and October 1 contained detectable pandemic (H1N1) 2009 viral RNA. Because virus isolation was not performed, true infectivity remains unresolved, but intermittent detection suggests ongoing replication, such as that seen in other immunocompromised patients (*2*,*3*).

By February 3, 2010, a total of 225 cases of oseltamivir-resistant pandemic (H1N1) 2009 had been identified worldwide; a high proportion of cases were in immunocompromised persons (*4*). A minority of these mutations were detected in treatment-naive patients. Immunocompromised, particularly lymphopenic, patients shed virus for prolonged periods leading to longer treatment courses and viral shedding reviving on termination of treatment. Viral shedding for up to 18 months has been reported for seasonal influenza, which has important implications for infection control (*5*). Our patient demonstrated that a single PCR-negative test does not reliably determine the end of viral shedding, which continued despite co-treatment with 2 neuraminidase inhibitors. Neuraminidase inhibitors interfere with the release of progeny influenza virus from their infected host cells. Effective treatment depends partially on immune system destruction of the foci of infection (*6*), or potential persistent viral particles can be released as soon as oseltamivir therapy is stopped. The low genetic barrier to oseltamivir means that resistance is a likely consequence of monotherapy in immunocompromised patients.

Concern about oseltamivir resistance has led to issuance of additional guidelines, especially in light of the transmission of resistant virus between immunocompromised patients on hospital wards in the United States and Wales (*7*,*8*). This finding suggests that immunocompromised patients should be treated with oseltamivir and zanamivir, or with zanamivir alone, for a

minimum of 10 d. Patients should be retested for ongoing viral secretion every 5 d and negative results confirmed with a follow-up sample after 48 h. Classic virus isolation in addition to molecular methods may also identify potentially infectious patients.

Prophylactic neuraminidase inhibitor use in such patients also needs to be addressed. Resistance is more likely with the reduced prophylactic dose of oseltamivir and is more likely to be a problem in immunocompromised patients. Zanamivir is now the drug of choice for prophylaxis for such patients, although some experts propose no prophylaxis and instead early treatment after symptom onset (*9*).

Immunocompromised patients are more likely to shed virus for prolonged periods and are more likely to develop oseltamivir-resistance, especially when this drug is used as monotherapy. Further clinical experience and trials will support or refute newer guidelines on the management of pandemic (H1N1) 2009 in such patients.

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Grant A. Hill-Cawthorne, Silke Schelenz, Matthew Lawes, and Samir Dervisevic

Author affiliations: University of East Anglia, Norwich, UK; and Norfolk and Norwich University Hospital, Norwich

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Address for correspondence: Samir Dervisevic, Virology Centre, Department of Microbiology, Norfolk and Norwich University Hospital, Bowthorpe Rd, Norwich NR2, UK; email: samir.dervisevic@nnuh.nhs.uk

Sapovirus in Adults in Rehabilitation Center, Upper Austria

To the Editor: Contrary to norovirus (NoV) infections, sapovirus (SaV) is believed to affect mainly young children (*1*), although recent studies show that SaV is present in all age groups (*2*,*3*). SaV has been classified into 5 genogroups, of which GI, GII, GIV, and GV affect humans (*4*). SaV can be transmitted in various ways, including person-to-person fecal–oral route, by aerosol, and by consumption of contaminated food or water (*5*). Outbreaks of SaV have been reported in various settings but are less common than NoV outbreaks (*1*,*6*).

During October 2–7, 2008, an outbreak of gastroenteritis occurred in a rehabilitation center in Upper Austria. Signs including diarrhea, vomiting, and fever developed in 21 adult patients and 12 staff members. The observed signs and the likely incubation period initially suggested NoV as the cause of the outbreak. Stool specimens of 10 patients were collected and submitted to the Institute for Medical Microbiology and Hygiene in Graz.

