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December 2010

Zoonoses

Bequest of John Ringling, 1936. Collection of The John and Mable Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

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On the Cover

Jacopo Bassano (c. 1510–1592)
Allegory of Water (16th century)
Oil on canvas (139.7 cm × 180.3 cm)

Bequest of John Ringling, 1936, Collection of The John and Mable Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

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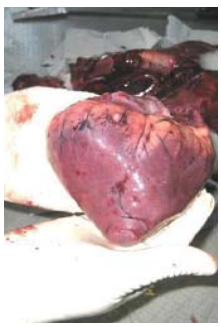

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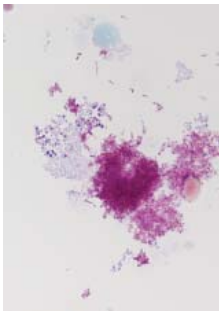

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About the Cover

Surveillance of Wild Birds for Avian Influenza Virus

Bethany J. Hoye, Vincent J. Munster, Hiroshi Nishiura, Marcel Klaassen, and Ron A.M. Fouchier

Recent demand for increased understanding of avian influenza virus in its natural hosts, together with the development of high-throughput diagnostics, has heralded a new era in wildlife disease surveillance. However, survey design, sampling, and interpretation in the context of host populations still present major challenges. We critically reviewed current surveillance to distill a series of considerations pertinent to avian influenza virus surveillance in wild birds, including consideration of what, when, where, and how many to sample in the context of survey objectives. Recognizing that wildlife disease surveillance is logistically and financially constrained, we discuss pragmatic alternatives for achieving probability-based sampling schemes that capture this host–pathogen system. We recommend hypothesis-driven surveillance through standardized, local surveys that are, in turn, strategically compiled over broad geographic areas. Rethinking the use of existing surveillance infrastructure can thereby greatly enhance our global understanding of avian influenza and other zoonotic diseases.

Avian influenza virus (AIV) gained a high profile after the unprecedented bird-to-human transmission of highly pathogenic AIV (HPAIV) subtype H5N1 in 1997. Originating in Asia, HPAIV (H5N1) subsequently caused widespread deaths among wild and domestic birds in Southeast Asia and westward throughout Europe and Africa in 2005 and 2006. After ≈50 years of research in wild birds, a wide range of low-pathogenicity AIV (LPAIV) subtypes is known to circulate in numerous species (*1*,*2*–*5*), and LPAIVs are believed to perpetuate in aquatic bird populations (*6*). In contrast, outbreaks of HPAIV are extremely rare in wild birds (*7*). Although the role of wild birds in HPAIV maintenance remains controversial (*8*), the magnitude of the subtype H5N1 epidemics increased the demand for early recognition of potential threats to humans and poultry and an understanding of the natural history of AIV in wild birds. Consequently, surveillance of aquatic bird populations surged (*9*).

Although surveillance for AIV often uses state-of-the-art storage, transport and diagnostics, these must be underpinned by appropriate survey design, sampling, and interpretation in the context of the host population. In the wake of such rapid growth in surveillance, we reviewed the literature to determine a scientifically and statistically sound approach to the design, conduct, and interpretation of surveillance for AIV and other wildlife diseases.

Current Surveillance

We reviewed 191 published reports of surveillance in wild birds (online Technical Appendix, http://www.cdc.gov/EID/content/16/12/1827-Techapp.pdf). The number of studies initiated per year rapidly increased after the first reports of HPAIV (H5N1) in Asia (Figure 1). All studies addressed 4 major lines of investigation: 1) early detection of HPAIVs; 2) ecology and epidemiology of LPAIV in host populations; 3) diversity and evolution of viral strains within wild birds; and 4) identification of the pathogens that infect individual birds or populations, often as part of multipathogen surveillance. Multiple aims can, and often are, addressed within the same surveillance program, albeit in a post hoc manner. However, identifying the aims in

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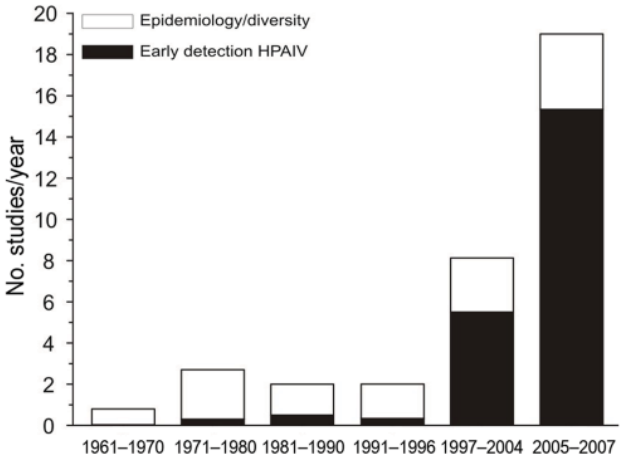

Figure 1. Average number of surveys of avian influenza in wild birds initiated per year in different awareness periods: each decade from the first discovery in 1961 until the outbreak of highly pathogenic avian influenza virus (HPAIV) (H5N1) in Asia in 1997; the period after the first outbreak, 1997–2004; and the period after mass deaths of wild birds from HPAIV (H5N1) (2005–2007). Black bar sections indicate studies citing the detection of contemporary HPAIV strains as one of the main aims of their survey are indicated in black; white bar sections indicate studies investigating other aspects of the wild bird–avian influenza system without mention of monitoring HPAIV.

advance is vital, because what, when, and where to sample will critically depend on the purpose of the survey (*10*,*11*).

Early Detection of HPAIV

More than half of the studies reviewed, and all but a handful initiated since the mass bird deaths in 2005–2006, cited early detection of HPAIV as one of the main goals of conducting the research (Figure 1). Such early warning systems question whether HPAIV exists in a population at a given location and point in time. The global rarity of HPAIV in wild birds and apparent clustering of such cases (*7*) present additional challenges to addressing this aim.

Ecology and Epidemiology

Greater understanding of transmission cycles, reservoirs, and the role of wildlife in the dynamics of AIV invoke questions related to the epidemiology and ecology of the virus, including host range and spatial and temporal variation in infection (*12*,*13*). Elucidating such questions requires investigating not just presence or absence of infection in a specific host, but also prevalence over space and time.

Viral Diversity

Influenza viruses are highly diverse and capable of rapid genetic alteration. Understanding the pathogenic and antigenic properties of AIVs circulating in the host population and the rate and direction of genetic alterations could become a powerful tool for identifying transmission parameters, reservoir populations (*14*), viral maintenance in the face of host immunity (*12*,*15*), and factors promoting disease emergence (*10*). Such information also facilitates compilation of comprehensive diagnostic reference panels and generation of potential vaccines (*13*). Investigation of variation in the viral population requires isolates that represent the entire circulating virus pool.

Host Health

Almost 15% of the studies reviewed aimed to ascertain whether certain individuals or populations had been infected with AIV as part of broader health surveys within the context of conservation programs, or in an attempt to understand causes of death. Although these studies often have a predefined host population of interest, they are likely to be sensitive to the underlying spatial and temporal patterns of disease.

Critical Assessment

To characterize the specific features required for rigorous wildlife disease surveillance, it is critical to highlight methods that encumber our current approach. Our assessment therefore aims to foster the development of more objective and scientifically sound disease surveillance networks.

Maximizing Viral Yield

A successful surveillance program is often perceived as one that identifies a high number of positive samples. Moreover, exploitation of spatial, temporal, phylogenetic, and demographic differences in viral prevalence have been advocated to maximize the proportion of positive samples collected (*12*,*16*). Minimizing the number of negative samples is expedient from a laboratory perspective, particularly when labor-intensive virus isolation techniques are being used. However, a key tenet of surveillance is that the sampling scheme is representative: infection characteristics of the host population and genetic diversity of the viral population are sufficiently captured, and results can be interpreted on the basis of statistical probability (*11*,*17*). A study designed to maximize the number of positive samples by sampling historically high cohorts, populations, times, and locations can confirm the presence of the disease in the sampled cohort. However, such samples cannot be used to conclude the absence of AIV in the population or to estimate prevalence or diversity of circulating viral strains (*17*).

Host Range

Although AIVs have been isolated from >100 species, several species from the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds) are thought to

act as the reservoir community for AIV (*6*), primarily because AIVs have been most frequently isolated from these groups (*9*). Yet, surveillance is rarely representative of the diversity of wild birds or their relative abundance at the time and location of sampling. Considerable bias exists toward species that are easily caught or are present in accessible areas at high concentrations (*9*,*13*). Surveys that have included a wide range of species often obtained samples in a highly opportunistic manner, resulting in few species being sampled in reasonable numbers (*12*,*13*). For instance, despite sampling >56,000 birds in the Netherlands from 1998 to 2009, only 20 of the 174 species were sampled >300 times. Moreover, prevalence in a given species may vary over space and time. Although passerines have often been found negative for AIV, recent evidence suggests that, when sampled in or near waterfowl-rich bodies of water, a high proportion of individuals from 8 different passerine families show infection (*18*,*19*). Current surveillance may, therefore, overlook many potential reservoir or transient host species and their role in the introduction, transmission, maintenance and diversity of AIV.

Temporal and Spatial Patterns

The prevalence of AIV infection has long been recognized to vary over time and space. Viruses have been most frequently isolated from duck populations in North America and Europe in late summer and early autumn (*5*,*15*,*20*), a pattern attributed to high concentrations of susceptible juvenile birds on premigratory staging grounds (*4*,*6*). Less frequent isolations from wintering populations have prompted suggestions that prevalence rapidly decreases over the course of autumn migration (*21*,*22*); thus, premigratory staging grounds in late summer and early autumn are considered the optimal time and location for conducting surveillance among waterfowl (*16*,*23*). Yet when samples have been collected elsewhere, high numbers of AIVs have been isolated in winter (*21*,*24*), spring (*20*), and summer (*25*). Several positive samples from birds in the tropics (*26*) have also been found, including unexpectedly high numbers in tropical Africa (*27*). The temporal and spatial bias in existing surveillance may therefore result in delayed detection of novel strains or an incomplete understanding of AIV transmission, maintenance, diversity, and evolution.

Age-dependent Patterns

Pioneering work by Hinshaw et al. (*4*) found significantly higher prevalence of AIV infection among juvenile birds than among contemporaneously sampled adult birds, leading to the suggestion that immunological naivety may make juvenile birds a high-risk group within waterfowl populations. Emphasis has subsequently been placed on sampling juvenile birds; accounting for ≈80% in some recent surveys. However, wild bird populations are rarely composed of ≥80% juvenile birds, and numerous infected adults have also been found (*4*,*24*). Given that recent experimental results indicate that age at the time of infection might also affect the extent of viral shedding (*28*), different age cohorts may play different roles in the introduction, transmission, maintenance, and diversity of AIVs.

Site of Infection

AIVs replicate in the gastrointestinal tract (sampled by swabbing the cloaca or collecting droppings) and in the respiratory tract (sampled by swabbing the oropharynx) (*16*). Individual mallards (*Anas platyrhynchos*) have historically shown higher detection probability from cloacal c.f. oropharyngeal swabs (*29*; Figure 2). Accordingly, 61% of studies investigating contemporary infection sampled the gastrointestinal tract alone. However, the site of infection may differ between species. As part of ongoing surveillance (*21*,*29*), free-living Eurasian wigeons (*Anas penelope*) showed no difference in detection probability between the cloacal and oropharyngeal swabs ($p>0.05$, McNemar test; Figure 2). In contrast, white-fronted geese (*Anser albifrons*) were roughly 2× as likely to have infection detected in the oropharynx (6.58%; 95% confidence interval 6.57–6.59) than in the cloaca (3.13%; 95% confidence interval 3.13–3.14; $p<0.001$); ≈60% of the infected birds were positive by oropharynx sample alone (Figure 2). Together with the apparent oropharynx affinity of HPAIV (H5N1) in experimental and natural infections (*30*), these

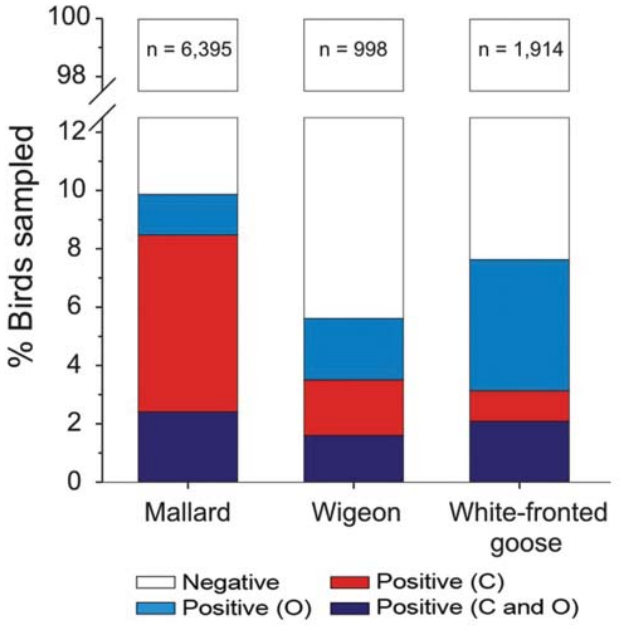

Figure 2. Proportion of wild mallards (*Anas platyrhynchos*), Eurasian wigeons (*Anas penelope*), and white-fronted geese (*Anser albifrons*) positive for low-pathogenicity avian influenza virus when sampled in the cloaca (C) and the oropharynx (O), the Netherlands, September 2006–March 2009.

findings have ramifications for the quantification of viral diversity, prevalence of infection, or absence of AIV.

Disease-free Populations and Prevalence Estimates

In general, survey sample sizes must be sufficiently large to draw appropriate inferences, and interpretations of AIV in wild birds based on many current sampling schemes may be hampered due to the limited number of samples collected (*9*). Studies have often concluded that AIV, particularly HPAIV (H5N1), was absent from a certain population or location. Infected birds may indeed have been present, but at a prevalence below the level of detection of the study (*17*). Only 3 of the studies that reported negative findings acknowledged a detection limit, yet such information is crucial to screening for HPAIV incursion. Similarly, 81 (42%) of the articles reviewed explicitly reported prevalence or seroprevalence; however, just 3 of these accounted for the uncertainty of their estimates (i.e., confidence limits). Such reports have fostered an impression that prevalence is a fixed property of a given host population, rather than a dynamic quantity, potentially influenced by many temporal, geographic, and biological interactions.

Utility of Birds Found Dead

Many surveillance programs aimed at the early detection of HPAIV (H5N1) focus on collections from sick or dead birds, often without surveillance of the living avian population (*31*). Although finding an HPAIV (H5N1) infection is statistically more likely in birds found dead (*31*), the absence of dead birds (or infection in dead birds) does not indicate freedom from disease. Dead birds fail to provide information on any animals that survived the infection, any animals that were not infected, or any viruses that were not lethal (*30*). Moreover, large numbers of carcasses may go undetected or unreported (*10*).

Screening for Only the Current Strain of Interest

Recently, some studies have only screened for H5 strains. Yet, none of the known genotypes can be ruled out as potential candidates for future pandemics. Additional information on all circulating gene segments is preferable as a novel-incursion warning system and in the broader context of AIV ecology, epidemiology, and evolution, particularly because no additional sample collection is necessary.

A Way Forward?

Although ≈50 years have passed since AIVs were first detected in wild birds, research is still in the exploratory phase, primarily because sampling wild animals is logistically challenging and expensive and techniques for high-throughput molecular surveillance have only recently become available. Wildlife disease surveillance regularly involves limited samples obtained in various ways that are already readily available, such as ornithologist-captured and hunter-collected birds. Although these methods of convenience sampling are often assumed to be representative of a population, sampling biases (most notably selection bias) do occur, making it difficult to develop statistically valid estimates of disease absence or prevalence, regardless of how many birds are sampled.

Our critique illustrates that to build on the findings of existing surveillance a scientifically sound approach is required. A study's aims need to be clearly identified at the outset, and appropriately designed sampling regimes and diagnostic techniques must be used. The global distribution of AIV and its avian hosts presents a major hurdle for such hypothesis-based research, making it difficult for individual research groups to tackle these questions in isolation. Our review highlights the need for global collation of existing wild bird AIV data and infrastructure, as well as the pooling of expertise and resources between epidemiologists, ornithologists, geneticists, and conservation organizations to unravel the complex interactions among diverse host and viral populations and the environments they utilize. Many such international initiatives exist in principle; however, there are currently several challenges in terms of data coverage, compatibility, management, and ownership. The following section outlines key considerations pertaining to the design, implementation, and interpretation of local surveys that could ameliorate data coverage and compatibility problems, paving the way for increasingly integrated studies of AIV and other wildlife diseases.

Sampling Unit

Target Virus

Particular strains, especially those with a history of HPAIV potential (H5 and H7), are of greatest interest when screening for HPAIV (*16*). However, screening for other virus subtypes by virus isolation, or targeting the matrix gene segment in molecular-based diagnostics, will simultaneously enhance our ecologic, epidemiologic, and virologic understanding of AIV.

Dead or Alive

Birds found dead may indicate rapid changes in host range, geographic range, viral pathogenicity, or disease emergence, and as such warrant swift investigation. However, to clarify the presence or absence of HPAIV, as well as trends in LPAIV presence, prevalence, and circulating strains, such surveys should be paired with active surveillance of the living wild bird population.

Sampling Site within the Bird

Viral strains of different host origin may differ in their affinity for either the digestive or respiratory tract and may

also differ between different host species. Sampling the cloaca/feces and oropharynx is therefore desirable when screening wild birds. Such differences also exemplify the need for experimental clarification of tract affinity and how this may influence interpretations based on a single sample type (e.g., droppings).

Which Populations Should Be Sampled?

Target Population

With >10,000 species of birds worldwide, careful selection of a local target population is critical to the design of any surveillance program. Because the prevalence of infection is generally low (requiring large sample sizes) and can vary over time and between locations within a species, it is difficult to make an initial assessment of the most important species to target on the basis of virus detection alone. Each of the surveillance aims outlined above may be most appropriately addressed by considering 1) populations with evidence of previous infection, or ecologic potential for infection (*32*), on the basis of not only existing literature and conventional monitoring but also serosurveillance in a large number of locally and regionally abundant species; and 2) Evidence of contemporary AIV infection in populations that were identified in step 1, and species in which AIV has historically been detected (for comparative purposes). Surveillance for emergent HPAIV may also benefit from targeting species displaying natural histories of interest, including species that link wild and human/agricultural populations or disparate locations.

Serologic studies have great potential for enhancing wildlife disease surveillance and understanding. However, in isolation, cross-sectional observations of seroprevalence provide insufficient information to interpret the degree to which a population has been infected with AIV. Without age specificity, high seroprevalence may indicate a recent outbreak of infection or long-term antibody maintenance rather than persistence of AIV infection in the population (*14*,*16*). Moreover, low seroprevalence may result from a high mortality rate among infected birds, a long time interval between infection and sampling, or species-specific differences in the sensitivity or specificity of the antibody diagnostics. Explicit interpretation of seroprevalence calls for age-specific sampling, longitudinal observations, understanding of the underlying epidemiologic dynamics, and experimental validation of antibody diagnostics.

Individual Birds within Populations

Within each species, infection may depend on multiple factors, including age and prior exposure to AIV (*4*), gender (*33*), and even nutrition or social status (*8*). Given that most capture methods inherently result in biases within these cohorts, a population should ideally be sampled to account for these differences. Experimental validation of such interindividual differences in infection could greatly enhance the design and interpretation of surveillance.

When, Where, and How Often to Sample?

When and where sampling is conducted will critically depend on the question at hand and should be representative of the biology of the hosts of interest. Single time or location studies may be sufficient to inform of novel incursions of HPAIV (Table) and may therefore be best matched to times/locations with a high risk for wild bird–poultry interaction. Changes in climatic conditions, host population dynamics, and host population immunity are likely relevant to understanding the ecology, epidemiology, and evolution of AIV in its natural host(s) (*34*). Enhancing our knowledge in these areas will require information from before, during, and after infection from ecologically connected populations (*35*), often over longer periods and across large spatial scales when studying migratory birds (*36*). Coordinated local surveys, both along flyways and over time, will greatly enhance these efforts.

How Many Individual Birds Should Be Sampled?

As prevalence decreases, an increasingly large number of birds need to be sampled to detect contemporary infection (Figures 3, 4). Deciding just how many is critically dependent on the study aim, with a clear distinction between surveys that aim to substantiate freedom from infection (presence or absence), and those that are designed to provide an estimate of disease prevalence.

Table. Data requirements for assessment of major questions regarding avian influenza in wild birds*

Aim	Type of question	Geographic range	Temporal range	Frequency
Early detection of HPAIV	Presence/absence	Local/regional	Period when birds present	Approximately weekly (average infection duration)
Ecology and epidemiology	Comparative prevalence	Local to flyway, depending on the process in question	1 to many epidemic seasons (multiple times/year)	Weekly to monthly (multiple times before, during, and after an epidemic)
Diversity and evolution	Comparative prevalence (of viral strains)	Flyway to global	Decades (multiple times/year repeated for multiple years)	Monthly to seasonally

*Larger-scale studies can be compiled over large geographic areas from relevant local surveys that are methodologically comparable and over long periods from relevant annual surveys that are likewise methodologically comparable. HPAIV, highly pathogenic avian influenza virus.

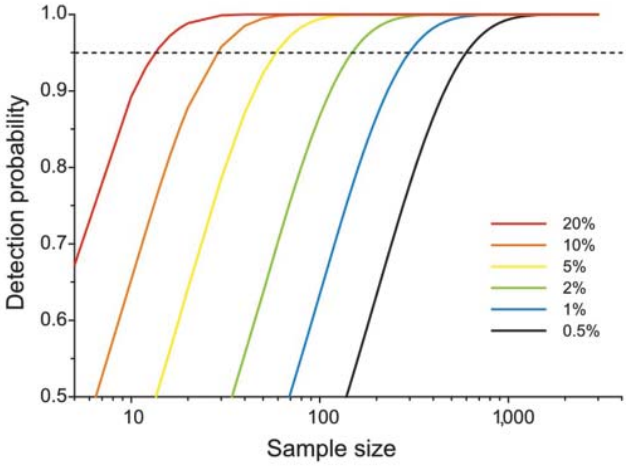

Figure 3. Probability of detecting ≥1 individual bird infected with avian influenza virus from a given number of samples selected at random from an extremely large population in which individual birds are infected at random at different prevalence levels. Although this nominal minimum detectable prevalence assumes binomial sampling, it can also be used for gaining a rough quantitative estimate of the minimum number of samples required before embarking on a surveillance program.