Along with microbiologic investigations, infection control measures were introduced by local authorities on each affected ward. The earliest reported onset of illness was on October 2, 2008, in a 52-year-old woman on the third floor. The next day 2 additional patients on the same floor and 1 member of the kitchen staff showed symptoms. Another 7 patients, on 3 different floors, and 2 of the medical staff suffered from symptoms the following day (October 4). The outbreak peaked with 11 cases 3 days after the initial episode of vomiting. An additional 9 persons became infected (5 patients, a doctor, janitor, psychotherapist, and kitchen worker) within the following 2 days. The affected patients were pre-

dominantly elderly; mean age was 54 years (range 20–81 years, male:female ratio 1:0.83). Clinical signs continued for 24 hours.

Routine microbiologic cultures for enteric bacterial pathogens were performed and showed negative results. A NoV-specific 1-tube real-time PCR assay (LightCycler 2.0; Roche Applied Science, Mannheim, Germany) with primers/probes reported by Hoehne et al. (*7*) and ELISAs (Ridascreen; R-Biopharm AG, Darmstadt, Germany) were conducted to detect rotavirus and adenovirus antigen. All tests yielded negative results. Subsequently, 5 of 10 samples were submitted to the Robert Koch Institute in Berlin for further investigation. SaV was identified in 4 samples by using the reverse transcription–PCR described by Oka et al. (*8*); mean viral load was 2.65×10^8 RNA copies/g stool (range 3.3×10^7–7.7×10^8 copies/g stool). Direct sequencing of the appropriate amplification product showed 100% identical nucleotide sequences, which indicated 1 causative strain.

Retrospective testing of the 10 specimens showed 9 SaV-positive samples. Subsequently, genotyping was conducted by using a 1,130-bp amplification product of the polyprotein gene open reading frame 1 spanning the recombination site at the junction between the polymerase gene and the capsid gene. One specimen was amplified by RT-nested PCR with sense primer SV 53a (5′-TAGACTACAGCAAGTGGGA-3′, nt position 4356–4374), antisense primer SV 63 (5′-ACACCATGT TGGACACGCTGC-3′, nt position 6901–6881), and SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, Paisley, UK) for the first-round PCR. For the second-round PCR, the HotStarTaq Master Mix Kit (QIAGEN, Hilden, Germany) and primer SV 55a (5′-CCMTCKGGCATGCCATTCAC-3′, nt position 4529–4548) and SV60 (5′-ATGTTAAATGTGATAGGATCCA C-3′, nt position 5658–5636) were used (nucleotide positions according to GenBank accession no. DQ058829).

Phylogenetic analysis of the second-round PCR product (GenBank accession no. GU724600) showed 97% nucleotide identity to the strain Angelholm/SW278/2004/SE (GenBank accession no. DQ125333), which is a known intergenogroup recombinant virus (II.2/ IV) as seen in Japan and Sweden (*9*). Thus, by sequence analysis of the polymerase region, our strain Graz1561/2008/Austria was grouped into genogroup II; the capsid region belonged to genogroup IV.

Diarrhea and vomiting were the most common signs in patients, 97% and 73%, respectively. Fever was recorded for only 1 case-patient. Our findings are consistent with those of SaV outbreak studies in adults reported by Johansson et al. (*3*), who reported diarrhea in 72% and vomiting in 56% of the case-patients they studied.

The new genetic background of the recombinant virus may have enhanced host susceptibility by evading the immune response and is therefore able to affect adults. Our study shows that SaV causes outbreaks of gastroenteritis in adults; consequently, the role of SaV in the adult population should be reconsidered. We suggest that diagnostics for SaV should be included in the study of gastroenteritis outbreaks in adults, especially when clinical signs suggest NoV as the causative agent but no diagnostic confirmation can be achieved.

Claudia Mikula, Burkhard Springer, Sandra Reichart, Katharina Bierbacher, Alfred Lichtenschopf, and Marina Hoehne

Author affiliations: Austrian Agency for Health and Food Safety, Graz, Austria (C. Mikula, B. Springer, K. Bierbacher); Public Health Authority Upper Austria, Linz, Austria (S. Reichart); Rehabilitation Center Weyer, Weyer, Austria (A. Lichtenschopf); and Robert Koch-Institute, Berlin, Germany (M. Hoehne)

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Address for correspondence: Claudia Mikula, Österreichische, Agentur für Gesundheit und Ernährungssicherheit GmbH, Institut für Medizinische Mikrobiologie und Hygiene, Beethovenstrasse 6, Graz A-8010, Austria; email: claudia.mikula@ages.at

Inside the Outbreaks: The Elite Medical Detectives of the Epidemic Intelligence Service

Mark Pendergrast

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In this book, Mark Pendergrast, a journalist by training, tries to present a comprehensive history of the Epidemic Intelligence Service (EIS). He successfully tells the stories of the elite group, but in attempting to cover so many of the outbreaks in which they have been involved, he loses the essence of their story.