Presence/Absence

In practice, it is not possible to confirm disease freedom in a large population by any direct observational method. Instead, appropriate sampling and analysis can demonstrate that at that time and location, prevalence was below a nominal detection threshold (online Technical Appendix) (*17*). Although this nominal minimum detectable prevalence assumes binomial sampling, it can also be used for gaining a rough quantitative estimate of the minimum number of samples required before embarking on a surveillance program (Figure 3; online Technical Appendix). Given that information on the absence of pathogens is crucial to understanding disease dynamics (*10*), postsurveillance reporting of such maximum undetected prevalence is highly desirable for all studies with negative findings.

Prevalence

The proportion of positive findings among a given number of samples is rarely sufficiently precise to inform population prevalence. Thus, the confidence intervals of any observed proportion should be calculated and reported alongside any prevalence estimates when reporting surveillance results. Such confidence limits depend on the number of samples taken and the underlying true (unbiased) prevalence of infection (Figure 4).

Achieving Effective Surveillance

Each of the points above highlight the need for surveillance that captures the underlying temporal, spatial, demographic, and phylogenetic variation in the wild bird population, often requiring detailed information on host population size, density, demographic structure, rates of recruitment and attrition, habitat utilization, and species composition. However, wildlife surveillance is also faced with substantial logistical and financial constraints. Effective surveillance, therefore, requires a compromise between sampling that is based on probability and the constraints of sample collection, transport and analysis, the details of which will depend on the specific objectives of the survey. To this end, it is critical to have active, investigator-defined surveillance designs based on probability on a larger scale while using convenience sampling within these units (*11*). For instance, probability methods could be used to plan the species, locations, and months of the year to sample, and a certain number of individual birds within these units could be sampled by ornithologists and hunters, with additional top-up sampling where necessary. Such convenience-within-probability surveillance could provide statistically valid estimates of disease absence and prevalence by reducing the effect of bias generated by sampling on a first-come-first-served basis. It facilitates stipulation of an upper limit to the use of convenience samples, allowing targeted allocation of limited sampling, diagnostic, and financial resources.

To employ such convenience-within-probability surveillance, samples will often need to be collected from times, places, and species that are not currently covered by ornithologists and hunters. Preferably, individual birds should be sampled to confirm species, gender, age, and body mass, and sampling of digestive and respiratory tracts. However, when it is logistically and/or financially difficult to capture live birds several alternatives exist. Swabbing

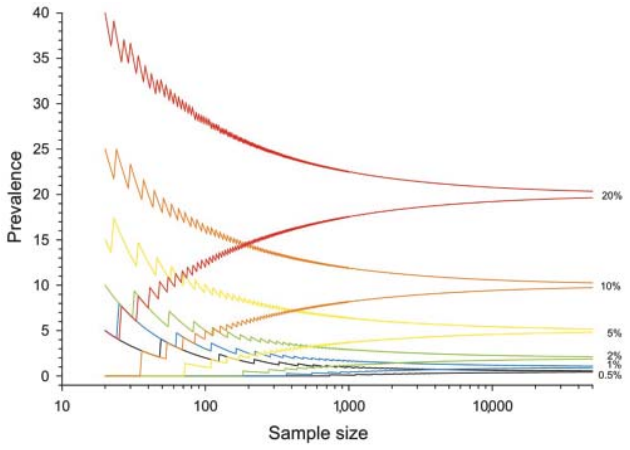

Figure 4. The 95% confidence intervals for prevalence in an independent population for a given number of samples, derived from the binomial distribution. Confidence intervals depend on the number of samples taken and unbiased prevalence of infection; they should be calculated and reported along with prevalence estimates when reporting surveillance results.

of fresh, species-specific feces is 1 method for collecting a regulated number of samples (*16*). Species should be identified through careful presampling observation of flocks, or, when sampling mixed-species flocks, through DNA barcoding of the fecal samples (*37*). Given that AIV can be detected from the same nucleic acid extract used in species identification (*37*), and substantially more samples can be collected at a much higher frequency than traditional trapping methods, dropping samples may greatly enhance our capacity to detect AIV in the population. Other, more proximate surveillance methods include sampling surface water that is, has been, or is about to be inhabited by wild birds (*16*), as well as regular sampling of sentinel species (*38*). Both methods are likely to yield insight into infection in the broader host population (*16*), although their usefulness for understanding infection in specific populations must be carefully assessed.

Conclusions

Surveillance for wildlife diseases is an inherently arduous task. However, as the vanguard of our understanding of these diseases, surveillance warrants a scientific approach. To make major inroads into the broader understanding of AIV ecology, epidemiology, and evolution, as well as risks associated with HPAIV, an integrated sampling strategy with clearly defined aims and appropriate methods is required. The financial and logistical constraints of covering vast spatial and temporal scales call for concerted efforts among our combined virologic, ecologic, and genetic expertise.

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Cyprinid Herpesvirus 3

Benjamin Michel, Guillaume Fournier, François Lieffrig, Bérénice Costes, and Alain Vanderplasschen

The recently designated cyprinid herpesvirus 3 (CyHV-3) is an emerging agent that causes fatal disease in common and koi carp. Since its emergence in the late 1990s, this highly contagious pathogen has caused severe financial losses in common and koi carp culture industries worldwide. In addition to its economic role, recent studies suggest that CyHV-3 may have a role in fundamental research. CyHV-3 has the largest genome among viruses in the order Herpesvirales and serves as a model for mutagenesis of large DNA viruses. Other studies suggest that the skin of teleost fish represents an efficient portal of entry for certain viruses. The effect of temperature on viral replication suggests that the body temperature of its poikilotherm host could regulate the outcome of the infection (replicative vs. nonreplicative). Recent advances with regard to CyHV-3 provide a role for this virus in fundamental and applied research.

The common carp (*Cyprinus carpio carpio*) is a freshwater fish and one of the most economically valuable species in aquaculture; worldwide, 2.9 million metric tons are produced each year (*1*). Common carp are usually cultivated for human consumption. Koi (*C. carpio koi*) are an often-colorful subspecies of carp, usually grown for personal pleasure and competitive exhibitions. In the late 1990s, a highly contagious and virulent disease began to cause severe economic losses in these 2 carp industries worldwide (*2*) (Figure 1). The rapid spread was attributed to international fish trade and koi shows around the world (*3*). The causative agent of the disease was initially called koi herpesvirus because of its morphologic resemblance to viruses of the order Herpesvirales (*3*). The virus was subsequently called carp interstitial nephritis and gill necrosis virus because of the associated lesions (*4*). Recently, on the basis of homology of its genome with previously described cyprinid herpesviruses (*5*), the virus was assigned to family *Alloherpesviridae,* genus *Cyprinivirus*, species *Cyprinid herpesvirus 3* and renamed cyprinid herpesvirus 3 (CyHV-3). Because of the economic losses caused by this virus, CyHV-3 rapidly became a subject for applied research. However, recent studies have demonstrated that CyHV-3 is also useful for fundamental research. We therefore summarized recent advances in CyHV-3 applied and fundamental research.

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Characterization of CyHV-3

Classification

CyHV-3 is a member of the order Herpesvirales and newly designated family *Alloherpesviridae* (*5*,*6*) (Figure 2, panel A). *Alloherpesviridae* viruses infect fish and amphibians. The common ancestor of this family is thought to have diverged from the common ancestor of the family *Herpesviridae* (herpesviruses that infect reptiles, birds, and mammals) (*6*). According to phylogenetic analysis of specific genes, the family *Alloherpesviridae* seems to be subdivided into 2 clades (*6*) (Figure 2, panel B). The first clade comprises anguillid and cyprinid herpesviruses, which possess the largest genomes in the order Herpesvirales (245–295 kb). The second clade comprises ictalurid, salmonid, acipenserid, and ranid herpesviruses, which have smaller DNA genomes (134–235 kb).

Structure

The CyHV-3 structure is typical of viruses of the order Herpesvirales. An icosahedral capsid contains the genome, which consists of a single, linear, double-stranded DNA molecule. The capsid is covered by a proteinaceous matrix called the tegument, which is surrounded by a lipid envelope derived from host cell trans-golgi membrane (*7*) (Figure 3). The envelope contains viral glycoproteins (*3*). The diameter of the entire CyHV-3 particle is 170–200 nm (*3*,*8*).

Figure 1. Mass deaths of common carp caused by cyprinid herpesvirus 3 infection in Lake Biwa, Japan, 2004. A) Dead wild common carp; deaths occurred throughout the lake. B) Dead carp (>100,000) collected from the lake in 2004. An estimated 2–3× more carp died but were not collected from the lake. Reproduced with permission from Matsui et al. (*2*).

Molecular Structure

Genome

The genome of CyHV-3 is a 295-kb, linear, double-stranded DNA molecule consisting of a large central portion flanked by two 22-kb repeat regions, called the left and right repeats (*9*). The genome size is similar to that of CyHV-1 but larger than that of other members of the order Herpesvirales, which are generally 125–240 kb.

The CyHV-3 genome encodes 156 potential protein-coding open reading frames (ORFs), including 8 ORFs encoded by the repeat regions. These 8 ORFs are consequently present as 2 copies in the genome (*9*). Five families of related genes have been described: ORF2, tumor necrosis factor receptor, ORF22, ORF25, and RING families. The ORF25 family consists of 6 ORFs (ORF25, ORF26, ORF27, ORF65, ORF148, and ORF149) encoding related, potential membrane glycoproteins. The expression products of 4 of the sequences were detected in mature virions (ORF25, ORF65, ORF148, and ORF149) (*10*). CyHV-3

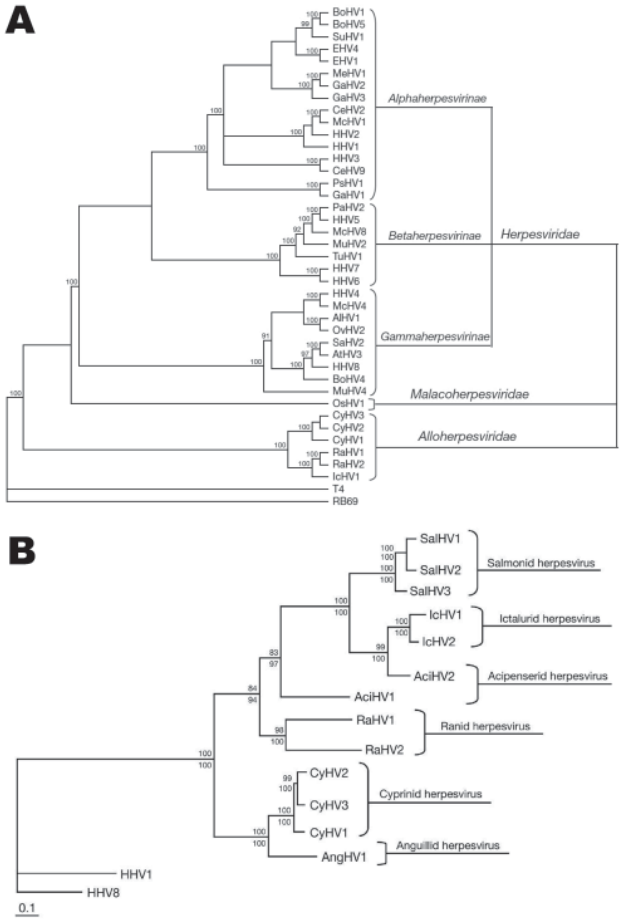

Figure 2. A) Cladogram depicting relationships among viruses in the order *Herpesvirales*, based on the conserved regions of the terminase gene. The Bayesian maximum-likelihood tree was rooted by using bacteriophages T4 and RB69. Numbers at each node represent the posterior probabilities (values >90 shown) of the Bayesian analysis. B) Phylogenetic tree depicting the evolution of fish and amphibian herpesviruses, based on sequences of the DNA polymerase and terminase genes. The maximum-likelihood tree was rooted with 2 mammalian herpesviruses (human herpesviruses 1 and 8). Maximum-likelihood values >80 and Bayesian values >90 are indicated above and below each node, respectively. Scale bar indicates branch lengths, which are based on the number of inferred substitutions. AlHV-1, alcelaphine herpesvirus 1; AtHV-3, ateline herpesvirus 3; BoHV-1, -4, -5, bovine herpesviruses 1, 4, 5; CeHV-2, -9, cercopithecine herpesviruses 2, 9; CyHV-1, -2, cyprinid herpesviruses 1, 2; EHV-1, -4, equid herpesvirus 1, 4; GaHV-1, -2, -3, gallid herpesvirus 1, 2, 3; HHV-1, -2, -3, -4, -5, -6, -7, -8, human herpesvirus 1, 2, 3, 4, 5, 6, 7, 8; IcHV-1, ictalurid herpesvirus 1; McHV-1, -4, -8, macacine herpesvirus 1, 4, 8; MeHV-1, meleagrid herpesvirus 1; MuHV-2, -4, murid herpesvirus 2, 4; OsHV-1, ostreid herpesvirus 1; OvHV-2, ovine herpesvirus 2; PaHV-1, panine herpesvirus 1; PsHV-1, psittacid herpesvirus 1; RaHV-1, -2, ranid herpesvirus 1, 2; SaHV-2, saimiriine herpesvirus 2; SuHV-1, suid herpesvirus 1; and TuHV-1, tupaiid herpesvirus 1. Adapted with permission from Waltzek et al. (*6*).

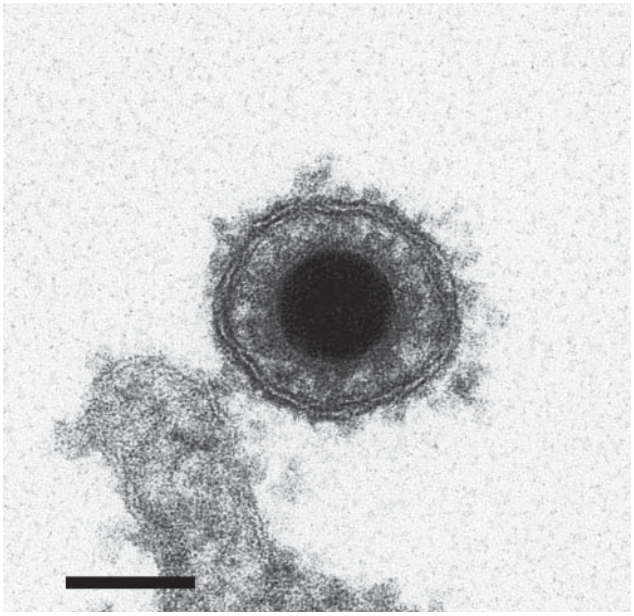

Figure 3. Electron micrograph image of cyprinid herpesvirus 3 virion. Scale bar = 100 nm. Adapted with permission from Mettenleiter et al. (*7*).

encodes several genes that could be involved in immune evasion processes, such as ORF16, which codes for a potential G-protein coupled receptor; ORF134, which codes for an IL-10 homolog; and ORF12, which codes for a tumor necrosis factor receptor homolog.

Within the family *Alloherpesviridae*, anguillid herpesvirus 1 is the closest relative of CyHV-3 that has been sequenced (*11*). Each of these viruses possesses 40 ORFs exhibiting similarity. Sequencing of CyHV-1 and CyHV-2 will probably identify more CyHV-3 gene homologs. The putative products of most ORFs in the CyHV-3 genome lack obvious relatives in other organisms; 110 ORFs fall into this class. Six ORFs encode proteins with closest relatives in virus families such as *Poxviridae* and *Iridoviridae* (*9*). For example, CyHV-3 genes such as B22R (ORF139), thymidylate kinase (ORF140), thymidine kinase (ORF55), and subunits of ribonucleotide reductase (ORF23 and ORF141) appear to have evolved from poxvirus genes (*9*). Neither thymidylate kinase nor B22R has been identified previously in a member of the order Herpesvirales.

Three unrelated strains of CyHV-3, isolated in Israel (CyHV-3 I), Japan (CyHV-3 J), and the United States (CyHV-3 U), have been fully sequenced (*9*). Despite their distant geographic origins, these strains exhibit high sequence identity. Low diversity of sequences among strains seems to be a characteristic of the CyHV-3 species. Despite this low diversity, molecular markers enabling discrimination among 9 genotypes (7 from Europe and 2 from Asia) have been identified (*12*).

Because CyHV-3 possesses the largest genome among members of the order Herpesvirales, it provides a model for mutagenesis of large DNA viruses. Recently, the CyHV-3 genome was cloned as a stable and infectious bacterial artificial chromosome, which could be used to produce CyHV-3 recombinants (*13*).

Structural Proteome

The structural proteome of CyHV-3 was recently characterized by using liquid chromatography tandem mass spectrometry (*10*). A total of 40 structural proteins, comprising 3 capsid, 13 envelope, 2 tegument, and 22 unclassified proteins, were described. The genome of CyHV-3 possesses 30 potential transmembrane-coding ORFs (*9*). With the exception of ORF81, which encodes a type 3 membrane protein expressed on the CyHV-3 envelope (*10*,*14*), no CyHV-3 structural proteins have been studied. ORF81 is thought to be one of the most immunogenic (major) membrane proteins of CyHV-3 (*14*).

In Vitro Replication

CyHV-3 is widely cultivated in cell lines derived from koi fin, *C. carpio* carp brain, and *C. carpio* carp gill (*3*,*4*,*8*,*15*–*17*) (Table 1). Other cell lines have been tested, but few have been found to be permissive for CyHV-3 infection (Table 1).

The CyHV-3 replication cycle was recently studied by use of electron microscopy (*7*). Its morphologic stages suggested that it replicates in a manner similar to that of members of the family *Herpesviridae.* Capsids leave the nucleus by budding at the inner nuclear membrane, resulting in formation of primary enveloped virions in the perinuclear space. The primary envelope then fuses with the outer leaflet of the nuclear membrane, thereby releasing nucleocapsids into the cytoplasm. Final envelopment occurs by budding into trans-golgi vesicles. Because CyHV-3 glycoproteins have little or no similarity with those of members of the family *Herpesviridae*, identification of the CyHV-3 glycoproteins involved in entry and egress will require further study.

Table 1. Cyprinid herpesvirus 3–susceptible cell lines

Cell type (cell line)	Cytopathic effect (reference)
Cyprinus carpio brain (CCB)	Yes (*8*,*15*)
C. carpio gill (CCG)	Yes (*8*)
Epithelioma papulosum cyprinid (EPC)	No (*3*,*4*,*15*,*16*); Yes (*8*)
Koi fin (KFC, KF-1)	Yes (*3*,*4*,*15*,*17*)
Carp fin (CFC, CaF-2)	Yes (*8*)
Fathead minnow (FHM)	No (*3*,*15*); Yes (*16*)
Chinook salmon embryo (CHSE-214)	No (*16*)
Rainbow trout gonad (RTG-2)	No (*16*)
Goldfish fin (Au)	Yes (*15*)
Channel catfish ovary (CCO)	No (*15*)
Silver carp fin (Tol/FL)	Yes (*15*)

Because fish are poikilotherms and because CyHV-3 only affects fish when the water temperature is 18°C–28°C, the effect of temperature on CyHV-3 replication growth in vitro has been investigated. Replication in cell culture is restricted by temperature; optimal viral growth is at 15°C–25°C. Virus propagation and virus gene transcription are turned off when cells are moved to a nonpermissive temperature of 30°C (*18*). Despite the absence of detectable virus replication, infected cells maintained for 30 days at 30°C preserve infectious virus, as demonstrated by viral replication when the cells are returned to permissive temperatures (*18*) (Figure 4). These results suggest that CyHV-3 can persist asymptomatically for long periods in the fish body when the temperature prevents virus replication; bursts of new infection occur after exposure to permissive temperatures.

Disease Caused by CyHV-3

History

In 1998, the first mass deaths of common and koi carp were reported in Israel and the United States (*3*). However, analyses of samples from archives determined that the virus had been in wild common carp since 1996 in the United Kingdom (*19*). Soon after the first report, outbreaks of CyHV-3 were identified in countries in Europe, Asia, and Africa. Currently, CyHV-3 has been identified everywhere in the world except South America, Australia, and northern Africa (*20*). Worldwide, CyHV-3 has caused severe financial and economic losses in the koi and common carp culture industries.

Host Range

Common and koi carp are the only species known to be affected by CyHV-3 infection (*21*). Numerous fish species, cyprinid and noncyprinid, were tested for their ability to carry CyHV-3 asymptomatically and to spread it to unexposed carp (*21–23*) (Table 2). CyHV-3 DNA was recovered from only 2 other fish species: goldfish and crucian carp. Cohabitation experiments suggest that goldfish, grass carp, and tench can carry CyHV-3 asymptomatically and spread it to unexposed common carp. Hybrids (koi–goldfish and koi–crucian carp) die of CyHV-3 infection (*24*).

Susceptibity

CyHV-3 affects carp of all ages, but younger fish (1–3 months, 2.5–6 g) seem to be more susceptible to infection than mature fish (1 year, ≈230 g) (*16*,*21*). Recently, the susceptibility of young carp to CyHV-3 infection was analyzed by experimental infection (*25*). Most infected juveniles (>13 days posthatching) died of the disease, but the larvae (3 days posthatching) were not susceptible.

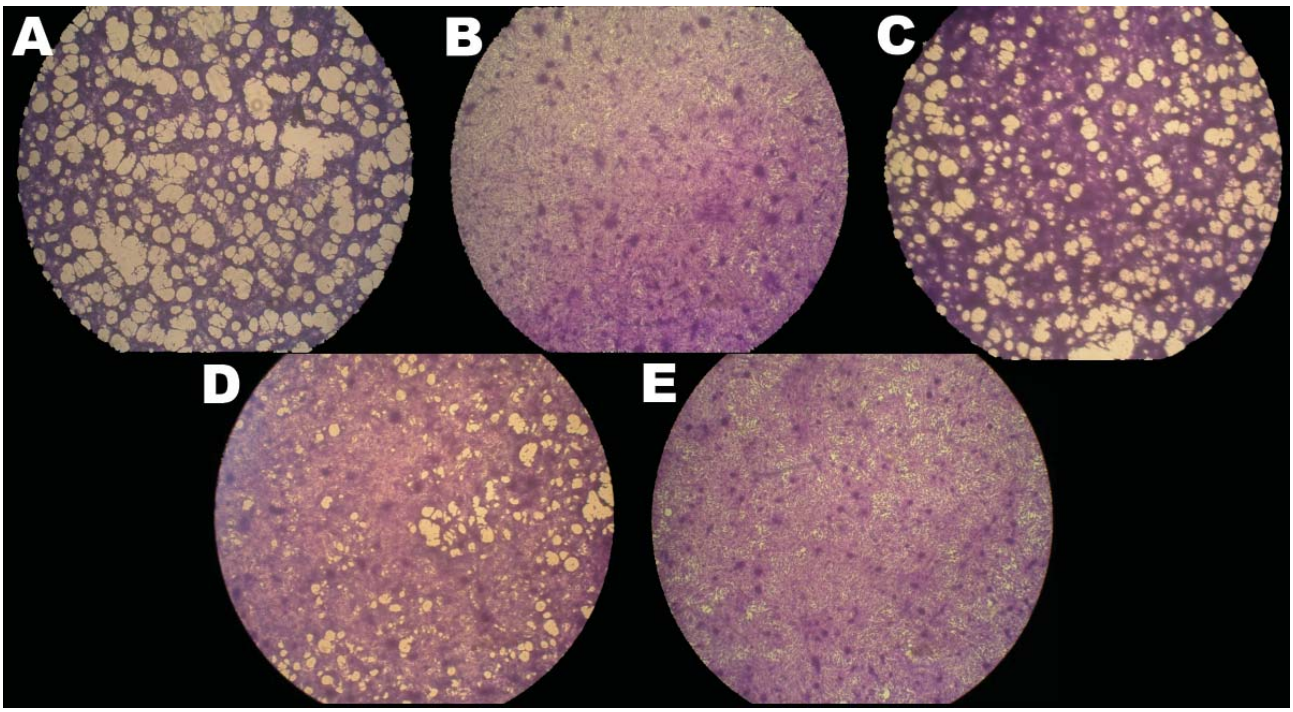

Figure 4. Effects of temperature on cyprinid herpesvirus 3 replication in Cyprinus carpio carp brain cells. After infection, cells were kept at 22°C (A) or shifted to 30°C (B–D); some cells were returned to 22°C at 24 hours (C) or 48 hours (D) postinfection. Uninfected control cells (E) and infected cells at 9 days postinfection were fixed, stained, and photographed. Viral replication was highest in cells maintained at 22°C and lowest in those maintained at 30°C. Original magnification ×20. Adapted with permission from Dishon et al. (*18*).

Table 2. Fish tested for cyprinid herpesvirus 3 infection*

Species (common name)	Inoculated fish (reference)			Carp deaths during cohabitation (reference)
	DNA	Protein	Clinical signs	
Carassius auratus (goldfish)	Yes (*23*)	Yes (*23*)	No (*21*); Yes (*23*)	No (*21*); Yes (*22*)
Ctenopharyngodon idella (grass carp)	NT	NT	No (*21*)	No (*21*); Yes (*22*)
Carassius carassius (crucian carp)	NT	NT	NT	No (*22*)
Hypophthalmichthys molitrix (silver carp)	NT	NT	No (*21*)	No (*21*,*22*)
Aristichtys nobilis (bighead carp)	NT	NT	NT	No (*22*)
Bidyanus bidyanus (silver perch)	NT	NT	No (*21*)	No (*21*)
Oreochromis niloticus (Nile tilapia)	NT	NT	No (*21*)	No (*21*)
Tinca tinca (tench)	NT	NT	NT	Yes (*22*)
Silurus glanis (sheatfish)	NT	NT	NT	No (*22*)
Vimba vimba (vimba)	NT	NT	NT	No (*22*)
Acipenser ruthenus (sterlet)	NT	NT	NT	No (*22*)
Acipenser gueldenstaedtii (Russian sturgeon)	NT	NT	NT	No (*22*)
Acipenser oxyrinchus (Atlantic sturgeon)	NT	NT	NT	No (*22*)

*NT, not tested.

Pathogenesis

Several researchers have postulated that the gills might be the portal of entry for CyHV-3 (*17, 26–28*); however, this hypothesis was recently refuted (*29*). Bioluminescent imaging and an original system for performing percutaneous infection restricted to the posterior part of the fish showed that the skin covering the fin and body mediated entry of CyHV-3 into carp (*29*) (Figure 5). This study, together with an earlier study of the portal of entry of a rhabdovirus (infectious hematopoietic necrosis virus) in salmonids (*30*), suggests that the skin of teleost fish represents an efficient portal of entry for certain viruses. The skin of teleost fish is a stratified squamous epithelium that, unlike its mammalian counterpart, is living and capable of mitotic division at all levels, even the outermost squamous layer. The scales are dermal structures. More extensive studies are needed to demonstrate that the skin is the only portal of entry of CyHV-3 into carp.

After initial replication in the epidermis (*29*), the virus is postulated to spread rapidly in infected fish, as indicated by detection of CyHV-3 DNA in fish tissues (*27*). As early as 24 hours postinfection, CyHV-3 DNA was recovered from almost all internal tissues (including liver, kidney, gut, spleen, and brain) (*27*), where viral replication occurs at later stages of infection and causes lesions. One hypothesis regarding the rapid and systemic dissemination indicated by PCR is that CyHV-3 secondarily infects blood cells. Virus replication in organs such as the gills, skin, and gut at the later stages of infection represents sources of viral excretion into the environment. After natural infection under permissive temperatures (18°C–28°C), the highest mortality rates occur 8–12 days postinfection (dpi) (*21*). Gilad et al. suggest that death is due to loss of the osmoregulatory functions of the gills, kidneys, and gut (*27*).

All members of the family *Herpesviridae* exhibit 2 distinct life-cycle phases: lytic replication and latency. Latency is characterized by maintenance of the viral genome as a nonintegrated episome and expression of a limited number of viral genes and microRNAs. At the time of reactivation, latency is replaced by lytic replication. Latency has not been demonstrated conclusively in members of the family *Alloherpesviridae*. However, some evidence supports existence of a latent phase. CyHV-3 DNA has been detected by real-time PCR at 65 dpi in clinically healthy fish (*27*). Furthermore, the virus persisted in a wild population of common carp for at least 2 years after the initial outbreak (*31*). Finally, St-Hilaire et al. demonstrated the possibility of a temperature-dependent reactivation of CyHV-3 lytic infection several months after initial exposure to the virus (*32*). This finding suggests that the temperature of the water could control the outcome of the infection (replicative/nonreplicative). Whether the observations described above reflect latent infection, as described for the family *Herpesviridae*, or some type of chronic infection, remains to be determined. Similarly, the carp organs that support this latent or chronic infection still need to be identified.

Transmission

Horizontal transmission of CyHV-3 in feces (*26*) and secretion of viral particles into water (*21*) have been demonstrated. The skin of carp acts as the portal of entry of CyHV-3 and the site of early replication (*29*). The early replication of the virus at the portal of entry could contribute not only to the spread of the virus within infected fish but also to the spread of the virus throughout the fish population. As early as 2–3 dpi, infected fish rubbed against other fish or against objects. This behavior could contribute to a skin-to-skin mode of transmission. Later during infection, this mode of transmission could also occur when uninfected fish pick at the macroscopic herpetic skin lesions on infected fish. To date, no evidence of vertical transmission of CyHV-3 has been found.

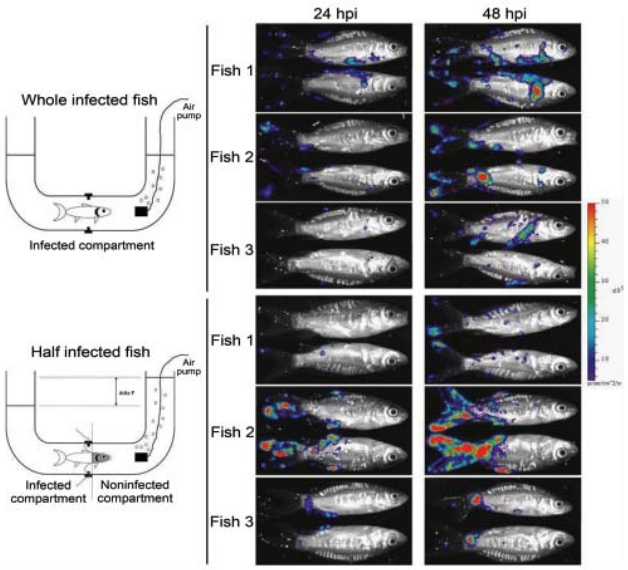

Figure 5. Skin of carp as a portal of entry for cyprinid herpesvirus 3. A schematic representation of the system used to restrict viral inoculation to the fish skin is shown on the left. The lower drawing shows the conditions under which 6 fish were inoculated by restricted contact of the virus with the skin located posterior to the anterior part of the dorsal fin. The upper drawing shows control conditions under which 6 fish were inoculated in the system but without the latex diaphragm dividing the fish body into 2 isolated parts, enabling virus to reach the entire fish body. The fish were infected by bathing them for 24 h in water containing 2×10^3 PFU/mL of a recombinant cyprinid herpesvirus 3 strain able to emit bioluminescence. All fish were analyzed 24 h postinfection (hpi) by bioluminescence imaging. After an additional incubation period of 24 h in individual tanks containing fresh water, they were reanalyzed by bioluminescence imaging at 48 hpi. Three representative fish are shown. The images are shown with standardized minimum and maximum threshold values for photon flux. Adapted with permission from Costes et al. (*29*).

Clinical Signs

The first signs appear at 2–3 dpi. The fish exhibit appetite loss and lethargy and lie at the bottom of the tank with the dorsal fin folded. Depending on the stage of the infection, the skin exhibits different clinical signs, such as hyperemia, particularly at the base of the fins and on the abdomen; mucus hypersecretion; and herpetic lesions (Figure 6). The gills frequently become necrotic and hypersecrete mucus, which suffocates the fish. Bilateral enophthalmia is observed in the later stages of infection. Some fish show neurologic signs in the final stage of the disease, when they become disoriented and lose equilibrium (*3*,*19*,*21*).

Histopathologic Findings

In CyHV-3 infected fish, prominent pathologic changes occur in the gill, skin, kidney, liver, spleen, gastrointestinal system, and brain (*3*,*17*,*21*,*28*). Histopathologic changes appear in the gills as early as 2 dpi and involve the epithelial cells of the gill filaments. These cells exhibit hyperplasia, hypertrophy, and/or nuclear degeneration (*3*,*17*,*21*,*28*). Severe inflammation leads to the fusion of respiratory epithelial cells with cells of the neighboring lamellae, resulting in lamellar fusion (*17*,*28*). In the kidney, a weak peritubular inflammatory infiltrate is evident as early as 2 dpi and, along with blood vessel congestion and degeneration of the tubular epithelium in many nephrons, increases with time (*17*). In the spleen and liver, splenocytes and hepatocytes, respectively, are the most obviously infected cells (*28*). In brain of fish that showed neurologic signs, congestion of capillaries and small veins are apparent in the valvula cerebelli and medulla oblongata, associated with edematous dissociation of nerve fibers (*28*).

Diagnosis

Diagnosis of CyHV-3 infection is described elsewhere (*20*). Suspicion of CyHV-3 infection is based on clinical signs and histopathologic findings. Since initial isolation of CyHV-3 in 1999, complementary diagnostic methods have been developed. Virus isolation from infected fish tissues in cell culture (*C. carpio* carp brain and koi fin cells) was the first method to be developed (*3*). This time-consuming approach is still the most effective method for detecting infectious particles during an outbreak of CyHV-3 infection. A complete set of techniques for detecting viral genes—including PCR (*20*), nested PCR (*33*), TaqMan PCR (*27*), and loop-mediated isothermal amplification (*34*)—has been developed. Real-time TaqMan PCR has been used to detect CyHV-3 in freshwater environments after concentration of viral particles (*2*). Finally, ELISAs have been developed to detect specific anti-CyHV-3 antibodies in the blood of carp (*35*) and to detect CyHV-3 antigens in samples (*17*,*26*).

Immune Response

Immunity in ectothermic vertebrates differs in several ways from that of their mammalian counterparts. Environmental temperature has drastic effects on the fish immune system. In carp, for example, at <14°C, adaptive immunity is inhibited, but the innate immune response remains functional (*36*). As mentioned above, host temperature also has an effect on CyHV-3 replication, which can occur only at 18°C–28°C. In carp that are infected and maintained at 24°C, antibody titers begin to rise at ≈10 dpi and plateau at 20–40 dpi (*37*). In the absence of antigenic reexposure, the specific antibodies gradually decrease over 6 months to a level slightly above or comparable to that of unexposed fish. Although protection against CyHV-3 is proportional to the titer of specific antibodies during primary infection, immunized fish, even those in which antibodies are no longer detectable, are resistant to a lethal challenge, possibly because of the subsequent rapid response of B and T memory cells to antigen restimulation (*37*).

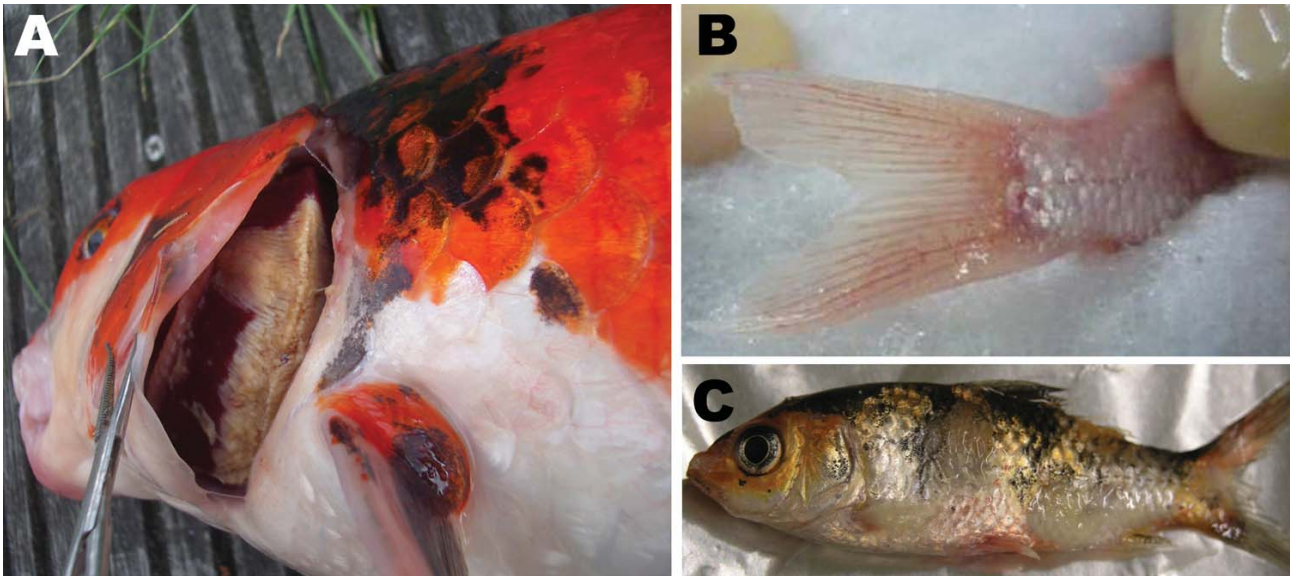

Figure 6. Clinical signs in cyprinid herpesvirus 3–infected fish. A) Severe gill necrosis; B) hyperemia at the base of the caudal fin; C) herpetic skin lesions on the body and fin erosion.

Prophylaxis and Control

For CyHV-3 control, 3 approaches are being developed. They are 1) management and commercial measures to enhance the international market of certified CyHV-3 –free carp and to favor eradication of CyHV-3, 2) selection of CyHV-3–resistant carp, and 3) development of safe and efficacious vaccines.

Selection of CyHV-3–Resistant Carp

Carp resistance to CyHV-3 might be affected by host genetic factors. Shapira et al. demonstrated differential resistance to CyHV-3 (survival rates 8%–60%) by crossbreeding sensitive domesticate strains and a resistant wild strain of carp (*38*). Further supporting the role of host genetic factors in CyHV-3 resistance, major histocompatibility class II genes were recently shown to affect carp resistance (*39*).

Vaccination of Carp

Soon after the characterization of CyHV-3, a protocol to induce a protective adaptive immune response in carp was developed. This approach is based on the fact that CyHV-3 induces fatal infections only when the water temperature is 18°C–28°C.

According to this protocol, healthy, uninfected fish are exposed to CyHV-3 infected fish for 3–5 days at permissive temperature (22°C–23°C) and then transferred for 30 days to ponds at a nonpermissive temperature (≈30°C). After this procedure, 60% of fish become resistant to further challenge with CyHV-3 (*4*). Despite its ingenuity, this method has several disadvantages: 1) increasing the water temperature to 30°C makes the fish more susceptible to secondary infection by other pathogens and requires a large amount of energy in places where the water is naturally cool; 2) the protection is observed in only 60% of fish; 3) carp that are "vaccinated" by using this protocol have been exposed to wild-type virulent CyHV-3 and could therefore represent a potential source of CyHV-3 outbreaks if they later come into contact with an unexposed carp.

Attenuated live vaccine appears to be the most appropriate for mass vaccination of carp. Attenuated vaccine candidates have been produced by successive passages in cell culture (*4*). The vaccine strain candidate was further attenuated by UV irradiation to increase the mutation rate of the viral genome (*4*,*37*). A vaccine strain obtained by this process has been produced by KoVax Ltd. (Jerusalem, Israel) and has been shown to confer protection against a virulent challenge. However, this vaccine is available in only Israel and has 2 main disadvantages: 1) the molecular basis for the reduced virulence is unknown, and consequently, reversions to a pathogenic phenotype cannot be excluded; and 2) under certain conditions, the produced attenuated strain could retain residual virulence that could be lethal for a portion of the vaccinated fish (*37*).

An inactivated vaccine candidate was described by Yasumoto et al. (*40*). It consists of formalin-inactivated CyHV-3 trapped within a liposomal compartment. This vaccine can be used for oral immunization in fish food. Protection efficacy for carp is 70% (*40*).

Conclusions

Because CyHV-3 causes severe financial losses in the common carp and koi culture industries worldwide, it is

a useful subject for applied science. Safe and efficacious vaccines adapted to mass vaccination of carp and efficient diagnostic methods need to be developed. Several aspects of CyHV-3 make it also useful for fundamental science. These aspects are its large genome, the relationship between CyHV-3 infectivity and temperature, and the low similarity between CyHV-3 genes and the genes of other members of the order Herpesvirales that have been studied. Further studies are needed to identify the roles of CyHV-3 genes in viral entry, egress, and disease pathogenesis.

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etymologia

Cyprinid [sip′ri nid] **Herpesvirus** [hur′pēz vi′rəs]

Cyprinids, members of the large freshwater fish family *Cyprinidae*, take their name from the Greek *Kypris*, also another name for the Aphrodite, Greek goddess of love and beauty. It refers to the island of Cyprus, alleged to be the site of her birth. The term herpesvirus derives from Greek *herpes*, a spreading eruption, and the Latin word for poison. This virus is an emerging infection in common carp (*Cyprinus carpio carpio*) and koi (*C. carpio koi*).

Source: Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders Elsevier; 2007; www.statemaster.com/encccyclopedia/Cyprinids.

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Reassortant Group A Rotavirus from Straw-colored Fruit Bat (*Eidolon helvum*)

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Bats are known reservoirs of viral zoonoses. We report genetic characterization of a bat rotavirus (Bat/KE4852/07) detected in the feces of a straw-colored fruit bat (*Eidolon helvum*). Six bat rotavirus genes (viral protein [VP] 2, VP6, VP7, nonstructural protein [NSP] 2, NSP3, and NSP5) shared ancestry with other mammalian rotaviruses but were distantly related. The VP4 gene was nearly identical to that of human P[6] rotavirus strains, and the NSP4 gene was closely related to those of previously described mammalian rotaviruses, including human strains. Analysis of partial sequence of the VP1 gene indicated that it was distinct from cognate genes of other rotaviruses. No sequences were obtained for the VP3 and NSP1 genes of the bat rotavirus. This rotavirus was designated G25-P[6]-I15-R8(provisional)-C8-Mx-Ax-N8-T11-E2-H10. Results suggest that several reassortment events have occurred between human, animal, and bat rotaviruses. Several additional rotavirus strains were detected in bats.

Rotaviruses are members of the family *Reoviridae* and genus *Rotavirus* and contain 3 primary species: *Rotavirus A*, *Rotavirus B*, and *Rotavirus C* (*1*). The rotavirus genome contains 11 segments of double-stranded RNA encoding 6 structural viral proteins (VP1–VP4, VP6, and VP7) and 6 nonstructural proteins (NSP1–NSP6). Rotavirus A strains are associated with acute infectious diarrhea in humans and animals (*2*). The segmented nature of rotavirus genomes enables reassortment events in which novel rotavirus strains are produced with new combinations of genome segments derived from parental virus strains (*3*). Reassortment is a major mechanism for generating genetic diversity of rotaviruses and driving rotavirus evolution. New rotavirus strains emerge every year as a result of genomic reassortment among cocirculating rotaviruses. Although most rotaviruses appear to be host restricted, interspecies transmission of rotaviruses has been documented (*4*–*8*). Recently, new genotypes found in a variety of animals have been reported (*9*). Thus, monitoring rotaviruses in domesticated and wild animals can potentially identify emerging human and veterinary pathogens.

Species A rotaviruses have been traditionally classified by using a binomial nomenclature based on serotype and genotype specificities of the outer capsid antigens, VP7 (G-type) and VP4 (P-type). Recently, Matthijnssens et al. (*10*) proposed a classification system based on all 11 genome segments (*4*,*7*,*10*). This scheme uses specific nucleotide sequence identity cutoff values for the complete open reading frame (ORF) of each gene segment to delineate genotypes, and new genotypes are formally assigned by the Rotavirus Classification Working Group (RCWG) (*10*).

Bats of many species are being recognized as reservoir hosts for viruses that can cross species barriers to infect humans (*11*). Such viruses include Ebola and Marburg viruses, Nipah and Hendra viruses, severe acute respiratory syndrome–like coronavirus, rabies and other lyssaviruses, togaviruses, flaviviruses, bunyaviruses, and members of the family *Reoviridae* (*11*–*13*). The straw-colored fruit bat (*Eidolon helvum*, family Pteropodidae, order Chiroptera) (*11*) is widely distributed and ranges from the southwestern Arabian Peninsula to the forest and savanna zones (south of the Sahara Desert) and offshore islands of Africa. Detection of Lagos bat virus (*Lyssavirus*)

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(*14*) and Ife virus (*Orbivirus*) (*15*) in *E. helvum* bats demonstrates their potential as reservoirs of viruses that cause zoonotic diseases. Rotaviruses have not been detected in bats, although other viruses in the family *Reoviridae* have been detected in bats (*11*).