Some of the outbreaks clearly have more detail than others. The story of the Cutter polio vaccine incident in 1955, the 1976 Ebola virus outbreak in Zaire, smallpox eradication, and the beginning of the AIDS epidemic in the United States are well documented. Unfortunately, even for these famous outbreaks, the stories are told better in other books singularly devoted to these topics rather than in this volume devoted to the role played by the EIS. Furthermore, most of the other outbreaks discussed in the book are covered in less than a page, and the author provides far too little detail. For example, the 1994 *Salmonella enteritidis* outbreak associated with Schwann's ice cream that sickened >200,000 persons and was the largest common-source outbreak in the United States warranted only 2 paragraphs of coverage. The way in which Pendergrast describes the investigation—"He [Minnesota-based EIS officer Tom Hennessy] discovered that truck drivers were "back-hauling" liquid raw eggs on their return trip, and they were not cleaning their tankers sufficiently to prevent cross-contamination."—makes the investigation seem simple and easy when it was, in fact, anything but simple and easy. Even though the individual outbreaks are well researched and written, the brevity with which each is portrayed makes light of the enormous effort involved in determining the source and controlling the outbreak. An outbreak investigation that may have taken weeks or months might be covered in the book in a mere 2 or 3 paragraphs, with little indication of how much the EIS officer(s) struggled to determine the etiologic agent and implement appropriate control measures.

If taken as a story of the history of the EIS, rather than as a story of the outbreaks themselves, the book achieves its primary goal. However, how interesting the history of the EIS is to persons who were not EIS officers or who do not aspire to be one is questionable. On the other hand, given that the book has a retail price of $28.00, and Pendergrast interviewed such an impressive number of EIS alumni that any public health professional is bound to recognize at least 1 name, even with its flaws, the book is still a fast and entertaining read.

Shira C. Shafir
Author affiliation: University of California Los Angeles School of Public Health, Los Angeles, California, USA

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Address for correspondence: Shira C. Shafir, Department of Epidemiology, UCLA School of Public Health, Box 951772, Los Angeles, CA 90095, USA; email: sshafir@ucla.edu

Prince Twins Seven-Seven (b. 1944), ***The Lazy Hunters, and the Poisonous Wrestlers, Lizard Ghost and the Cobra*** **(1967)**
Ink, paint, and chalk on plywood (124.5 cm × 78.1 cm), National Museum of African Art, Washington, DC, USA,
Gift of Mr. and Mrs. Sean Kelly, 75–28–3, Photo by Franko Khoury

There Is Always Something New Out of Africa

—Pliny the Elder, Natural History (c. 2,000 years ago)

Polyxeni Potter

Lone survivor of seven sets of twins born to his mother, he started life as a legend among the Yoruba in Nigeria, who have one of the highest rates of twin births and infant deaths in the world. According to family lore, he was the spirit that kept trying to be born and kept being turned back, the child "born to die." Bamidele to his father, Olaniyi to his grandmother, Prince Twins Seven-Seven renamed himself in adulthood to commemorate newly found royal lineage and the circumstances of his birth, which colored his life and shaped his art. Deeply conscious of these aspects of his nature, he has conveyed in art his personal understanding of a world teeming with spirits and populated with myths.