The purpose of this study was to examine *E. helvum* bats for rotaviruses and characterize any strains found. We detected a novel rotavirus A species in *E. helvum* bats. This isolate contained a VP4 gene that probably originated from a human rotavirus, an NSP4 gene of likely human or animal rotavirus origin, and otherwise a unique genetic background requiring establishment of new genotypes for at least 7 genes.

Materials and Methods

Samples

During 2007, fecal swabs were collected from bats during field surveys conducted in Kenya as described (*14*). Samples tested for rotaviruses were first screened for lyssaviruses and coronaviruses (*13*).

Nucleic Acid Extraction and Reverse Transcription–PCR

Suspensions of fecal swabs obtained from *E. helvum* bats were prepared in phosphate-buffered saline. Total nucleic acid was extracted by using the QIAamp Mini Viral Elute Kit (QIAGEN, Valencia, CA, USA). After denaturing extracted nucleic acid at 95°C for 5 min, reverse transcription–PCR (RT-PCR) for amplification of the rotavirus VP6 gene was performed by using a One-Step RT-PCR Kit (QIAGEN). VP6F and VP6R primers and cycling conditions have been described (*16*). For VP6-positive samples, attempts were made to amplify different rotavirus gene segments (complete or partial) by using published rotavirus-specific consensus primers for VP4 and VP7 (*17–19*) and VP1, VP2, VP6, NSP1, NSP2, NSP3, NSP4, and NSP5 (*5*). New internal oligonucleotide primers for VP1, VP2, VP7, NSP2, NSP3, NSP5, and VP6 genes were designed on the basis of initial bat sequences obtained and sequences of published mammalian and avian rotavirus strains and used for sequencing (Table). RT-PCR products were analyzed by performing electrophoresis on 1% agarose gels containing GelRed Gel Stain (Biotium, Inc., Hayward, CA, USA) and viewed by using UV transillumination.

Nucleotide Sequencing

Specific RT-PCR amplicons were excised from agarose gels and purified by using the QIAquick Gel Extraction Kit (QIAGEN). Cycle sequencing of each amplicon was performed with the same consensus primers used for RT-PCR by using a Big Dye Terminator 1.1 or 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Cycle sequencing products were purified by using a BigDye XTerminator Purification Kit (Applied Biosystems) or an in-house magnetic bead purification method (S. Mijatovic-Rustempasic, unpub. data). Sequencing was performed by using an ABI 3130XL Sequencer (Applied Biosystems). Primer walking sequencing was performed to cover each gene segment on both strands.

Nucleotide and Protein Sequence Analysis

Sequence chromatogram files were edited and sequence contigs were assembled by using Sequencher 4.8 software (Gene Codes Corporation, Inc., Ann Arbor, MI,

Table. Newly designed primers used for amplification and sequencing of bat rotavirus genes*

Primer	Sequence, 5′ → 3′	Gene	Nucleotide position, strand
VP1_Bat	GCT TCG AAT GGA GAA TCG CG	VP1	596–576, –
VP1_Bat	CTC CTG GTG TGT ACC TAC C	VP1	485–504, +
VP1_Bat	CCT CGT GTG TAA ATA CGG ACA	VP1	1130–1151, +
VP2_Bat	CAG AGC AGG CTA AGA AGC AGA CTA	VP2	393–370, –
VP2_Bat	GCA GCA CCA ATT TGG GTT GAG	VP2	810–830, +
VP2_Bat	CTC AAC CCA AAT TGG TGC TG	VP2	830–810, –
VP2_Bat	CTG AAT CTG AGC TTC AGT TG	VP2	1206–1187, –
VP2_Bat	GAT GTA GCT AGA GTG CCA G	VP2	2247–2265, +
VP2_Bat	CTG GCA CTC TAG CTA CAT C	VP2	2265–2247, –
VP2_Bat	GGT GGC GAA TTA TGA TTG G	VP2	2723–2741, +
VP6_Bat	TCG GTC TAT GGA ATG TGA AAC CTG TTC	VP6	380–354, –
VP7_Bat	CAG ATG TCG TCG ATA ATG	VP7	693–710, +
VP7_Bat	TGG CGT ACG CAG TGT CCA TTG AC	VP7	218–196, –
VP7_Bat	CGA GTT GGC GTA CGC AGT G	VP7	258–240, –
NSP2_Bat	CAA CTT CCA CAT TGT AAA CGC	NSP2	574–555, –
NSP2_Bat	CTT TCG CGA ATA CAC TGG T	NSP2	1087–1068, –
NSP2_Bat	CT GAT AGA GTG TAT GCG AC	NSP2	727–747, +
NSP3_Bat	CCT CAC TCT CAT CTT TCG GGT CTT C	NSP3	401–376, –
NSP3_Bat	GGA GTT ACC GAG TGA AGC GAA GG	NSP3	673–695, +
NSP5_Bat	ACG CCA GCA TCT GCA TTT GTC	NSP5	279–258, –

*VP, viral protein; NSP, nonstructural protein.

USA). Nucleotide sequences were translated into amino acid sequences and manually aligned by using GeneDoc software (*20*). Nucleotide and protein similarity searches were performed by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search GenBank databases (*21*). Nucleotide and amino acid identities were calculated by using the *P*-distance algorithm of MEGA 4.0 software (*22*). Multiple sequence alignments were performed by using GeneDoc software (*20*). Phylogenetic relationships were inferred from aligned nucleotide sequences and amino acid sequences (VP1 and VP7 proteins only) by Bayesian analysis in MrBayes 3.1.2 (*23*,*24*). Each nucleotide sequence analysis was run for 500,000–700,000 generations until the average SD of split frequencies was <0.01. Bayesian analysis of amino acid data was run for 100,000 generations, after which the average SD of split frequencies was <0.05. Tree files were visualized by using TreeDyn 198.3 (*25*).

Results

We initially screened 6 samples; 1 sample, Bat/KE4852/07, obtained from an *E. helvum* bat trapped in Vihiga, Kenya, was positive for rotavirus by VP6 RT-PCR. Using primers annealing to noncoding regions of each segment and internal primers, we then obtained full-length ORF sequences for VP2, VP6, VP7, NSP2, NSP3, NSP4, and NSP5, except for VP1 and VP4, for which partial-length gene sequences were obtained (for the remainder of this report, we will refer to each ORF, from ATG to stop codon, as a gene). None of the sequences reported in this study were inferred from primer sequences. Sequences were not obtained for VP3 and NSP1, despite repeated attempts to obtain amplicons by using panels of rotavirus A–specific primer pairs. Nucleotide sequences for VP1, VP2, VP4, VP6, VP7, NSP2, NSP3, NSP4, and NSP5 were deposited in GenBank under accession nos. GU983672–GU983680. Genetic analyses of Bat/KE4852/07 indicated that 7 genes were unique and 2 were similar to described rotavirus genotypes. Results are summarized below and in Figure 1.

VP7 Gene

The putative VP7 gene of strain Bat/KE4852/07 was 981 bp and encoded a 326 aa protein. The nucleotide sequence of Bat/KE4852/07 VP7 showed low levels of identity to the 24 established G genotypes (range 55.9%–67.4%) (Figure 1, panel A). The VP7 gene of Bat/KE4852/07 was classified into a novel VP7 genotype, G25, by the RCWG (*10*). When compared with other mammalian rotavirus VP7 protein sequences, amino acid dissimilarity was >17% overall (Figure 1, panel A) and exceeded 20% in antigenic regions A, B, and C (*26*).

VP4 Gene

An 829-bp region of the VP4 gene of Bat/KE4852/07 strain was amplified and sequenced by using standard VP4 RT-PCR primers (*18*). The sequence corresponded to bases 24–851 of strain US1205 VP4 (GenBank accession no. AF079356.) BLAST searches indicated that the bat rotavirus VP4 sequence was closely related to human P[6] strains from Africa (6809/ARN) and eastern Asia (CAU214) and showed 99% nt and 98% aa identities (Figure 1, panel B). Relatively low sequence identities were found when compared with P[6] sequences of animal origin (81.6% nt and 85.2% aa identities) (Figure 1, panel B).

VP6 Gene

The complete VP6 gene (1,194 bp), with a protein of 397 aa, of Bat/KE4852/07 strain was determined. Overall nucleotide identity with reference genotypes I1-I13 strains ranged from 68.1% to 80.6% (Figure 1, panel C). These nucleotide identity values fell below the VP6 genotype cutoff value of 85% (*10*), indicating that Bat/KE4852/07 strain belongs to a novel VP6 genotype designated I15 by the RCWG. Bat/KE4852/07 VP6 amino acid sequence shared 69%–92.7% identity with other rotavirus VP6 sequences (Figure 1, panel C).

VP1 Gene

A fragment of the VP1 gene was obtained for Bat/KE4852/07. The sequenced region was 1,198 nt, which was one third the expected full length of the VP1 gene and corresponded to bases 252–1451 of rotavirus strain S2 VP1 sequence (GenBank accession no. DQ870485). Comparison of the partial VP1 nucleotide and amino acid sequences of Bat/KE4852/07 with those of other mammalian and avian rotavirus strains showed low levels of identity similar to those of other VP1 genotypes (<59% and <38%, respectively; Figure 1, panel D).

Despite the uniqueness of the bat rotavirus VP1 sequence, BLAST search and alignment results with amino acid sequence displayed the highest degree of identity with rotavirus A VP1 sequences (48% conservation of similar amino acid residues) (Figure 2). The Bat/KE4852/07 VP1 gene partial sequence includes the region encoding the 19 residue polymerase F motif described (*27*), but within this domain, the percent amino acid identity with other group A rotaviruses is <37% (53% similarity), and none of the 3 arginine residues (RR452, R457, R460) of a predicted functional role in rotaviruses are present in Bat/KE4852/07 VP1 (Figure 2). The partial gene nucleotide sequence identity was well below the cutoff value of 83% that has been used to classify VP1 genotypes. The amino acid sequence divergence (>62%) exceeds that observed when group A and C rotavirus VP1 proteins are compared (>53%) (*27*).

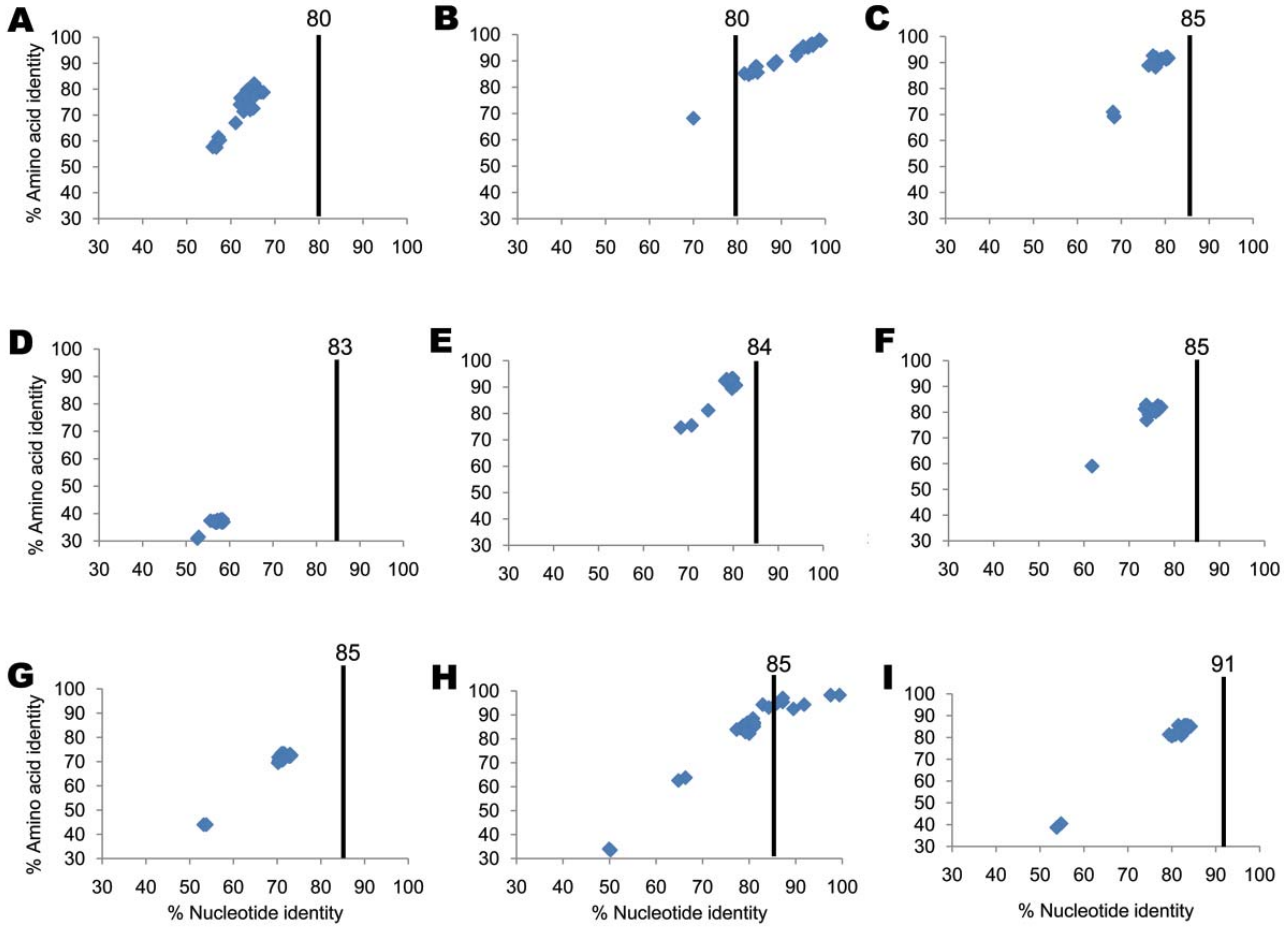

Figure 1. Percentage nucleotide and deduced amino acid homologies of A) viral protein 7 (VP7), B) VP4, C) VP6, D) VP1, E) VP2, F) nonstructural protein 2 (NSP2), G) NSP3, H) NSP4, and I) NSP5 gene segments of bat rotavirus strain Bat/KE4852/07 from Kenya compared with respective genes deposited in GenBank. Vertical lines indicate nucleotide percentage identity cutoff values defining genotypes for 11 rotavirus gene segments (*7,10*). Blue diamonds indicate coordinates for each pairwise comparison when percentage nucleotide identity is plotted against percentage amino acid identity. GenBank accession numbers used in this comparison are listed in the online Technical Appendix (www.cdc.gov/EID/content/16/12/1844-Techapp.pdf).

Although the minimal sequence length set by the RCWG for new candidate genotypes does not permit official designation of a new genotype derived from the partial gene sequence of Bat/KE4852/07, we showed that this bat rotavirus contains a highly divergent VP1 gene provisionally designated genotype R8.

VP2 Gene

The VP2 gene of strain Bat/KE4852/07 was 2,712 bp and encoded a deduced protein of 903 aa. The sequence of the VP2 gene segment of Bat/KE4852/07 strain was longer than those of most rotavirus strains because of several nucleotide insertions within the ORF near the 5′ end of the gene. As a result of these insertions, the predicted VP2 protein was 21 aa longer than that of most mammalian and avian rotaviruses. Nucleotide sequence comparisons indicated that the VP2 sequence of Bat/KE4852/07 was distantly related to all 6 established VP2 genotypes; identities ranged from 68.3% to 80.6% (Figure 1, panel E). These values were below the cutoff value of 84% nt sequence identity that has been used to classify VP2 genotypes (*10*). Thus, Bat/KE4852/07 was assigned into novel genotype C8 by the RCWG. VP2 amino acid identities between Bat/KE4852/07 and other rotaviruses were <94% (Figure 1, panel E).

NSP2 Gene

The NSP2 gene of strain Bat/KE4852/07 was 954 bp and encoded a polypeptide of 317 aa. Bat/KE4852/07 NSP2 gene sequence shared <78% identity with other rotavirus NSP2 strains (Figure 1, panel F). This value was below the cutoff value of 85% nt sequence identity that has

Figure 2. Alignment of viral protein 1 (VP1) amino acid sequence of bat rotavirus strain KE4852/07 from Kenya with cognate VP1 sequence of reference rotavirus A strain S2. The consensus line shows conserved amino acid residues and similar residues (indicated by +). The motif F region (*27*) is shaded.

been used to classify NSP2 genotypes (*10*). The RCWG assigned novel genotype N8 to the NSP2 gene of strain Bat/KE4852/07.

NSP3 Gene

The NSP3 gene of strain Bat/KE4852/07 was 936 bp and encoded a deduced protein of 311 aa. Bat/KE4852/07 strain NSP3 nucleotide and amino acid sequences exhibited <74% identity with other NSP3 genotypes (Figure 1, panel G). Because this value is below the cutoff value of 85% nt identity used to differentiate T genotypes (*10*), this gene was assigned to a novel NSP3 genotype (T11) by the RCWG.

NSP4 Gene

The putative NSP4 gene was 528 bp and encoded a polypeptide of 175 aa. Analyses of nucleotide and deduced amino acid sequences indicated that the Bat/KE4852/07 NSP4 gene shared >99% nt and >98% aa identities with human (I321, B1711 and DS-1), ovine (OVR762), simian (PTRV), and bovine (WC3) rotavirus strains of the NSP4 genotype E2 (Figure 1, panel H).

NSP5 Gene

The NSP5 gene of strain Bat/KE4852/07 was 630 bp and encoded a 209-aa polypeptide. This gene was longer than NSP5 genes of most rotavirus strains because of several nucleotide insertions within the ORF. Thus, the putative NSP5 is 11 aa longer than that of most mammalian and avian rotaviruses. The NSP5 gene of strain Bat/KE4852/07 shared <85% nt identity with other NSP5 genotypes (*10*) (Figure 1, panel I) and was assigned to a novel NSP5 genotype (H10). Within the NSP5 gene of strain Bat/KE4852/07, there was a second ORF at nucleotide positions 80–346 (+1 reading frame) which corresponded to a putative NSP6 gene.

Phylogenetic Relationships of Bat/KE/4852/07 Genes to other Rotavirus Strains

Bayesian phylogenetic analyses of Bat/KE4852/07 and other rotavirus strain sequences indicated that 2 genes (VP4 and NSP4) were closely related to described mammalian rotaviruses; that 6 bat rotavirus genes (VP2, VP6, VP7, NSP2, NSP3, NSP5) were more distantly related and represented novel genotypes; and that the VP1 gene was distant from all known cognate genes of mammalian and avian rotaviruses (Figures 3, 4). In the phylogeny estimated from VP7 nucleotide sequences, Bat/KE4852/07 occupied a well-supported (posterior probability 0.94) terminal group with G9 rotavirus strain t203 (AY003871), although the bat strain was connected by a long branch to the terminal node (Figure 3, panel E). Bayesian analysis of VP7 amino acid sequences yielded the same result.

The VP6 phylogenetic estimate grouped Bat/KE4852/07 with the I9 genotype TUCH strain (EF583013) (posterior probability 0.81) and a long terminal branch (Figure 3, panel D). Phylogenetic analysis of VP2 sequences resulted in well-resolved phylogeny with Bat/KE4852/07 and occupied an intermediate lineage between C1 genotype rotaviruses and a clade containing C2, C3, and C5 viruses (Figure 3, panel B). Analysis of NSP3 sequences indicated weak support (posterior probability 0.57) for monophyly of the bat rotavirus with T1 and T6 genotype viruses, and Bat/KE4852/07 was separated from these other mammalian viruses by a relatively long terminal branch (Figure 4, panel B). Bayesian analysis of NSP5 sequences indicated strong support (posterior probability 1.0) for monophyly of the bat rotavirus with mammalian rotaviruses in genogroups H1, H2, H3, and H5. However, intergenogroup relationships within this clade were not resolved (Figure 4, panel D).

Bayesian analysis of partial VP4 sequences yielded a phylogenetic estimate that places the bat rotavirus strain within a clade containing all genotype P[6] viruses (posterior probability 1.0) and occupying a lineage shared with human P[6] strains 6809/ARN and CAU214 (Figure 3, panel C). The phylogenetic estimate obtained by using NSP4 gene nucleotide data supported the monophyletic origin of Bat/KE4852/07 with human strain I321 (AF165066 genotype E2), bovine strain WC3 (AY050273, genotype E2),

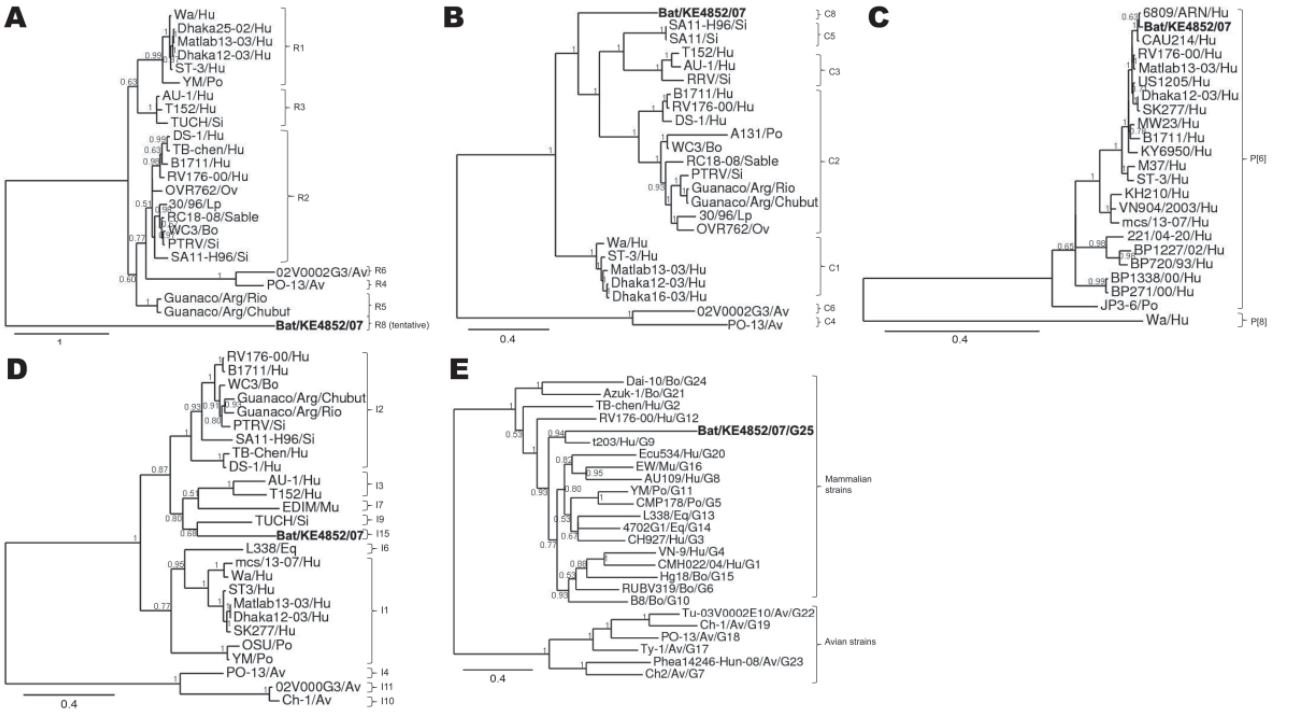

Figure 3. Phylograms indicating genetic relationships of partial or complete nucleotide sequences of A) viral protein 1 (VP1) partial, B) VP2, C) VP4 partial, D) VP6, and E) VP7 of bat rotavirus strain Bat/KE4852/07 (**boldface**) from Kenya with representatives of known human and animal rotavirus genotypes. Posterior probability values are indicated at each branch node. Scale bars indicate nucleotide substitutions per site. GenBank accession numbers of all strains used are listed in the online Technical Appendix (www.cdc.gov/EID/content/16/12/1844-Techapp.pdf). Genotypes of each gene segment characterized in this study are listed to the right of each tree. Hu, human; Po, porcine; Si, simian; Ov, ovine; Lp, lapine; Bo, bovine; Av, avian; Eq, equine.

and simian strain PTRV (FJ422140, genotype E2) (Figure 4, panel C).

For partial VP1 sequences, Bayesian analyses of nucleotide and amino acid data did not produce well-resolved phylogenies. However, in each tree the longest terminal branch was between the bat rotavirus and the ancestral node (Figure 3, panel A). Genetic distance separating Bat/KE4852/07 from the other rotaviruses was longer than the distance between avian rotaviruses (genotypes R4 and R6) and mammalian rotaviruses. For NSP2, the bat rotavirus gene occupied an intermediate position between avian rotaviruses (genotypes N4 and N6) and mammalian rotaviruses (genotypes N1, N2, N3, and N5) and showed maximum posterior probability support (Figure 4, panel A).

Additional Screening Results

After characterization of Bat/KE4852/07, we processed an additional 39 *E. helvum* bat fecal swab samples from Kenya and screened them for rotaviruses by using VP6 RT-PCR. Three additional samples (Bat/KE5096/07, Bat/KE5105/07, and Bat/K5175/07) were positive for rotavirus. These 3 samples were obtained in Maseno, Kenya, which is ≈20 km from Vihiga. Given the population dynamics and migratory patterns of this species, bats from both roosts likely interact, at least during certain times of the year. VP6 sequences for Bat/KE5096/07, Bat/KE5105/07, and Bat/K5175/07 samples were 100% identical to VP6 sequence of Bat/KE4852/07. In an attempt to obtain a complete genomic sequence for the bat rotavirus, we will analyze this virus by using sequence-independent deep sequencing.

Discussion

We detected and genetically characterized a bat-associated rotavirus strain. Although NSP1 and VP3 gene sequences remain undefined, this incomplete genome sequence provides insight into rotavirus diversity, evolution, classification, and ecology. The RCWG has classified bat strain Bat/KE4852/07 as G25-P[6]-I15-R8(provisional)-C8-Mx-Ax-N8-T11-E2-H10. The finding that the Bat/KE4852/07 VP4 gene was nearly identical to human P[6] strains suggests that it has been introduced as a result of a ressortment event between human and bat rotaviruses. The NSP4 gene of this bat rotavirus is also likely to have been introduced into the bat rotavirus genome by reassortment with human or animal strains. The possibility that the VP4 and NSP4 gene segments were originally bat rotavirus genes, which have evolved and become ubiquitous among human rotaviruses for several years, cannot be excluded.

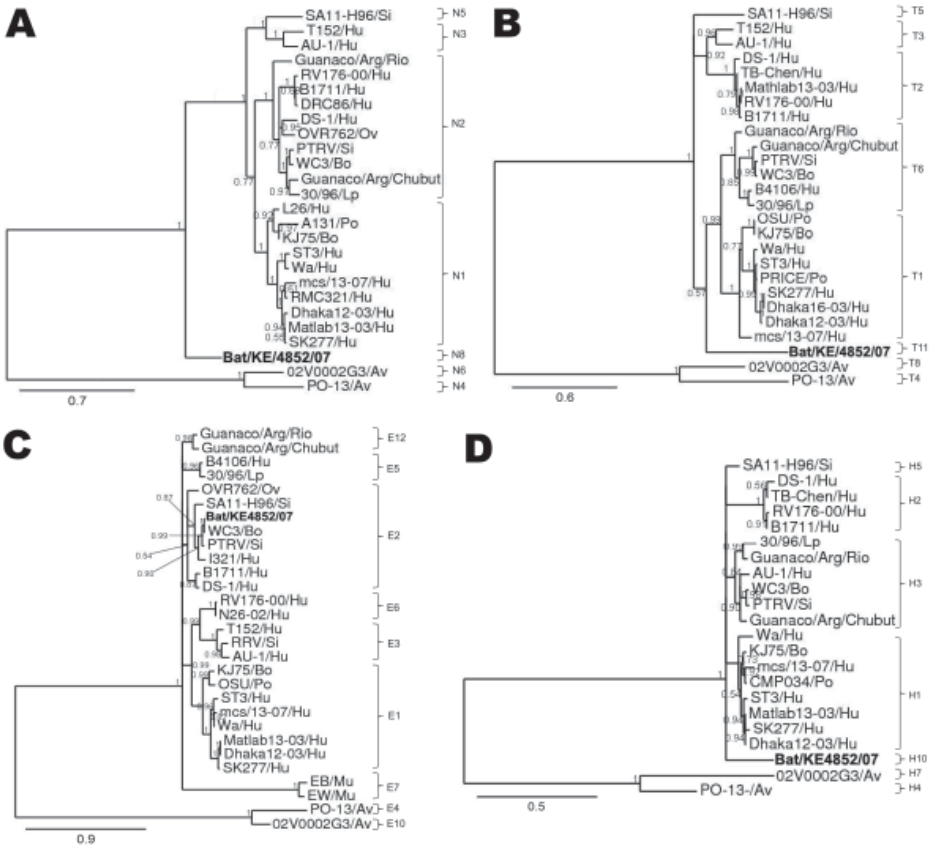

Figure 4. Phylograms indicating genetic relationships of partial or complete nucleotide sequences of A) nonstructural protein 2 (NSP2), B) NSP3, C) NSP4, and D) NSP5 of bat rotavirus strain Bat/KE4852/07 (**boldface**) from Kenya with representatives of known human and animal rotavirus genotypes. Posterior probability values are indicated at each branch node. Scale bars indicate nucleotide substitutions per site. GenBank accession numbers of all strains used are listed in the online Technical Appendix (www.cdc.gov/EID/content/16/12/1844-Techapp.pdf). Genotypes of each gene segment characterized in this study are listed to the right of each tree. Si, simian; Hu, human; Ov, ovine; Bo, bovine; Lp, lapine; PO, porcine; Av, avian; Mu, murine.

However, this possibility is less likely because 7 of the Bat/KE4852/07 genes are genetically divergent from any previously reported rotavirus genotype for humans or animals.

This study provides evidence that human rotavirus gene segments can reassort naturally into an animal rotavirus backbone. Reassortment of animal rotavirus segments onto human strain backbones has been documented (*28*), and experimental insertion of human rotavirus strains onto animal strain backbones was the basis for construction of reassortant rotavirus vaccines Rotashield and RotaTeq (*29*). Detection of human rotavirus strain components in domestic animals has been reported (*30*,*31*) but it is not known whether these strains were reassortant viruses or human strains that infected animals because only G and P types of these viruses were reported. Documentation of a human-to-bat transmission event of any infectious virus is unprecedented, and the genetic bases of such interactions deserve additional studies (*8*).

Human and fruit bat activities provide ample opportunities for *E. helvum* bats and humans to contact each other and their respective rotaviruses. Fruit bats often live near human habitats (*11*) as a result of deforestation and expanded settlement of human populations. Fruit bats must forage for food over wide areas and often feed in orchards. A proposed mechanism for bat infection with human rotaviruses would be through use of night soil (human feces) as fertilizer on farmlands and orchards, a common practice throughout Africa and Asia (*32*,*33*). Night soil contains pathogenic bacteria, viruses, and parasites linked with disease endemicity in these countries (*32*–*34*). Human rotaviruses have been detected in surface water, reservoirs, and sewage; and viable virus has been isolated from drinking water (*35*,*36*). *E. helvum* bats have has been observed skimming bodies of water in Africa, presumably to collect water for drinking (*37*), and surface water could be contaminated with viable human rotaviruses by night soil fertilizer or inadequate sanitation practices. Contact with human feces during drinking or feeding provides a mechanism by which fruit bats can ingest human rotaviruses, which would serve as the source of heterologous rotavirus genes acquired by reassortment. Additional information is needed about the actual mechanisms and frequency of rotavirus transmission between humans and bats.

The partial VP1 gene sequence obtained for Bat/KE4852/07 is more divergent than other group A rotavirus VP1 genes and does not contain several conserved motifs in VP1 proteins of group A, B, and C rotaviruses (*27*). The level of divergence suggests that the origin of this gene may be outside group A rotaviruses, perhaps from a yet-to-be-defined rotavirus group distinct from groups A, B, C,

and the ADRV-N/J19 group (*38*). Group A and C rotaviruses can replicate each other's plus-strand RNAs, and it has been speculated that rotaviruses from these 2 groups can exchange genomic segments if they co-infect the same cell (*27*), although this phenomenon has not been demonstrated empirically. We propose that the Bat/KE4852/07 VP1 gene (segment 1) originated from a nonspecies A rotavirus, most likely a currently undefined or unrecognized rotavirus group.

Each year, group A rotaviruses cause ≈600,000 deaths among children worldwide (*39*). Current surveillance studies are focused mainly on humans, and to a lesser extent, on domesticated animals and pets. Recent studies have identified new rotavirus genotypes in many captive and free-ranging wildlife (*9*). However, relatively little is known about the prevalence of rotaviruses among wildlife. Results of our study reinforce previous initiatives to enhance animal rotavirus surveillance, including corresponding information about wildlife rotaviruses, not only to investigate the genetic variability of wildlife rotaviruses, but also to understand the origin of unusual rotavirus strains in various hosts, including humans, especially within the One World, One Health concept described by the Wildlife Conservation Society (*40*).

In summary, although a limited amount of stool sample resulted in our inability to complete the full genome of Bat/KE4852/07, discovery of a rotavirus in bats and genetic analysis of this virus provide new insight into the ecology and evolution of rotaviruses. Our findings not only reinforce the potential role of bats as reservoirs of zoonotic viruses that are threats to human health (*11*), but also suggest that humans can serve as reservoirs of virus, which can result in anthropozoonotic transmission of rotavirus genes. Global surveillance for rotaviruses in bats and other wildlife is needed to corroborate, elaborate, and more fully understand the origin, adaptation, and evolution of bat rotavirus strains. In addition, further research is needed to establish the precise role of bats in the natural history of this rotavirus.

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Hantavirus Pulmonary Syndrome in Argentina, 1995–2008

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We report a large case series of hantavirus pulmonary syndrome (HPS) in Argentina that was confirmed by laboratory results from 1995 through 2008. The geographic and temporal distribution of cases by age, sex, fatality rate, and risk factors for HPS was analyzed. A total of 710 cases were unequally distributed among 4 of the 5 Argentine regions. Different case-fatality rates were observed for each affected region, with a maximum rate of 40.5%. The male-to-female ratio for HPS case-patients was 3.7:1.0; the case-fatality rate was significantly higher for women. Agriculture-associated activities were most commonly reported as potential risk factors, especially among men of working age. Although HPS cases occurred predominantly in isolation, we identified 15 clusters in which strong relationships were observed between members, which suggests ongoing but limited person-to-person transmission.

Hantavirus pulmonary syndrome (HPS) was first recognized in 1993 during an outbreak of acute respiratory distress syndrome in the southwestern United States (*1*,*2*). Previously, only Old World hantaviruses had been associated with illness in humans as the causative agents of hemorrhagic fever with renal syndrome. After recognition of HPS, cases in other countries of Central and South America were quickly identified, along with the associated virus and rodent reservoirs (*3*–*10*).

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Although serologic studies provided the initial evidence of hantavirus circulation in Argentina (*11*,*12*), the etiologic agent of HPS in Argentina was first described in 1995 after an outbreak occurred in the Andean sector of Patagonia where Andes virus (ANDV) was characterized (*4*). Several reports have been published since then, describing HPS cases in 4 regions of the country: Northwest, Northeast, Central, and Patagonia. Six lineages of ANDV were associated with HPS in the 4 regions of Argentina: AND-Oran, AND-Bermejo, AND-BsAs, AND-Lechiguanas, AND-Plata, and AND-South (*10*,*13*–*16*). Juquitiba virus (JUQV) and Laguna Negra (LN)–like virus were also found in the Northeast and Northwest regions, respectively (*14*,*17*).

We describe the epidemiologic features of a large proportion of confirmed HPS cases in Argentina. Detailed data were compiled for analysis of age, sex, onset of symptoms, clinical signs, case-fatality rates, geographic origin, and the most probable risk activities.

Materials and Methods

Study Site

Argentina is located at the southern extreme of South America. It has a large longitudinal extension, 3,779 km, and the highest altitudinal range of the continent. With a continental extension of 2,791,810 km^2, Argentina is the second largest country in South America and the eighth largest in the world. The size of the country supports multiple natural ecosystems. Argentina has been divided into 5 epidemiologic regions: Northwest, Northeast, Cuyo, Central, and Patagonia (Figure 1). It has been also classified into 18 ecoregions on the basis of geographic, climatic, and biologic factors (*18*).

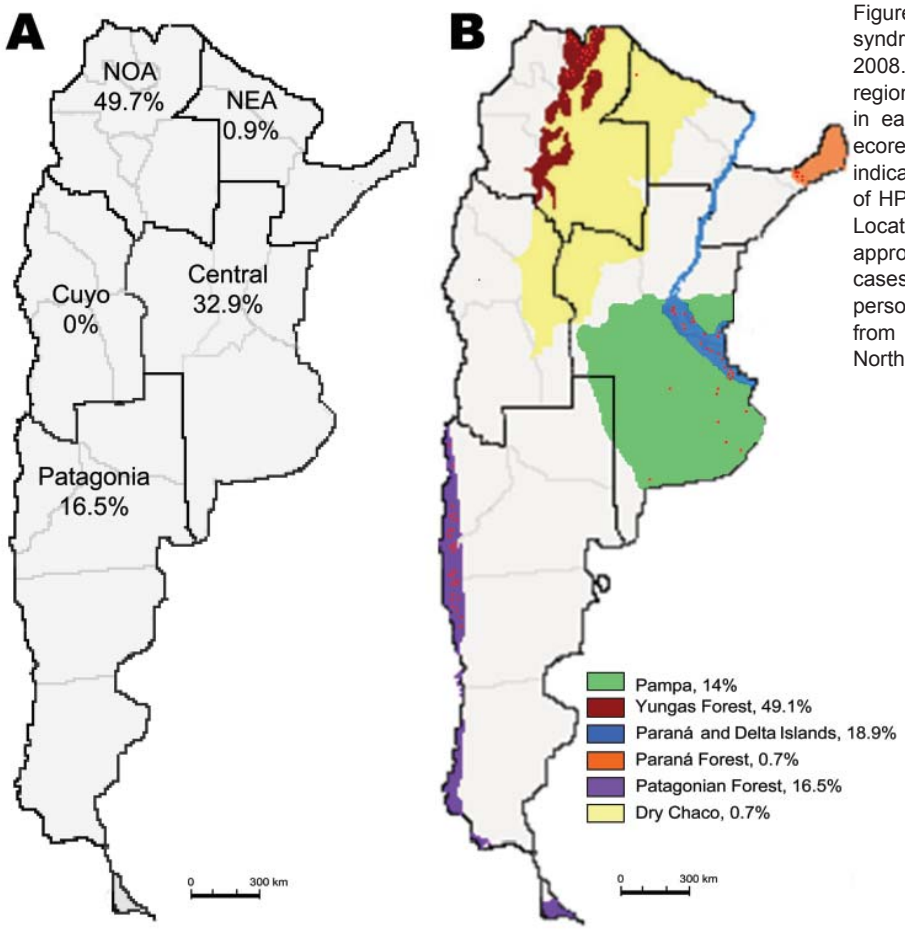

Figure 1. Distribution of hantavirus pulmonary syndrome (HPS) cases in Argentina, 1995–2008. A) The 5 Argentine epidemiologic regions and percentages of HPS cases in each one are shown. B) Six of the 18 ecoregions (*18*) represented by the colors indicated in the reference key; percentages of HPS cases in each ecoregion are shown. Location of HPS cases is represented approximately by point density. Total no. of cases analyzed: 692; confirmed cases of person-to-person transmission were excluded from this analysis. NOA, Northwest; NEA, Northeast.

Study Population

We analyzed suspected HPS cases from Argentina that occurred during 1995–2008 and for which samples were submitted to our laboratory for diagnostic confirmation. Standardized information was required for each suspected case (obtained by completion of a clinical/epidemiologic HPS form designed by the National Ministry of Health, Epidemiology Department). Samples were received from regional epidemiology units or directly from hospitals or private health systems. HPS diagnosis in Argentina during this period was performed in 2 validated national institutions: the Instituto Nacional de Enfermedades Virales Humanas (INEVH) and the Instituto Nacional de Enfermedades Infecciosas (INEI), components of the National Administration of Laboratories and Institutes of Health (ANLIS "Dr. C.G. Malbrán"). The selection of either of these 2 institutions was based on the convenience of the sender institution, without directions from national authorities, so samples were sent without distinction to either of them. We reviewed available data from all laboratory-confirmed HPS cases analyzed at INEI (n = 710), which represents 77.6% of the total cases submitted to the ANLIS "Dr. C. G. Malbrán" that fit the definition of laboratory-confirmed HPS cases. An acute febrile illness (>38.5°C) and any sign of respiratory compromise were required to meet the case definition of a suspected HPS case. The development of prodromal signs in contacts of previously confirmed HPS case-patients was enough to include them as suspected HPS case-patients.

HPS Case Confirmation

Clinical diagnoses were confirmed if laboratory testing detected hantavirus-specific immunoglobulin (Ig) M or rising titers of hantavirus-specific IgG or detected viral genomic material in any tissue. Serum or whole blood samples were tested by ELISA for specific IgM (μ-capture technique) and IgG against ANDV as previously described (*19*). Viral RNA detection was performed by reverse transcription–PCR for the detection of the S and M segments, followed by nucleotide sequencing as previously described (*10*).

Risk Factors

To analyze the type of exposure, we categorized information provided by 410 case-patients about risk activi-

ties during a 30-day period before illness: rural (persons who used to work in rural settings), wild (persons who performed activities in natural, nondisturbed, nonrural environments), peridomestic (rural or suburban residents without defined events of exposure in other places, considering housing and the surrounding land the source of infectious rodents), and undefined (persons with urban residence and no reported rural or recreational activities) (Table 1). Case-patients with a history of recent travel outside the country were excluded from the analysis (n = 12).

Results

From 1995 through 2008, a total of 8,522 suspected HPS cases were submitted from the 5 Argentinean epidemiologic regions to our laboratory for diagnosis, and HPS was confirmed for 710 (8.3%). Prodromal symptoms did not differ from the HPS clinical picture previously reported (*20*–*24*); the prodromal phase was followed by different degrees of respiratory compromise, usually rapid and acute respiratory distress. However, 4 cases were confirmed in patients without respiratory manifestations. One of these cases was detected in the daughter of a woman with confirmed HPS during monitoring of the case-patient's contacts. At the time of hospitalization, for all patients, platelet counts ranged from 15,000 to 305,000/mL (mean value 82,340; median value 68,000; n = 510); therefore, platelet count resulted to be an important laboratory indicator of a probable case of HPS.

Of the 710 HPS cases, 708 were laboratory confirmed by detection of IgM, and 2 were confirmed by detection of IgG and the viral genome by reverse transcription–PCR. Of the 708 IgM-positive samples, 606 (85.6%) were also IgG positive. Samples were received at the laboratory for diagnosis 11.6 days after onset of disease (mean value, n = 470), and definite laboratory-confirmed diagnosis was obtained within 2 days.

During the first 3-year period (1995–1997), the annual number of cases increased and then varied, ranging from 42 to 82 cases per year (Figure 2, panel A). Of the 710 confirmed cases, 183 deaths caused by HPS were reported, with an overall mortality rate of 25.8%. Case-fatality ratios declined during the first 4 years of the period (1995–1998) (χ^2 test for trend, p = 0.0372).

An accurate analysis of the occurrence of HPS cases showed that their distribution was limited to small areas inside 4 of the 5 Argentine epidemiologic regions: Northwest, Northeast, Central, and Patagonia (Figure 1). The Northwest region accumulated the highest case number (49.7%), but in the Northeast region, only 6 cases have been confirmed since 2003 (0.87%). In the second most frequently affected region, the Central (32.9%), most cases occurred in Buenos Aires Province. In Patagonia, the affected area (16.5%), comprised the forested southern Andes Mountains. The southernmost case occurred >800 km from the disease-endemic area of Patagonia (*25*) (Figure 2, panel B). Comparative analysis of case distribution in epidemiologic regions and ecoregions showed that location of cases fit better within ecoregions. Cases occurred in 6 of the 18 Argentine ecoregions: Yungas Forest, Paraná Forest, Dry Chaco, Pampa, Paraná Delta and Islands, and Patagonian Forest (Figure 1, panel B).