A young man in his early 20s, he went on the road as a dancer and musician. "I was born with music." He found himself in the town of Oshogbo, in the heart of southwestern Nigeria's Yoruba-speaking people. "I was…doing entertainment for traders to boost their sales. They would hire me and I would dance to attract people." Oshogbo was home to a namesake artists group, progenitor of modernism. German scholars Ulli and Georgina Beier promoted literature and the arts there and conducted workshops for young talent outside the academic art scene. Twins was invited to stay and soon showed artistic promise with imaginative works free of form, perspective, proportion, or convention. "I don't want to do what everybody does." From the start, his works were instead vibrant and rich in linear and color motifs and were informed by the surrounding culture, one of the oldest, largest, and most influential in Africa, with a long tradition of sculpture, wood and metal carving, textiles, and beadwork.

The Yoruba acknowledge a supreme deity, Olorun, and view the universe as two halves of a closed calabash. One filled with living beings—animals, people, and plants; the other with spirits, deities, and ancestors connected with natural forces—thunder, rain, disease. When the sky god sent his son to earth, the son became the first ruler of the Yoruba kingdom. As his direct descendants, the first kings and their offspring became divine kings. In this world of

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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complicated and constant interaction, living beings draw on surrounding forces to realize their personal destiny. Their descendent artist does no less. Steeped in these traditions, he brings through his art their authenticity and purity to the world.

"In order to make people look, you have to make it extraordinary." Twin's interpretation of this tightly woven cultural fabric had immediate and wide popular appeal. His works, usually intense personal variations of Yoruba myth often interspersed with his own life story, were well received at home and abroad and inspired many followers and imitators. He exhibited in France, Finland, and Japan. But he also experienced the downside of fame, along with financial and other difficulties. He fled Nigeria and traveled to the United States, where he settled for a while in Philadelphia. His work, again recognized and appreciated, is now found in museums around the world.

"I was doing it because that was what came from my mind," the artist said referring to his subject matter, the unseen. His approach was syndetic, circuitous, and surprising. He started working on one end of the canvas and continued until every bit of the surface was covered, leaving the impression that more could always be added. He reveled in complexity and used it to engage the viewer. "I want to make a picture where the more you look, the more you see." Still and always a musician, he found a rhythm and danced to it, creating dynamic and repetitive patterns in a seemingly endless continuous design. "It is helpful to have something else to do after you've made a mistake," he advised. "You come back, and the mistake is fixed. Your subconscious will solve it." Continuous engagement opens up the world.

The title *The Lazy Hunters, and the Poisonous Wrestlers, Lizard Ghost and the Cobra* of the painting on this month's cover suggests a scene from some Yoruba tale but offers no further explanation. Like the plot in a folk opera with spoken parts along with music and dance, such as some that the artist himself may have participated in, it involves the common people, hunters, bumping into the extrahuman in the "bush of ghosts." They are clearly at a disadvantage as they huddle in the corner under the serpent in the ferocious lizard claws of his imagination, affirming that African myths with animals are often about the strengths or foibles of people.

The farming town of Oshogbo was likely founded in the early 18th century by hunters from a nearby village languished by famine. Details are scant and mixed with legendary deals with local deities to secure water and leadership rights. In a culture that rewarded self-reliance and judged its members by their achievements, hunters had to survive in the forest, fully expecting to take on the role of warrior or scout in time of conflict. Yet in Twins' version the hunters are branded lazy and appear powerless, their implements strewn in the foreground. Tiny against the animals, they seem frozen in fright, wild-eyed, and panic stricken.

Stacked on the left are dwellings, framing a community in the background. Nature is present in elusive forms since everything is woven with the same thread. Lizard fins double as mountains, flowers as oracles, beast attire as staring eyelets. The inflated lizard and serpent, an extraordinary spectacle, are inhabited by ancestral or other spirits. The scene has the static glow of a dream, but one thing is certain. Humans here are not the dominant species. They have lost control of the village to the lizard and cobra now locked mouth-to-mouth in a poisonous embrace.

Twins' vision cuts through daily realities to expose a most inclusive web of interconnectedness. And through myth he gently navigates the web, guiding the viewer. He unveils mysterious forces unleashed in the forest. And while the beasts wrestle, he delivers more about this struggle than meets the eye.