Seasonal occurrence, determined on the basis of onset of symptoms and considering the 4 regions together, showed a decrease of cases only in winter (13.2%); a higher proportion of cases occurred in spring (35%), followed by summer (28.6%) and autumn (23.1%) (Figure 3). The highest peak occurred in autumn, mostly representing cases in the Northwest (64% of cases within the season in the Northwest region). Both the Northwest and Central regions showed similar peaks in spring (49% and 31%, respectively) and summer (43.4% and 42.4%, respectively). Although few cases occurred in the 2 remaining regions each month, a similar pattern could be inferred for both regions.

Age range of case-patients was 0–77 years; mean age was 30 years and median was 28 years. Age frequency distribution in groups with a 10-year age range showed that

Table 1. Demographic characteristics of case-patients who had laboratory-confirmed HPS, by region, Argentina, 1995–2008*

Characteristic	North	Central	Patagonia	Northeast
No. case-patients (no. deaths)†				
Total	345 (59)	234 (72)	126 (51)	6
M	290 (45)	170 (49)	89 (33)	4
F	52 (14)	64 (23)	37 (18)	2
Case-fatality rate for all case-patients, %	17.1	30.8	40.5	0
Type of exposure				
Rural	111	34	22	1
Wild	34	20	17	1
Peridomestic	28	26	13	2
Contact with previous HPS case-patient	–	6	23	–
Other	25	30	6	–
Total	208	116	81	4

*HPS, hantavirus pulmonary syndrome; –, no reported events.
†No. HPS cases in each region with confirmed diagnosis (n = 710). Type of exposure was classified on the basis of the most probable activities of risk (n = 410).

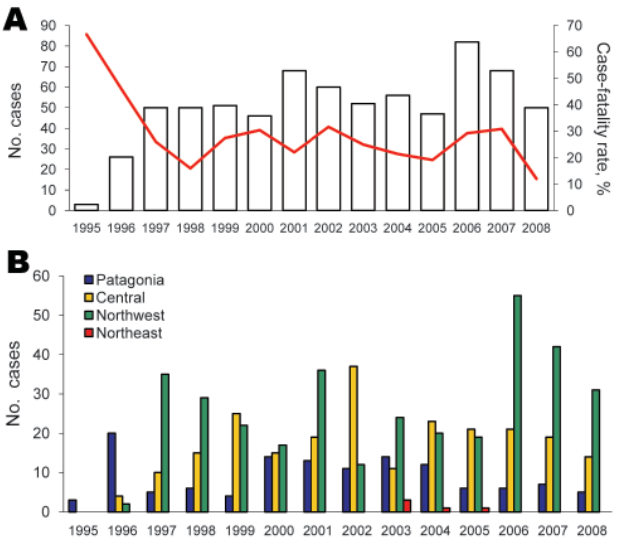

Figure 2. Annual hantavirus pulmonary syndrome case distribution and case-fatality rate, Argentina, 1995–2008. A) Annual case numbers (bars) and case-fatality rate (red line). B) Annual case distribution by region.

the group of those 21–30 years accounted for the highest number of cases (Figure 4); 9.3% of cases were in children <14 years of age. The HPS-case population had a higher proportion of male patients than female patients in all 4 regions (78.8% vs. 21.2%; Table 1).

Analysis of case-fatality rates by region showed that values increased as the region's location became more southern: Northwest (0%), Northeast (17.1%), Central (30.8%), and Patagonia (40.5%) (Table 1). Among these regions, the difference in case-fatality rates between the northern regions and those of the Central and Patagonia regions were significant (χ^2; p = 0.0001 and p<0.0001). However, the difference among the Central and Patagonia regions was not significant (χ^2; p = 0.064).

The case-fatality rate was significantly higher for female than for male patients: 34% versus 21% (χ^2; p = 0.0013); it was also higher for patients without an IgG response than for the IgG-positive group, 51% versus 21% (χ^2; p<0.0001), whereas no difference was found between the elapsed time from symptom onset to sample collection among patients with fatal and nonfatal cases (mean 5.28 and 5.78, respectively; p<0.093 by *t* test). This analysis was performed with >572 acute-phase samples.

In the rural category, the most frequently reported activities were agriculture associated or general work on farms, mainly preparing land for cultivation, clearing weeds, planting and harvesting of field crops, and cleaning out barns or other outbuildings. The wild category included case-patients with probable exposures in natural environments during recreational activities, tourism, or activities related to occupations such as sanitary agent, security personnel, or truck driver. The peridomestic category included primarily housewives and children.

In regard to particular activities of risk, only 129 (32.8%) of 393 case-patients reported a defined event of exposure. The most frequently reported activities were the following: occasional recreational activities in wild areas, cleaning outbuildings in rural areas, and having contact with an HPS case-patient. Thirty-five case-patients had recent contact with a previously confirmed HPS case-patient. Among these, the suspicion of human-to-human transmission was based on the patient's close relationships with other case-patients, the lack of risk or low risk in other activities, and on the length of time that had elapsed between the onset of symptoms in both related case-patients.

The main indicator that raises suspicion of human-to-human transmission was a long period before symptom onset for both case-patients. In previous studies, based on confirmed cases of person-to-person transmission, the incubation period ranged from 12 to 27 days (*10*), which suggested that viral spread likely occurred near the end of the prodromal phase (*26*). Considering the length of the prodromal phase, a difference in time of symptom onset between 2 related case-patients (16–35 days) is used to raise suspicion. Case-patients who are exposed to the same permanent infectious source are not considered to demonstrate human-to-human transmission. To differentiate human-to-human transmission from common exposure to infectious rodents, after an accurate epidemiologic investigation, comparing long viral nucleotide sequences from patients has been useful (*10*,*26*). Fifteen of 35 clustered cases corresponded to a unique cluster in which person-to-person transmission was first demonstrated (*27*). The remaining 20 cases belonged to 14 additional clusters (Table 2). Nine clusters occurred in Patagonia, 5 in the Central region, and the remaining cluster had cases from both regions. We have previously confirmed person-to-person transmission in 2 of these 14 clusters (*26*,*27*).

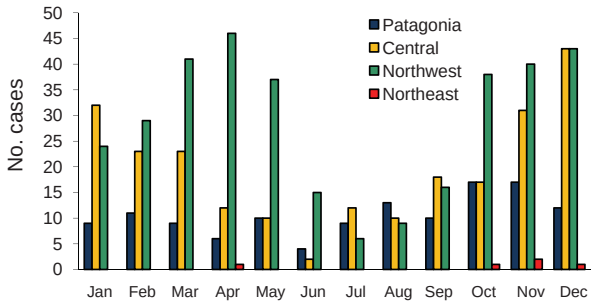

Figure 3. Hantavirus pulmonary syndrome case distribution, according to month of disease onset in disease-endemic regions, Argentina, 1995–2008.

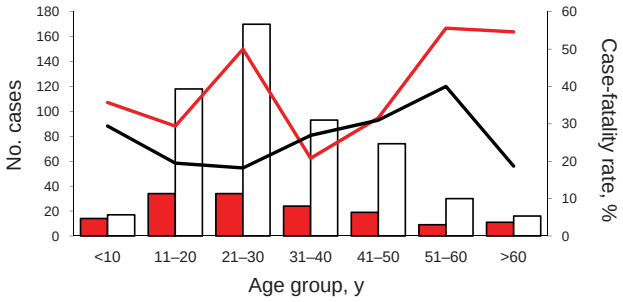

Figure 4. Hantavirus pulmonary syndrome cases and case-fatality rate, by age and sex distribution (n = 685), Argentina, 1995–2008. White bars and black line indicate male patients, red bars and line female patients.

Discussion

This report summarizes epidemiologic characteristics of 77.6% of HPS cases in Argentina since the disease was detected. The considerable extension, especially in the longitudinal direction, and multiple natural settings represented a great limitation to collecting the information and designing and conducting an epidemiologic study. Most of the data presented here were collected as part of local routine surveillance or emergency outbreak investigations without a consensus strategy or general design. HPS has affected 4 of 5 Argentine epidemiologic regions (Cuyo is the only region without reported cases) (Figure 1, panel A). This finding was consistent with the report of National Ministry of Health (www.msal.gob.ar/htm/site/sala_situacion/PANELES/boletines/bepAnual/BEPanual2006_Zoonosis.pdf). The Northwest and Central regions were the most affected. The distribution of HPS cases fit better inside ecoregions rather than inside epidemiologic regions, so the classification of the country in ecoregions could be more appropriate for understanding HPS occurrence (Figure 1, panel B). In northern Argentina, most of the cases grouped in the Yungas Forest (49.1%). Few cases occurred in Dry Chaco and Paraná Forest (0.7% each). In the Central region, cases accumulated in Humid Pampa (14%) and in Paraná Delta and Islands (18.1%), mainly in large suburbs. With regard to Patagonia, HPS cases accumulated in the cooler, high, Andean forested zone, a narrow strip along the Andes Mountains, which corresponded with the Patagonian Forest (16.5%). Each ecoregion is formed by particular ecosystems with specific plant and animal species especially adapted to them. Because the present analysis does not represent all of the registered cases in the country (22.4% were diagnosed in the other national institution), we recognize this as a limitation of our work. We consider that the real proportion of reported cases in each of the epidemiologic regions, as well as other epidemiologic factors included in this descriptive study, could differ slightly.

Since the first description of HPS in southwestern Argentina, new cases have been reported, and new disease-endemic areas have been gradually recognized. However,

Table 2. Epidemiologic characteristics of clustered HPS cases, Argentina, 1995–2008*

Cluster no.	Contact case-patient no.	Region	Case-patient sex/age, y		Relationship with index case-patient	Ambient risk†	Days between symptom onsets
			Index	Contact			
1‡	C1-s	Central	M/41	M/14	Son	No	27
2	C2-d2	Central	F/12	F/11	Sister	High	4
	C2-s	Central	F/12	M/ND	Brother	High	ND
	C2-m	Central	F/12	F/40	Mother	High	12
3	C3–2	Central	M/28	M/27	Friend, roommate	High	2
	C3–3	Central	M/28	M/21	Friend, roommate	High	15
	C3–4	Central	M/28	M/30	Friend, roommate	High	19
4‡	C4-b	Central/ Patagonia	M/39	M/58	Friend, roommate	No	15
	C4-c	Central	M/39	M/39	Friend, roommate	No	23
5	BA04–2	Central	F/24	F/0	Daughter	ND	28
6	BA06–2	Central	M/53	F/28	Wife	High	31
7	NQ00–2	Patagonia	M/46	M/10	Son	Low	22
8	NQ01–2	Patagonia	M/40	F/44	Ex-wife	No	22
9	NQ06–2	Patagonia	M/48	F/53	Wife	Low	30
10	RN95–2	Patagonia	M/38	F/25	Girlfriend	No	21
	RN95–3	Patagonia	M/38	F/15	Daughter	No	29
11	RN00–2	Patagonia	F/25	M/64	Husband	Low	20
12	RN03–2	Patagonia	M/31	F/28	Wife	Low	40
13	CH00–2	Patagonia	F/36	F/3	Daughter	Low	–
14	CH00–4	Patagonia	M/28	F/24	Wife	Low	22

*HPS, hantavirus pulmonary syndrome; ND, not determined; –, secondary case-patient did not show symptoms of illness.
†Ambiental risk was classified in as follows: no, without ambient risk, urban residence; low, rural residence and no evident rodent exposure; high, rural residence and evident rodent exposure.
‡Clusters with confirmed person-to-person transmission.

the current distribution of HPS cases does not imply that no other areas are affected. Samples from patients with suspected HPS were submitted mainly from the disease-endemic areas, but we also received samples from other areas. Among these, we confirmed a few HPS cases, which illustrates that new areas are potentially disease endemic. This is the case for the Dry Chaco region. Recently, we confirmed 1 case from the northern Paraná Delta and Islands ecoregion; the patient had no history of travel outside the area of residence. In Patagonia, outside the Patagonian Forest, several seroprevalence studies have been conducted in rodent populations in which no hantavirus-positive animals were found (*25*,*28*,*29*). We also received samples from suspected case-patients from this area, and no HPS cases were confirmed. The situation of the Cuyo region is less clear because few samples from persons with suspected HPS and few rodents were analyzed. Thus, future change in HPS case distribution might be expected.

HPS is a zoonotic disease, and its distribution is expected to be similar to that of its rodent reservoirs. In northwest Argentina, 3 pathogenic hantaviruses, AND-Orán and AND-Bermejo lineages and LN-like-virus, have been associated with *Oligoryzomys longicaudatus*, *O. chacoensis,* and *Calomys callosus* rodents, respectively (*13*,*15*,*17*). Inside the Paraná Forest ecoregion, AND-Lechiguanas was characterized from 1 HPS case, JUQV was characterized from 2 HPS cases, and *O. nigripes* rats were identified as the reservoir species of JUQV in the area (*14*). Three pathogenic lineages of ANDV have been identified in the Central region, AND-BsAs, AND-Lechiguanas, and AND-Plata (*10*,*16*,*26*). *O. flavescens* rice rats have been identified as the reservoir of AND-Lechiguanas and AND-Plata (*13*,*30*). In Patagonian Forest *O. longicaudatus* is the principal reservoir of AND-South lineage (*13*,*28*). Future studies will be necessary to determine the rodent reservoirs in Dry Chaco in both epidemiologic regions, Northwest and Northeast.

The increasing number of HPS cases during the first 3 years of the study period could be attributable to an improvement in detection of suspected cases. Cumulative frequency analysis showed that cases in the whole country occurred throughout the year; the highest number occurred during spring/summer. The case-fatality rate decreased during the first years of the period analyzed; the decrease could be attributed to the medical experience acquired during clinical treatment, to an earlier recognition of suspected cases, especially in disease-endemic areas, and to recognition of less severe cases. Currently, the clinical picture for a suspected HPS case is not necessarily associated with a severe respiratory disease. However, differences were observed in virus lethality between each Argentine region; the proportion of deaths increased as the region considered became more southern. The high case-fatality rate in Patagonia could be associated with higher viral load input because of climatic conditions that supported the maintenance of aerosolized infectious virus. However, multiple factors such as different rodent reservoirs and different lineages in each region could influence the proportion of deaths. Another possibility is that these differences could be because fewer mild cases are detected in the Patagonia and Central regions. Future research will be necessary to assess whether these or other factors could be considered predictors of the likelihood of death.

Humoral immune response seems to be early and strong in HPS case-patients from South America; however, low IgG titers or the absence of IgG has been associated with a higher mortality rate (*10*). In that study (*10*), by means of a stratified analysis in 91 cases, we showed the usefulness of IgG titers as a predictor of outcomes. In the present study, we confirmed that IgG titer was a strong predictor of outcomes for 572 case-patients in Argentina. Similar observations were described for HPS caused by Sin Nombre virus infections in 2 studies of 26 case-patients (*31*) and, more recently, in 51 HPS case-patients (*32*).

Changes in natural ecosystems have altered the abundance and distribution of rodent species and might have favored colonization of agro-ecosystems by hantavirus reservoir species (*25*). All 5 affected Argentine ecoregions have different degrees of anthropogenic disturbances (*33)*. The Yungas Forest and the Pampas are the most disturbed ecoregions. Future studies will be necessary to find factors associated with HPS emergence in each region, such as anthropogenic disturbance, population density, etc.

HPS is strongly associated with rural activities, which are performed mainly by men of working age. This is the probable reason for the predominance of young men among HPS patients. Although agricultural activities seem to be the main cause of exposure for men, peridomestic exposures were most frequently reported for women and children. Contact with rodents is extremely common in many rural locations. Clusters of cases were rare in general and usually more frequent in the Patagonia region, but in the Central region, several clustered cases were reported. Human-to-human transmission was previously confirmed on the basis of strong epidemiologic and genetic evidence (*26*,*27*). For other cases, secondary cases after the index case were classified as suspected of having person-to-person transmission because such transmission could not be either confirmed or rejected. Two ANDV lineages have previously been identified in events of human-to-human spread: AND-South and AND-BsAs lineages (*26*,*27*). Confirmed instances of person-to-person transmission represent 2.5% of the total case number during 1995–2008. Future events or outbreaks of person-to-person transmission would be expected, especially in the Patagonia and Central regions.

The reason the death rate is higher for women is a matter of speculation. It is well established that the sex of a

host can significantly affect susceptibility to infections with several pathogens. Differences in male and female immune responses have been recognized for some time. Gender-determined differences in susceptibility to virus infections have been reported for encephalomyocarditis virus (*34*), vesicular stomatitis virus (*35*), and coxsackievirus B3 (*36*). Gender-dependent differences in plasma cytokine responses have been found in patients infected with Puumala virus and Old World hantavirus (*37*). Further studies will be necessary to determine whether different cytokine profiles or other immune factors could explain the higher proportion of deaths in female patients with HPS.

The present analysis of HPS in Argentina contributes to an understanding of its distribution and transmission. Although HPS is a relatively rare disease, it is among the most pathogenic of human viral infections. As more cases are recognized and risk factors are better identified, it will be possible to enhance surveillance efforts and to evaluate prevention measures for HPS.

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Anatomy of Bluetongue Virus Serotype 8 Epizootic Wave, France, 2007–2008

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The introduction of bluetongue virus serotype 8 into northern Europe at the end of summer 2006 initiated one of the most widespread epizootics of bluetongue infection ever to occur. In winter 2007–2008, a cross-sectional serologic study was conducted in France along a transect perpendicular to the epizootic wave. Cattle herd-level seroprevalence varied from 4% to 100%, and animal-level seroprevalence from <1% to 40%. Only a low proportion of seropositive herds reported clinical cases in 2007. Sheep flocks were less frequently affected than cattle herds. The local occurrence of clinical cases and environmental indicators linked to forests were seropositivity risk factors, whereas the local density of cows had a protective effect. Overall results suggest that amplification of virus circulation in affected herds played a limited role in the epizootic wave diffusion and that bluetongue virus serotype 8 circulation in natural ecosystems could have played a substantial role in this progression.

Bluetongue is a vector-borne viral disease of wild and domestic ruminants caused by *Bluetongue virus* (BTV; family *Reoviridae*, genus *Reovirus*). Twenty-four serotypes of this virus are described, principally transmitted by several species of biting midges belonging to the genus *Culicoides* (Diptera: Ceratopogonidae). Until 1998, Europe was considered BTV free except for occasional incursions into the Iberian Peninsula, Cyprus, and Greek islands. In 1998, an unprecedented series of successful introductions of BTV serotypes occurred in countries in southern, western, and central Europe (*1*). Unexpectedly, in 2006, BTV serotype 8 (BTV-8) was introduced in Belgium, close to the borders with Germany and the Netherlands (*2*,*3*), and quickly spread in these 3 countries. By the end of 2009, BTV-8 had spread to most countries in western and central Europe, including the United Kingdom, Denmark, Norway, Sweden, Czech Republic, Hungary, Austria, Italy, Luxembourg, Spain, and France.

In France, the first clinical case was reported in late August 2006 near the border with Belgium. In July 2007, bluetongue reappeared there (*4*) and quickly progressed westward and southward, with the virus causing clinical cases in 10,500 herds in 2007 and in 26,500 herds in 2008. By the end of 2007, BTV-1 was introduced in southern France, resulting in a second epizootic wave that progressed northward during 2008; by late 2008, most of the French territory had been affected by BTV-1, BTV-8, or both serotypes (online Appendix Video, www.cdc.gov/EID/content/16/12/1861-appV.htm). A vaccination campaign launched in 2008 stopped the epizootic in 2009. During the winter of 2007–2008, the end of BTV transmission (during the vector inactivity period) offered the opportunity to study the epizootic wave. The objectives of this study were to describe the first of these 2 epizootic waves and to analyze the respective parts played by within- and between-herd dynamics in BTV-8 progression, the relationship between the progression of infection and that of clinical cases, and the environmental features that influenced the progression of BTV-8.

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Materials and Methods

Seroprevalence rates were estimated at the herd and animal levels in cattle and sheep along an east–west transect perpendicular to the epizootic wave. By comparing serologic results with clinical outbreaks, we estimated the proportion of silently infected herds or flocks (i.e., herds in which BTV-8 had circulated without any reported clinical cases) and variations in the outbreak along the transect. Main herd-level seropositivity risk factors were investigated (species, occurrence of clinical cases), as well as local seropositivity risk factors (animal density, land cover and landscape indices, occurrence of clinical cases).

Study Area and Sampling Design

The study area comprised 7 departments in France (Figure 1); 6 departments situated on an east–west transect from the center of the country to Brittany (codes 18, 41, 36, 37, 49, and 35) and, as a reference, the department where the first outbreak in France was reported in late 2006 (code 08). Sample size was calculated for a herd-level design prevalence of 10% and a precision of ±10%. Within-herd design prevalence was set to 10% with a detection probability (≥1 seropositive animal) of 95%. We sampled 50 herds per department, with 30 animals tested in each herd. This sampling protocol was applied to the beef cattle population in the 7 selected departments and to the sheep population in 4 of these departments selected because of the linear decrease (on a logarithmic scale) of the number of outbreaks reported in 2007 (department 08: 1,405 outbreaks, department 18: 104 outbreaks, department 36: 14 outbreaks, and department 35: 1 outbreak).

Biological Samples and Laboratory Analyses

The study was conducted in close collaboration with local veterinary laboratories. The laboratories were in charge of randomly drawing samples of herds and animals from their banks of serum. The sampling base was the set of serum samples taken during winter 2007–2008 for brucellosis detection, which is mandatory in France for beef cattle >12 months of age and for small ruminants >6 months of age. The sampling protocol was satisfactorily implemented, except in department 36 where only few sheep serum samples were available (Tables 1, 2). Overall, in the 7 departments, 9,888 serum samples were tested from 360 beef cattle herds, and 2,465 serum samples were tested from 157 sheep flocks (Tables 1, 2). All the local veterinary laboratories that participated in the study were accredited for detection of BTV-8 antibodies. Serum was analyzed by using a commercial ELISA kit according to the recommendations of the manufacturer (ID-Vet, Montpellier, France). ELISA specificity is high in clinically suspected disease in sheep and cattle (*5*,*6*), the lower sensitivity in these animals being attributed to early stages of infection (*6*). No estimate is available for healthy cattle and sheep. We thus assumed that sensitivity and specificity were perfect; this may have induced a slight underestimate of seroprevalence rates.

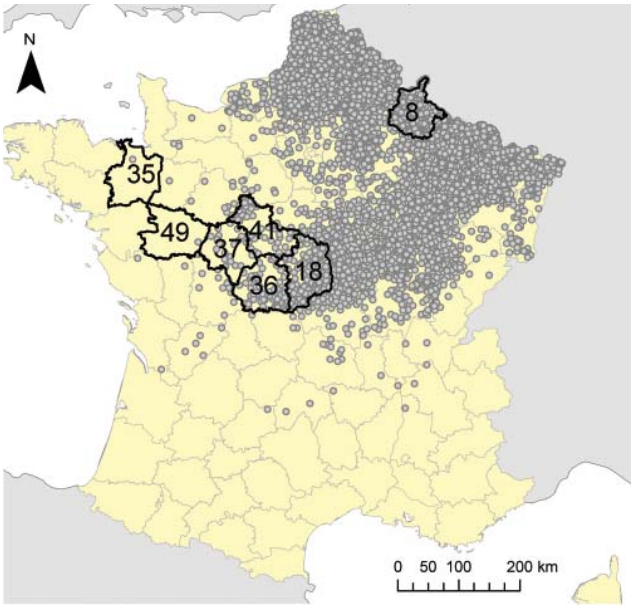

Figure 1. Locations included in a serologic study of the 2007 epizootic wave of bluetongue virus serotype 8 (BTV-8) among cattle herds in France. Black lines indicate the 7 departments included in the study: 6 departments aligned on an east–west transect (codes 18, 41, 36, 37, 49, and 35); and the first department to report BTV-8 infection in 2006 (code 08). Dots represent locations of BTV-8 outbreaks during 2007.

Outbreak, Population, and Land Cover Data

The database of BTV-8 outbreaks reported in France in 2007 was screened to determine whether confirmed clinical disease cases had been reported in the herds included in the study. Confirmed clinical cases were defined as disease in animals showing BTV-8 clinical signs and for which BTV-8 had been isolated or a positive BTV-8–specific PCR result had been obtained. For each herd we defined a binary variable, an outbreak status of 1 if a clinical case had been reported in 2007 and 0 if otherwise.

In the 6 departments of the east–west transect (Figure 1), each tested herd was geo-referenced at the municipality level (the smallest French administrative subdivision). For each municipality, we obtained from the French national database the number of cows and the number of flocks of small ruminants. Municipality-specific land cover data were extracted from the 2006 version of the CORINE (Coordination de l'Information sur l'Environnement) Land Cover database (CLC), provided by the European Environment Agency (*7*), at a 1:100,000 working scale (resolution of 100 m). The 44 classes of CLC nomenclature aim at describing perennial structures of land occupation and are organized

Table 1. Herd-level anti–bluetongue virus serotype 8 seroprevalence rate and clinical expression of bluetongue disease in 7 departments, France, winter 2007–2008*

Species	Department code	No. positive†/no. tested	Herd-level seroprevalence (95% CI‡)	No. outbreaks§	Reporting rate¶ (95% CI)
Cattle	08	63/63	1.00 (0.94–1.00)	37	0.59 (0.46–0.71)
	18	49/49	1.00 (0.93–1.00)	4	0.08 (0.02–0.20)
	41	42/50	0.84 (0.71–0.93)	3	0.07 (0.01–0.19)
	36	29/48	0.60 (0.45–0.74)	1	0.03 (0.00–0.18)
	37	31/50	0.62 (0.47–0.75)	0	0.00 (0.00–0.11)
	49	1/50	0.02 (0.00–0.11)	0	0.00 (0.00–0.98)
	35	2/50	0.04 (0.00–0.14)	0	0.00 (0.00–0.84)
Sheep	08	42/44	0.95 (0.85–0.99)	23	0.55 (0.39–0.70)
	18	14/59	0.24 (0.14–0.37)	2	0.14 (0.02–0.43)
	36	0/4	0.00 (0.00–0.60)	NA	NA
	35	0/50	0.00 (0.00–0.07)	NA	NA

*CI, confidence interval; NA, no seropositive flock.
†No. seropositive herds: >1positive result among tested serum samples.
‡Exact binomial 95% CI.
§No. seropositive herds with reported confirmed clinical disease in 2007.
¶Proportion of seropositive herds with reported confirmed clinical cases in 2007.

into a general-purpose 3-level hierarchy. For our study, the first level of this hierarchy was used, except for the second class of this first level (agricultural areas) for which the second level (more detailed) nomenclature was used. In addition, themes related to water were separated into 2 classes for inland versus marine wetlands and water bodies. These modifications resulted in a nomenclature with 8 classes (Table 3). For each municipality, we computed the proportion of the area covered by each class (8 variables) and, for each pair of classes, the edge density: the length of edges between the 2 classes divided by the municipality area (28 variables expressed in 100 m/ha).

Data Analysis

Descriptive Analyses

For each department and species, we computed the herd-level seroprevalence rate (proportion of seropositive herds, i.e., herds with ≥1 positive serum sample), the animal-level seroprevalence rate, and the distribution of within-herd seroprevalence rates in seropositive herds. For each department and species, we computed a disease reporting rate: the proportion of seropositive herds for which confirmed clinical disease had been reported in 2007 (outbreak status 1). The effects of department, species, and outbreak status on within-herd seroprevalence rate were analyzed with a logistic regression model. A quasi-likelihood estimation procedure was used to account for overdispersion.

Seroprevalence in Cattle and Local Conditions in the East–West Transect

We studied the link between local conditions and seroprevalence rate for cattle in the 6 departments of the east–west transect. Seroprevalence data were aggregated at the municipality level. The validity of this aggregation was verified by testing the homogeneity of within-herd seroprevalence rates in municipalities with >1 herd tested. Fisher exact tests were used and Bonferroni correction applied

Table 2. Animal-level anti–bluetongue virus serotype 8 seroprevalence rate and distribution of within-herd seroprevalence rates in seropositive herds or flocks of 7 departments, France, winter 2007–2008*

Species	Department code	No. positive/no. tested	Animal-level seroprevalence (95% CI†)	Median within-herd seroprevalence in seropositive herds (25%–75% quartiles)
Cattle	08	1,563/1,573	0.99 (0.99–1.00)	1.00 (1.00–1.00)
	18	642/1,530	0.42 (0.39–0.44)	0.40 (0.23–0.57)
	41	203/1,500	0.16 (0.14–0.17)	0.15 (0.07–0.27)
	36	103/1,470	0.07 (0.06–0.08)	0.10 (0.07–0.17)
	37	103/1,500	0.07 (0.06–0.08)	0.10 (0.05–0.13)
	49	1/1,410	0.001 (0.000–0.003)	0.03‡
	35	2/905	0.002 (0.000–0.007)	0.03§
Sheep	08	478/833	0.57 (0.54–0.61)	0.70 (0.43–0.87)
	18	19/874	0.02 (0.01–0.03)	0.08 (0.06–0.10)
	36	0/326	0.00 (0.00–0.01)	NA
	35	0/432	0.00 (0.00–0.01)	NA

*CI, confidence interval; NA, no seropositive flock.
†Exact binomial 95% CI.
‡ A single seropositive herd.
§Two seropositive herds or flocks with the same within-herd seroprevalence.

Table 3. Land cover repartition in municipalities with at least 1 herd tested, in 6 French departments aligned on an east–west transect*

ID	Landcover classes	CLC classes	Department†					
			18	41	36	37	49	35
C_1	Artificial surfaces	111–142‡	0.02	0.02	0.01	0.03	0.04	0.06
C_2	Arable land	211–213	0.33	0.51	0.24	0.46	0.45	0.33
C_3	Permanent crops	221–223	0.02	0.00	0.00	0.02	0.02	0.00
C_4	Pastures	231	0.40	0.08	0.39	0.15	0.25	0.15
C_5	Heterogeneous agricultural areas	241–244	0.08	0.15	0.19	0.11	0.19	0.39
C_6	Forests and seminatural areas	311–335	0.15	0.23	0.15	0.23	0.05	0.06
C_7	Inland wetlands and water bodies	411–412, 511–512	0	0.01	0.02	0.01	0	0.01
C_8	Marine wetlands and water bodies	421–423, 521–523	0	0	0	0	0	0
	Total		1.00	1.00	1.00	1.00	1.00	1.00

*Source: Coordination de l'Information sur l'Environnment (CORINE) land cover database, 2006 version.; ID, identification code for land-cover class; CLC, CORINE land cover.
†Departments: 18, Cher; 41, Loir-et-Cher; 36, Indre; 37, Indre-et-Loire; 49, Maine-et-Loire; 35, Ille-et-Vilaine.
‡Range of CLC classes. Each CLC class is identified by 3 numbers.

for test interpretation. In case of significant seroprevalence differences between herds, the corresponding municipality was excluded from the dataset. In the remaining municipalities, we computed the municipality-level seroprevalence rate and studied the effects, on this seroprevalence rate, of the following municipality-level covariates: 1) the department, included as a proxy for the municipality relative location in the epizootic wave; 2) the spatial density of cows (number of animals per km^2), of sheep flocks, and of goat flocks (number of flocks per km^2); 3) the existence of confirmed clinical cases reported in the municipality in 2007 (binary variable); 4) the proportion of municipality area covered by each land cover class; and 5) the edge density for each pair of land cover classes.

Land cover data were restricted to the 5 classes significantly represented in each of the 6 departments (presence in >50% of the municipalities): C_1, C_2, C_4, C_5, and C_6 (Table 3). Variables associated with seroprevalence were identified through a univariate analysis by using logistic models in which the department was systematically included. Variables for which the associated p value was <0.20 were selected for further analysis. A backward elimination process (*8*) was then applied to the corresponding model: variables with the lowest partial F test were successively eliminated until the corresponding p value was <0.05 for each remaining variable. A quasi-likelihood estimation procedure was used throughout. Standardized deviance residuals of the resulting model were tested to detect an association with the department by using the Kruskal-Wallis test. The epidemiologic system studied here is not stationary and clearly isotropic; a significant level of spatial autocorrelation of seroprevalence was thus expected. Since a relatively coarse proxy (the department) was used for representing relative positions in the epizootic wave, regression residuals were likely to be spatially autocorrelated. These residuals were thus examined to determine whether the model captured (or not) the main determinants of local seroprevalence variations. The Geary C statistic was used to quantify spatial autocorrelation of residuals in neighboring municipalities (C = 1: no spatial autocorrelation; C<1: positive autocorrelation; C>1: negative autocorrelation). Significance of this spatial autocorrelation was tested by using a permutation test, with 10,000 random permutations. As a reference, the same test was applied to the residuals of the null model, in which the only independent variable was the department. All statistical analyses were performed by using R software (*9*).

Results

Descriptive Results

The herds tested in department 08 were seropositive (≥1 positive serum test result) except for 2 flocks of sheep (Table 1). Along the east–west transect (Figure 1), the herd-level seroprevalence rate for cattle decreased from 100% in the easternmost department to 4% in the westernmost one (Figure 2). In sheep, herd-level seroprevalence rates were lower than in cattle. For example, in department 18, whereas nearly 100% of cattle herds were seropositive, this proportion fell to 24% in sheep flocks (Table 1). Animal-level seroprevalence rate was close to 100% in cattle of department 08 (Table 2). Along the east–west transect, cattle seroprevalence rate decreased from 42% in the easternmost department to 0.2% in the westernmost one (Figure 2). Seroprevalence rates were lower in sheep: in department 08, nearly 100% of cattle were seropositive, whereas only 57% of sheep were (Table 2). Similar differences were also observed in 2 other departments, 18 and 36. Within seropositive herds, median seroprevalence rate was 100% for cattle herds of department 08, whereas along the east–west transect, it decreased from 40% in the easternmost department to 3% in the westernmost one (Table 2; Figure 2). For each department, this median within-herd seroprevalence rate was close to the animal-level seroprevalence rate (Table 2; Figure 2). Values obtained for sheep flocks were lower.

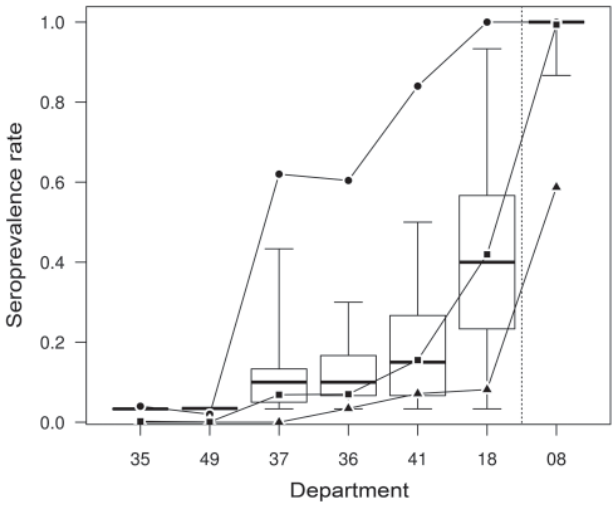

Figure 2. Results from a serologic study of the 2007 epizootic wave of bluetongue virus serotype 8 (BTV-8) in France among cattle herds from an east–west transect of 6 departments (codes 18, 41, 36, 37, 49 and 35) and from the first department to report BTV-8 infection in 2006 (code 08). Circles, herd-level anti–BTV-8 seroprevalence rate; squares, animal-level seroprevalence rate; triangles, proportion of seropositive herds having reported confirmed clinical cases in 2007; box plots, distribution of within-herd seroprevalence rates. Top and bottom of boxes indicate the 25th and 75th percentiles. Thick horizontal lines represent the median value of the distribution. Error bars represent the maximum and minimum values observed in the distribution.

All herds and flocks included in the study for which confirmed clinical cases had been reported in 2007 were seropositive herds, except for a sheep flock from department 18. In seropositive cattle herds of department 08, the disease reporting rate was 59%. It was much lower along the east–west transect, where it varied from 8% in the easternmost department to 0% in the westernmost departments; however, confidence intervals were wide (Table 1; Figure 2). For a given department, species-specific disease reporting rates were relatively homogeneous: ≈60% in department 08 (59% for cattle and 55% for sheep) and 10% in department 18 (8% for cattle and 14% for sheep).

The logistic model of within-herd seroprevalence rate showed a significant effect of the department ($p<0.0001$), as expected, because this variable was used as a proxy for the relative location in the epizootic wave. A significant effect of species was also observed with an odds ratio of 0.02 for sheep ($p<0.0001$, reference: cattle). No significant association between outbreak status and within-herd seroprevalence rate was observed ($p = 0.19$).

Seroprevalence in Cattle and Local Conditions in the East–West Transect

The 297 cattle herds of the 6 transect departments were located in 244 municipalities. Most of these contained a single herd, but 42 contained 2 or 3 herds. No significant difference of within-herd seroprevalence was observed in any of these 42 municipalities (Bonferroni correction: $p > 0.001$), except for 1 municipality in department 41 ($p<0.0001$). This municipality contained 3 herds, 2 of which had similar seroprevalences (3/30 and 1/30); the third had a higher seroprevalence (15/30). This municipality was excluded from the dataset. Seroprevalence data were then aggregated in the remaining 243 municipalities.

Besides the department, univariate analysis led to selection of the following variables: spatial density of cows, the spatial density of sheep flocks, confirmed clinical cases reported in 2007 in the municipality, proportion of municipality area covered by land cover classes C_4, C_5, and C_6, and edge densities for the following pairs of classes: C_1–C_4, C_1–C_6, C_2–C_6, C_4–C_5, C_4–C_6, and C_5–C_6 (see Table 3 for land cover classes definitions). Following the backward elimination process, only 5 variables were significantly associated with animal-level seroprevalence and kept in the final logistic model (Table 4). The main effect was attributed to the department, with the odds ratio decreasing progressively along the east–west transect. A significant protective effect was associated with the spatial density of cows; however, the strength of this association was moderate (Table 4). The existence of confirmed clinical cases reported in the municipality in 2007 was positively associated with seroprevalence. This positive association was also the case for 2 land cover variables: the edge densities between the forests and seminatural areas (C_6) class and 2 other classes: pastures (C_4) and arable land (C_2) (Table 4). Standardized deviance residuals did not differ significantly between departments (Kruskal-

Table 4. Logistic model of animal seropositivity in cattle according to the local conditions in 6 departments aligned on an east–west transect, France, winter 2007–2008*

Variable	Odds ratio	p value
Department		
18: Cher	Reference	
41: Loir-et-Cher	0.21	<0.0001
36: Indre	0.12	<0.0001
37: Indre-et-Loire	0.09	<0.0001
49: Maine-et-Loire	0.002	0.0009
35: Ille-et-Vilaine	0.007	0.0002
Confirmed clinical cases reported in 2007	1.49	0.01
Spatial density of cows in the municipality	0.84†	0.02
Spatial density of sheep flocks in the municipality	NS	0.15
Edge density between arable land and forests or seminatural areas	1.66‡	0.02
Edge density between pastures and forests or seminatural areas	2.06‡	0.001

*NS, not significant.
†Change in the odds of seropositivity when spatial density is increased by 10 cows/km^2.
‡Change in the odds of seropositivity when edge density is increased by 10 m/ha.

Wallis $\chi^2 = 1.96$; $p = 0.85$). No significant aggregation of these residuals was observed (Geary C = 0.58; p = 0.06). Conversely, for the null model (with the department as the only independent variable), the Geary C statistic was lower (indicating a stronger spatial autocorrelation), and a significant aggregation of the standardized deviance residuals was detected (Geary C = 0.46; p = 0.0007).

Discussion

Department 08 was the first infected area in France, with the first outbreaks reported there in August 2006. One year later, in winter 2007–2008, this department was behind the epizootic wave, and our results show that, at that time, nearly all cattle were seropositive. Such high seroprevalences have also been observed behind the epizootic wave in Belgium and in the Netherlands (*10*,*11*). Results from the east–west transect show that if the BTV-8 epidemiologic system was clearly saturated behind the epizootic wave, the system was not saturated in or in front of this wave. This transect started from the center of France (in department 18) where, although all the herds housed at least 1 seropositive animal, only 40% of cattle were seropositive (in this department ≈100 outbreaks were reported during October through the end of December 2007). The transect ended in Brittany (in department 35), where 4 of 2,370 cattle were seropositive; each of these 4 animals was located in a different herd (in this department a single outbreak was reported in late November 2007). Similar geographic variations of herd-level and animal-level seroprevalence rates have been observed in Belgium and in the Netherlands (*10*,*11*).

In our survey, seroprevalence variation along the transect showed that within infected areas, herd-level seroprevalence rate increased much earlier than animal-level seroprevalence rate. In cattle in each of the 7 studied departments, the median within-herd seroprevalence rate in seropositive herds was close to the animal-level seroprevalence rates. These results suggest that in cattle the animal infection probability is not particularly higher within infected herds than elsewhere, with seropositive animals being spatially scattered in infected areas rather than clustered inside some herds. The homogeneity of within-herd seroprevalence rates in neighboring cattle herds (located in the same municipality) also supports this hypothesis. Furthermore, if BTV-8 circulation level were more intense and rapid within than between infected herds, seroprevalence rate within seropositive herds should have been homogeneous (and high) along the east–west transect. This was not the case; this seroprevalence rate progressively decreased along the transect.

This absence of within-herd clustering of seropositive cattle could be explained by the fact that these animals spend a large part of the year on pastures. Under favorable temperature conditions, *Culicoides* spp. midges in general (*12*), and species of northern Europe in particular (*13*–*15*), are exophagic (i.e., they feed outside farm buildings) and exophilic (i.e., they rest outside farm buildings). This absence of clustering also suggests that the European epidemiologic system may differ from other systems described in countries where the disease is endemic. In Australia, for example, a close association exists between *C. brevitarsis* midges and cattle in whose dung *Culicoides* spp. midges lay eggs and larvae develop (*16*,*17*). Such a close association does not seem to exist in Europe, where contradictory results have been recently published about the role of cattle dung in the life cycle of local *Culidoides* spp. midges (*18*,*19*). Seroprevalence rates in sheep were similar to those obtained in the Netherlands during winter 2006–2007 (*20*). Sheep were globally less frequently infected than cattle. In department 08, the only fully seronegative herds were 2 sheep flocks, and logistic modeling attributed a strong protective effect to this species. These results reflect the trophic preferences of vectors (*21*).

Except in department 08, no clinical cases were reported in most seropositive herds or flocks in 2007. The proportion of these silently infected herds was 40% in department 08, and >90% in the east–west transect. At the herd level, no association was detected between occurrence of clinical cases and seroprevalence rate. However, this association was observed in cattle at the municipality level. In the east–west transect, confirmed clinical cases locally reported in 2007 (whatever the species of diseased animals) increased the animal-level seroprevalence risk. Clinical cases caused by a higher (and possibly longer) viremia could increase local viral circulation. This interpretation does not contradict the absence of association at the herd level. If clinical cases increase BTV-8 circulation not only inside the affected herd but also in the neighboring ones, association between seroprevalence rate and occurrence of clinical cases is then much more difficult to demonstrate at the herd level. A protective effect of the local density of cows was observed. The low infection rate of *Culicoides* spp. under experimental (*22*) or field conditions (*23*) could explain this result: increased cattle densities would dilute infective bites and decrease individual infection risk, with seroprevalence being consequently lower.

Two land cover variables were identified as seropositivity risk factors; both are edge densities. These landscape indices are increased when the interweaving of land cover themes increases because landscape is complex and fragmented. Fragmentation indices have been linked to abundance of vectors of BTV (*C. imicola* midges in southern France) (*24*), and the local density of forest and pasture edges have been linked to bluetongue disease risk in Corsica (*25*). More generally, ecotones are places where vegetal and animal species of different ecosystems meet and mix and thus where contact rates are increased. Therefore, land-

scape indices are considered as useful tools for biodiversity assessment (*26*), and species richness of several animal groups (in particular arthropod groups) has been linked to landscape heterogeneity (*27*). In our study, edges between pastures and forests could be areas where species richness is greater, both for vectors (*Culicoides* spp. midges) and for hosts (wild and domestic ruminants). This species richness could allow a more intense BTV circulation than elsewhere, inducing a higher seroprevalence in cattle.

The positive association between seroprevalence in cattle and the edge density between forests and arable land is more difficult to interpret. These edges could represent borders between resting (forest) and feeding (arable land) areas for wild ruminants. An increased density of these edges could then indicate an increased carrying capacity of wild ruminant species, in turn supporting an increased local BTV circulation. Several recent studies have demonstrated high anti-BTV seroprevalence levels in wild ruminants. In BTV-4–infected areas in Spain, a seroprevalence of 66% was observed in red deer (*Cervus elaphus*) (*28*). Interestingly, in this species, seroprevalence rate was higher in free-ranging animals than in captive ones (*29*). In Belgium, a seroprevalence of 52% was observed in red deer in 2007 (*30*). In France, a similar seroprevalence (41%) was observed in this species during winter 2008–2009; seroprevalence reached 70% in some areas (*31*). These field studies and the results of our study suggest that BTV circulation in Europe could involve complex epidemiologic cycles with several host and vector species. As suggested elsewhere (*28*), thorough research is needed on hosts and vectors involved in BTV circulation in natural ecosystems in Europe.