Magic and medicine share the same name in Yoruba language and both rely on natural objects with supernatural powers to prevent or cure disease, which occurs when a person's relationship with nature is disrupted. A balanced relationship is critical to life and health. Whether linked to twinning and infant deaths or to novel viruses and other causes of infection, the danger can become inflated out of proportion or curbed with public health measures. Having been tossed back and forth so many times, Twins rightly views himself as one charged with making images here from the other world and calling attention to lapses in vigilance.

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Hantavirus Infections in Humans and Animals, China

Clostridium difficile Bacteremia

Responses to Pandemic (H1N1) 2009, Australia

Bat Coronaviruses and Experimental Infection of Fruit Bats, the Philippines

Potential Drivers of West Nile Virus Expansion in British Columbia

Pandemic (H1N1) 2009 Surveillance and Response, New York, New York, USA

Scavenging Ducks and Transmission of Highly Pathogenic Avian Influenza, Indonesia

Corynebacterium pseudodiphtheriticum in Cystic Fibrosis Patients, France

Pandemic (H1N1) 2009 Viral Load and Disease Severity in Children

White-Nose Syndrome Fungus (*Geomyces destructans*) in Bats, Europe

Multiyear Surveillance for Avian Influenza Virus in Waterfowl, Texas, USA

Pearl Kendrick, Grace Eldering, and the Pertussis Vaccine

Quarantine Methods and Prevention of Secondary Influenza Outbreak

Pandemic (H1N1) 2009 Vaccination and Class Suspensions After Outbreaks, Taiwan

Bat Rabies in Massachusetts, USA, 1985–2009

Pandemic (H1N1) 2009 Virus and Down Syndrome

Lyme Borreliosis, Po River Valley, Italy

Picornavirus Salivirus/Klassevirus in Feces of Persons with Diarrhea, China

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

Upcoming Infectious Disease Activities

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

July 14–17, 2010
8th Biodefense Vaccines and Therapeutics
Almas Temple Club
Washington, DC, USA
http://www.infocastinc.com/biodef10

August 28–September 1, 2010
Infectious Disease 2010 Board Review Course – 15th Annual Comprehensive Review for Board Preparation
Hyatt Regency Crystal City
Arlington, VA, USA
http://www.IDBoardReview.com

November 6–10, 2010
APHA 138th Annual Meeting and Expo
Denver, CO, USA
http://www.apha.org/meetings

November 11–13, 2010
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Lisbon, Portugal
http://www.escaide.eu

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://cme.medscape.com/viewpublication/30063**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Extensive Drug Resistance in Malaria and Tuberculosis

CME Questions

1. You are seeing a couple who recently emigrated from Uganda. One of these patients, a 32-year-old woman, has a history of active tuberculosis (TB) and said she was treated with several drugs for 2 months, but she was told they were not working.

According to international consensus, multidrug-resistant (MDR) TB is defined by resistance to at least which of the following medications?

A. Rifampin only
B. Rifampin and isoniazid
C. Isoniazid and ethambutol
D. Ethambutol, pyrazinamide, and rifampin

2. Which of the following statements regarding the treatment of this patient with TB is most accurate?

A. Guidelines in the United States now recommend extending 4-drug treatment for TB based on the patients' symptoms alone
B. The World Health Organization recommends directly observed therapy (DOT) for treatment with rifampin
C. Previous TB treatment does not affect her risk of harboring resistant organisms
D. TB treatment should be changed when there is no clinical improvement after 2 weeks

3. The other patient is a 31-year-old man with intermittent fever and headache for 3 weeks. He is hospitalized, and malaria is diagnosed.

Which of the following general principles regarding the treatment of this patient is most accurate?

A. Multidrug resistance in *Plasmodium falciparum* is defined by resistance to more than 2 operational antimalarial compounds from 2 different classes
B. The use of combination antimalarial therapy has not improved efficacy or reduced resistance
C. The decision to treat MDR malaria is based on complicated laboratory data
D. Geography has little impact on the treatment choice of malaria

4. Which of the following statements regarding drug-resistant malaria is most accurate??

A. The simplest way to monitor the efficacy of a given therapeutic regimen is through molecular characterization
B. There is no known resistance to the combination of artesunate and mefloquine
C. Artemisinin resistance is now common in Africa
D. Rates of artemisinin-based combination resistance need to be monitored in Africa

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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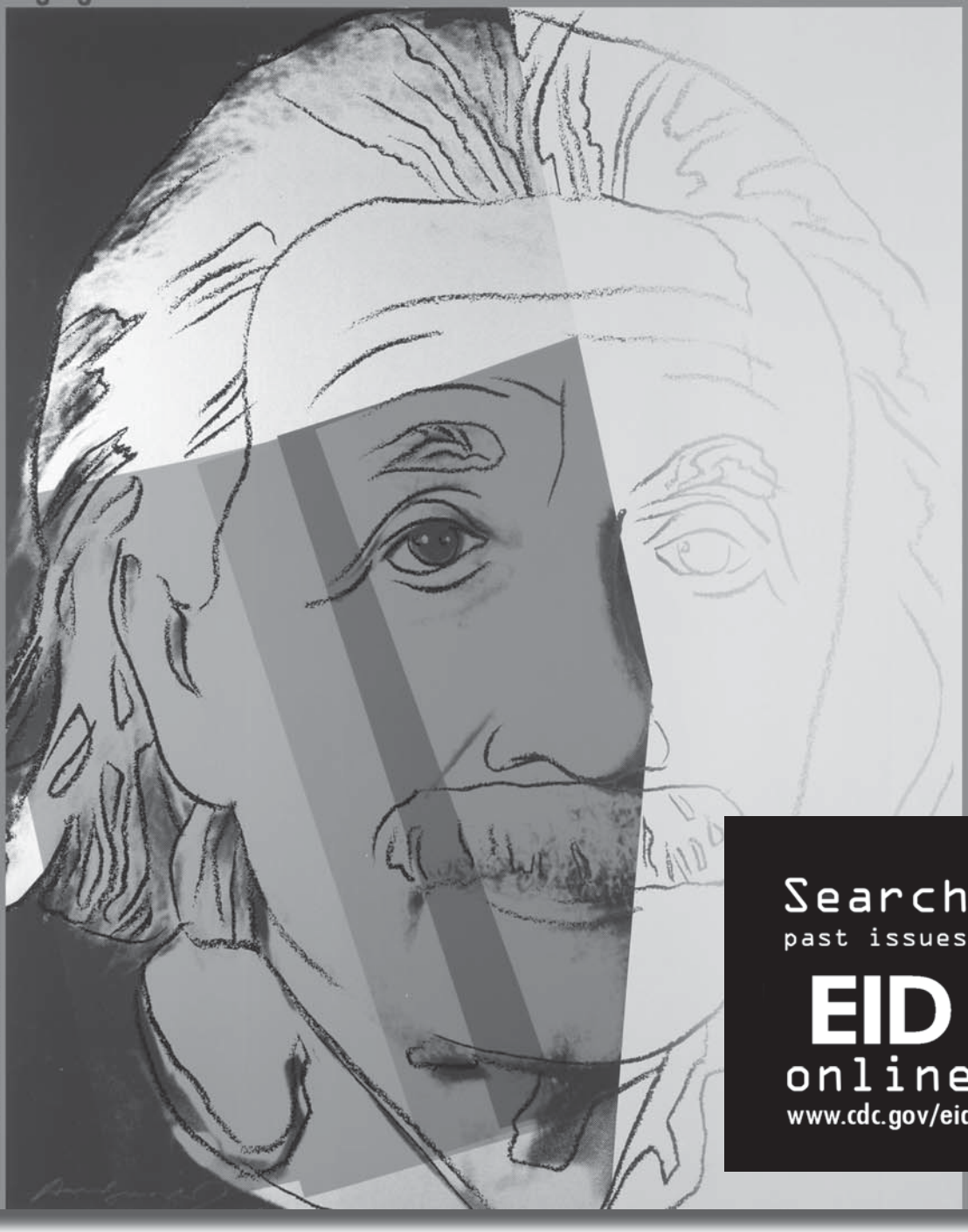
June 2010

Emerging Viruses



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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

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

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

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

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

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

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

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

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

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.