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Eastern Equine Encephalitis Virus in Mosquitoes and Their Role as Bridge Vectors

Philip M. Armstrong and Theodore G. Andreadis

Eastern equine encephalitis virus (EEEV) is maintained in an enzootic cycle involving *Culiseta melanura* mosquitoes and avian hosts. Other mosquito species that feed opportunistically on mammals have been incriminated as bridge vectors to humans and horses. To evaluate the capacity of these mosquitoes to acquire, replicate, and potentially transmit EEEV, we estimated the infection prevalence and virus titers in mosquitoes collected in Connecticut, USA, by cell culture, plaque titration, and quantitative reverse transcription–PCR. *Cs. melanura* mosquitoes were the predominant source of EEEV (83 [68%] of 122 virus isolations) and the only species to support consistently high virus titers required for efficient transmission. Our findings suggest that *Cs. melanura* mosquitoes are primary enzootic and epidemic vectors of EEEV in this region, which may explain the relative paucity of human cases. This study emphasizes the need for evaluating virus titers from field-collected mosquitoes to help assess their role as vectors.

During the past 6 years, eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) has reemerged in the northeastern United States and resulted in 26 human cases of infection and 9 deaths (ArboNET; Centers for Disease Control and Prevention, Atlanta, GA, USA). Virus transmission has intensified throughout this region, spread to locales where it had not been previously detected, and extended north into New Hampshire and Maine, USA, and Nova Scotia, Canada (*1*). Disease outbreaks caused by EEEV occur at irregular intervals when underlying ecologic conditions favor virus amplification and overflow into human and equine populations.

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EEEV is perpetuated in an enzootic cycle involving ornithophilic mosquitoes (primarily *Culiseta melanura*) and passerine birds in freshwater swamps (*2*,*3*). Human and equine cases occur infrequently despite relatively high rates of EEEV infection in *Cs. melanura* during virus amplification (ArboNET). Other mosquito species such as *Aedes vexans*, *Coquillettidia perturbans*, *Ochlerotatus canadensis*, and *Oc. sollicitans* have been implicated as epidemic/epizootic bridge vectors from viremic birds to horses and humans (*4*–*6*). These species are competent vectors of EEEV (*7*–*9*) and may acquire virus infection during disease outbreaks by feeding occasionally on birds but prefer mammalian hosts (*10*–*14*). Although *Cs. melanura* mosquitoes feed infrequently on mammals (*10*,*12*,*15*), their ability to serve as a bridge vector may be offset by a much higher prevalence of EEEV infection in this species.

One criterion used for incriminating enzootic and bridge vectors is based on the frequency of virus detection from each candidate species (*16*). Typically, mosquitoes are collected from disease-endemic sites, sorted into pools by trap location and species, and screened for virus by cell culture or molecular methods. This procedure provides critical information on the identity, spatial and temporal distribution, and proportion of virus-infected mosquitoes and forms the basis for many arbovirus surveillance programs. Nevertheless, virus titers may vary considerably within infected mosquitoes (*17*) and reflect the duration of the extrinsic incubation period and the ability of these mosquitoes to support virus replication, which is a necessary precondition for mosquitoes becoming infectious (*18*). The virus must undergo several rounds of replication in the mosquito midgut and salivary glands before being biologically transmitted to the vertebrate host when the mosquito salivates during blood feeding.

In Connecticut in 2009, EEEV activity increased substantially, and we isolated numerous viruses from *Cs. melanura* mosquitoes and potential bridge vectors. To evaluate the capacity of these mosquitoes to replicate and potentially transmit virus, we estimated EEEV titers from virus-positive mosquito pools with the expectation that the most efficient vectors will support consistently high virus titers.

Materials and Methods

Mosquito Collections

Mosquitoes were collected at 91 trapping locations statewide as a part of the Connecticut Mosquito and Arbovirus Surveillance Program during June–October 2009. Each trapping site was sampled on average weekly and at least every 10 days by means of CO_2-baited Centers for Disease Control light traps and gravid traps that were operated overnight. Adult mosquitoes were transported back to the laboratory alive and sorted on chill tables by trap location and according to species by using taxonomic keys (*19*). Mosquitoes containing visible blood in the abdomen were removed and not included in this study. Mosquitoes were combined into pools of ≤50 individuals, placed in microcentrifuge tubes containing a copper BB, and stored at –70°C until virus testing.

Virus Isolation and Identification

Mosquitoes were homogenized in 1.0–1.5 mL of phosphate-buffered saline supplemented with 30% heat-inactivated rabbit serum, 0.5% gelatin, and antibacterial and antifungal drugs by using a vibration mill set for 4 min at 25 cycles/s. Homogenates were centrifuged at 4°C for 7 min at 520 × *g*, and 100 μL of the supernatant was placed on confluent Vero cells growing in 25-cm^2 flasks containing minimal essential media, 5% fetal bovine serum, and antibacterial and antifungal drugs. Cell cultures were maintained at 37°C in an atmosphere of 5% CO_2 and monitored daily for cytopathic effect during days 3–7 postinfection. Infected cell supernatants were harvested and stored at –80°C.

Mosquito pools that yielded infectious virus in cell culture were directly tested for EEEV by quantitative reverse transcription–PCR (qRT-PCR). RNA was extracted from mosquito pool homogenates by using spin columns and reagents in a viral RNA kit (QIAGEN, Valencia, CA, USA). A total of 2.5 μL of this preparation was added to a 25-μL qRT-PCR by using the TaqMan One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) and primers/probe (9391/9459c/9414probe) (*20*). Amplification was performed as follows: 1 cycle at 50°C for 20 min and 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. Positive results were based on cycle threshold (C_t) values when the change in fluorescence increased above the baseline threshold value calculated by using IQ5 Optical System Software (Bio-Rad, Hercules, CA, USA). Samples that failed to amplify or yielded C_t values >30 were reconfirmed by reisolation of EEEV in cell culture. Supernatants from virus cultures were also tested for EEEV by qRT-PCR or by conventional RT-PCR and by sequencing a portion of the nonstructural protein gene of EEEV as described (*21*).

Plaque Titration

Plaque titrations were performed on confluent Vero cell cultures growing in 12-well plates. Ten-fold dilutions of mosquito pool homogenates were placed in triplicate onto cell monolayers and absorbed for 1 h at 37°C in an atmosphere of 5% CO_2. Cells were overlaid with 1% methylcellulose in minimal essential medium, 5% fetal bovine serum, and antimicrobial and antifungal drugs and returned to the incubator. After 3 days, cells were fixed overnight in 7.4% formaldehyde and stained with 1% crystal violet so plaques could be visualized.

Statistical Analyses

The Pooled Infection Rate add-in for Excel (Microsoft, Redmond, WA, USA) was used to calculate virus infection rates (per 1,000 mosquitoes) and 95% confidence intervals (CIs) on the basis of maximum likelihood estimation (MLE) (*22*). All other statistical tests were performed by using Instat version 3.06 (GraphPad Software, San Diego, CA, USA). The Mann-Whitney U test was used for comparing median virus titers in pools for *Cs. melanura* mosquitoes and 6 other species from which a minimum of 3 positive pools were obtained (*Ae. cinereus*, *Anopheles punctipennis*, *Culex salinarius*, *Oc. canadensis*, and *Uranotaenia sapphirina* mosquitoes). The relationship between C_t values and $\log_{10}$ virus titers were analyzed by regression analysis. Statistical significance was assigned at $p<0.05$ or by nonoverlapping 95% confidence intervals.

Results

In 2009, a total of 291,641 mosquitoes (35 species) were collected and processed as 16,909 pools for virus isolation. EEEV was isolated from 122 mosquito pools, which represented 14 species and 7 genera (Table), obtained during August 17–October 27 in 25 of 91 trapping locations. *Cs. melanura* mosquitoes yielded the greatest number of EEEV isolations (n = 83) and was followed by *Oc. canadensis* (n = 10) and *Ae. cinereus* (n = 6) mosquitoes. Relatively few (≤4) or no EEEV isolates were obtained from the remaining mosquito species. EEEV infection rates were higher in *Cs. melanura* mosquitoes (MLE 3.44, 95% CI 2.76–4.24) than in all other mosquito species tested except *An. quadrimaculatus* (MLE 3.27, 95% CI 0.59–10.55) and *Ur. sapphirina* (MLE 1.35, 95% CI 0.44–3.23) mosquitoes.

Table. Eastern equine encephalitis virus isolated and virus titers from mosquitoes obtained in Connecticut, USA, 2009*

Mosquito species	No. mosquitoes collected	No. virus isolates	Infection rate/1,000 mosquitoes, MLE (95% CI)	Mean C_t by qRT-PCR	Mean titer $\log_{10}$ PFU/ mosquito pool	% Mosquito pools >3.0 $\log_{10}$ PFU/ mosquito pool
Aedes cinereus	15,294	6	0.4 (0.2–0.8)	34.0	2.92	16.7
Ae. vexans	26,462	2	0.1 (0–0.2)	33.0	1.15	0
Anopheles punctipennis	5,573	4	0.7 (0.2–1.7)	34.6	1.69	0
An. quadrimaculatus	607	2	3.3 (0.6–10.6)	35.9	1.67	0
An. walkeri	2,381	2	0.8 (0.2–2.7)	35.1	1.43	0
Culex restuans	14,609	1	0.1 (0–0.3)	35.2	0.85	0
Cx. salinarius	12,605	3	0.2 (0.1–0.6)	32.1	1.31	0
Culiseta melanura	25,595	83	3.4 (2.8–4.2)	22.3	6.55	88.0
Ochlerotatus canadensis	40,543	10	0.2 (0.1–0.4)	32.9	2.82	10.0
Oc. cantator	4,457	1	0.2 (0–1.1)	31.8	1.54	0
Oc. triseriatus	3,000	1	0.3 (0–1.6)	>50	1.60	0
Oc. trivittatus	23,340	2	0.1 (0–0.3)	38.8	<0.8	0
Psorophora ferox	13,677	1	0.1 (0–0.35)	34.0	<0.8	0
Uranotaenia sapphirina	2,954	4	1.4 (0.4–3.2)	36.5	1.00	0
Remaining species†	100,544	0	–	–	–	–

*MLE, maximum-likelihood estimation; CI, confidence interval; C_t, cycle threshold; qRT-PCR, quantitative reverse transcription–PCR; –, not applicable. Mean C_t and PFU values were calculated for mosquito pools positive by qRT-PCR and plaque titration.
†n = 21.

Mosquito pools that yielded EEEV in cell culture were directly tested by qRT-PCR. A total of 108 mosquito pools had positive C_t values (≤37) on the basis of criteria established by Lambert et al. (*20*), 7 mosquito pools were equivocal (C_t 37.6–39.0), and 7 isolates failed to amplify after 50 amplification cycles. All mosquito pools that were negative or had C_t values >30 by qRT-PCR were retested in Vero cell culture and confirmed by reisolation of EEEV. Mean C_t values were lowest for *Cs. melanura* mosquitoes (C_t 22.3) and exceeded 30 for all other mosquito species (Table), which suggested species-specific differences in virus titer.

Concentration of infectious virus was estimated from positive mosquito pools by plaque titration in Vero cell culture. *Cs. melanura* mosquitoes had significantly higher virus titers (mean 6.55 $\log_{10}$ PFU/mosquito pool) than all other mosquito species for which statistical comparisons were possible ($p<0.01$ by Mann-Whitney U test). *Ae. cinereus* and *Oc. canadensis* had the next highest virus titers (2.92 $\log_{10}$ PFU/mosquito pool and 2.82 $\log_{10}$ PFU/mosquito pool, respectively), and mean titers ranged from <0.8 $\log_{10}$ PFU/mosquito pool to 1.69 $\log_{10}$ PFU/mosquito pool in the remaining species. The percentage of *Cs. melanura* mosquito pools with high virus titers (>3.0 $\log_{10}$ PFU/mosquito pool) was 88% compared with 0%–16.7% for the 13 other mosquito species (Table).

The relationship between C_t values and PFU estimated from EEEV-infected mosquito pools is shown in the Figure. A strong negative correlation was found between C_t values and PFU/mL in positive mosquito pools by regression analysis (slope –3.1, y-intercept 39; $p>0.0001$), which indicated a predictive relationship between these 2 measures of virus concentration.

Discussion

Our analysis of EEEV-positive mosquito pools showed major differences in virus titer among different mosquito species obtained in Connecticut. *Cs. melanura* was the only species in which titers developed that were associated with EEEV transmission, estimated to be 4–7 logs of virus in virus-transmitting mosquitoes in previous studies (*23*–*25*). This finding suggests that EEEV is transmitted primarily by *Cs. melanura* mosquitoes in this region of the northeastern United States, despite repeated virus isolations from other mosquito species.

Infrequent human and horse infections by EEEV may arise when *Cs. melanura* mosquitoes occasionally feed on mammals rather than by participation of another epidemic/epizootic bridge vector. Prior studies identified mammalian-derived blood meals in 1%–10% of *Cs. melanura* mosquitoes obtained in Massachusetts, Connecticut, and New York (*10*,*12*,*15*) and provided a direct conduit for virus transmission by this species to horses and humans. These analyses, in conjunction with observations on vector longevity (*26*), vector competence (*8*,*25*,*27*), and prevalence of EEEV infection in *Cs. melanura* mosquitoes (ArboNET), suggest that this species could serve as an enzootic and epidemic/epizootic bridge vector of EEEV. We provide additional support for this hypothesis by estimating virus titers in field-collected mosquitoes, which enabled us to determine which infected mosquitoes could potentially transmit virus.

In this study and previous studies, most EEEV isolations have been from either *Cs. melanura* or *Cs. morsitans* mosquitoes, depending on the region. These 2 species comprised 46%–100% of all isolations from field-collected mosquitoes in published studies (*1*,*28*–*35*) and 62%–92%

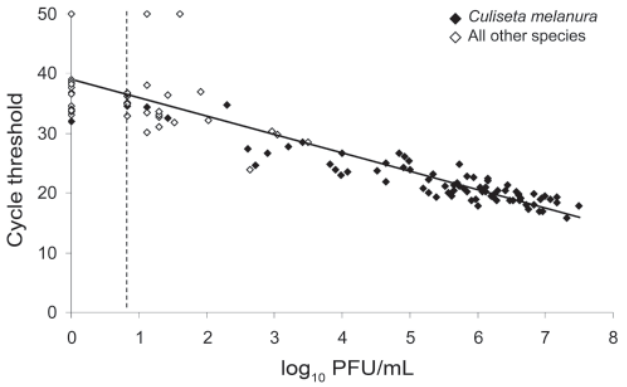

Figure. Relationship between cycle threshold value and PFU estimated from eastern equine encephalitis virus–infected mosquito pools, Connecticut, USA, 2009. Mosquito pools negative for virus by plaque titration were assigned a value of 0, and mosquito pools negative by quantitative reverse transcription–PCR were assigned a value of 50. Limit of detection by plaque titration (0.8 $\log_{10}$ PFU/mL) is indicated by the dashed vertical line.

of all EEEV-positive mosquito pools reported to the Centers for Disease Control and Prevention through ArboNET during 2004–2009. The remaining virus isolations come from a diversity of species, some of which were implicated as bridge vectors largely on the basis of local abundance, temporal and spatial distribution in relationship to human cases, and virus isolation during epidemics.

Ae. vexans mosquitoes are often mentioned as a possible bridge vector of EEEV in the northeastern United States (*1*,*3*,*5*,*12*,*33*). Their distribution and late season abundance overlap with distribution of human cases, and they will feed opportunistically on avian and mammalian hosts. However, vector competence trials have ranked this species as an inefficient vector in the laboratory (*9*); it failed to transmit virus in 1 study (*8*). Our results suggest a negligible role for this species. EEEV was isolated only twice from *Ae. vexans* mosquitoes, and the 2 positive pools showed low virus titers, which reinforced previous findings. A study by Nasci and Mitchell (*17*) reported low EEEV titers (<3.0 $\log_{10}$ PFU/mL) in all *Ae. vexans* mosquito pools tested (n = 4).

Oc. canadensis mosquitoes were the second major source of EEEV after *Cs. melanura* mosquitoes and accounted for 10 (8%) of 122 virus isolations in this study. EEEV has been detected in this species throughout the northeastern United States, including New Jersey, Rhode Island, New York, Massachusetts, and New Hampshire (*33*,*34*,*36*) (ArboNET) and is a moderately competent vector in the laboratory (*8*). *Oc. canadensis* is the most frequently trapped mosquito in Connecticut and is found in a variety of habitats that include freshwater swamps in which EEEV is found. Adult populations peak in late June–early July but extend into fall (*19*), particularly if a second hatch occurs during periods of heavy rainfall. Host-seeking females feed mainly on mammals, including horses and humans and occasionally birds (*11*,*14*). This finding suggests that *Oc. canadensis* mosquitoes may be a bridge vector in Connecticut, but its relative contribution appears to be minor when virus titers in field-collected mosquito pools are considered. We detected high virus titers in only 1 pool of *Oc. canadensis* mosquitoes (3.2 $\log_{10}$ PFU/mL), which is consistent with observations in which 0 of 2 EEEV-positive pools of *Oc. canadensis* mosquitoes contained high titers of virus (>3.0 $\log_{10}$ PFU/mL) (*17*).

EEEV was also isolated from 6 *Ae. cinereus* mosquitoes, which represented the third most common source of virus. Of these pools, 1 contained high titers of virus (3.5 $\log_{10}$ PFU/mL). This species may serve as a potential bridge vector on the basis of certain ecologic and behavioral criteria. Host-seeking females are abundant during June–October in many habitats throughout Connecticut (*19*). This species feeds opportunistically on mammals and birds but prefers mammals (*11*). The ability of this species to transmit EEEV has not been evaluated in the laboratory. Therefore, its contribution as a vector requires further evaluation.

We did not isolate EEEV from *Oc. sollicitans* or *Cq. perturbans*, 2 mosquito species implicated as likely bridge vectors in other epidemiologic settings (*4*,*5*,*34*,*37*). The eastern salt marsh mosquito, *Oc. sollicitans*, is an aggressive biter of humans that may transmit virus in the mid-Atlantic region but its coastal distribution does not overlap with that of human and equine cases in New England. *Cq. perturbans* mosquitoes are commonly trapped in Connecticut (35,389 females collected in 2009) and are found near EEEV foci. Host-seeking females emerge as 1 generation that peaks in early July and then decrease sharply by mid-August when EEEV begins to amplify in Connecticut (*19*). EEEV has been isolated from *Cq. perturbans* mosquitoes throughout the eastern United States, including Connecticut in other years (*6*,*28*,*33*,*37*) (ArboNET). In the study by Nasci and Mitchell (*17*), 13 (65%) of 20 pools of *Cq. perturbans* mosquitoes contained high titers of EEEV (>3.0 $\log_{10}$ PFU/mL), which suggested a bridge role for this species. However, its low abundance in late August and September when EEE activity is greatest argues against its involvement as a primary epidemic vector in this region.

Our results obtained by qRT-PCR corresponded to those estimated by plaque titration, which provided a basis for interpreting C_t values estimated directly from mosquito pools. On the basis of the fitted line estimated by regression analysis in the Figure, a C_t<29.8 corresponded to virus titers >3.0 $\log_{10}$ PFU/mL. We used this titer as a threshold for classifying low or high virus titers in mosquito pools for purposes of comparison and to discern whether a virus infection was established and replication occurred in the

mosquito vector. Competent vectors must support virus replication during the extrinsic incubation period to be able to transmit virus. However, quantitative data for the minimum virus titer necessary for transmission are limited or absent for most virus–vector systems. Despite these uncertainties, investigations showed that mosquitoes transmitted virus when their body titers exceeded 4–5 logs of virus for EEEV (*23*–*25*) and western equine encephalitis virus (*38*,*39*). Virus transmission was usually associated with mosquitoes containing >5 logs of virus. However, some females with high virus titers failed to transmit virus in these studies. On the basis of these considerations, we believe that mosquito pools exhibiting low virus titers (<3.0 log10 PFU/mosquito pool) would be highly unlikely to contain mosquitoes capable of transmitting virus at the time of sampling, whereas detection of high virus titers does not necessarily predict that infected mosquitoes are capable of virus transmission. The strength of the data in our study is based on consistent detection of high virus titers from only 1 competent mosquito vector (*Cs. melanura*).

Most of the pools that showed EEEV in cell culture also showed positive results by qRT-PCR ($C_t \leq 37$). However, our ability to reisolate EEEV from another 14 mosquito pools with either equivocal or negative results indicates that Vero cell culture is a highly sensitive assay for detection of EEEV. Mosquito pools containing infectious virus should be reconfirmed and quantified by another independent method; qRT-PCR is currently the most convenient method to accomplish this test.

Published data on virus titers from field-collected mosquitoes are currently limited to 1 study by Nasci and Mitchell (*17*), and their findings were consistent with our observations in *Cs. melanura*, *Ae. vexans*, and *Oc. canadensis* mosquitoes. Virus titer variation observed among mosquito species could reflect differences in vector competence. EEEV was shown to infect, replicate, and disseminate rapidly in *Cs. melanura* mosquitoes and was detected in the salivary glands 2–3 days after infection (*25*). Moreover, a larger proportion of *Cs. melanura* mosquitoes transmitted EEEV than *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, and *Ochlerotatus* mosquitoes in a direct laboratory comparison (*8*). Virus titers will also vary according to the duration of the extrinsic incubation period. One limitation of our study is that because we did not know the age structure of the mosquito population, we could not control this variable.

Low virus titers may have also been caused by contamination of infected mosquito fragments during sorting and testing procedures. Although we cannot preclude this possibility, we made concerted efforts to ensure accurate testing. Mosquitoes were sorted into mosquito pools on chill tables, and mosquito pools were processed for virus isolation in a separate facility according to standard practices. EEEV-positive pools were directly tested and quantified by using 2 independent tests (plaque titration and qRT-PCR). Our results were consistent with those of other studies (*1*,*28*–*35*), which showed that most EEEV isolations were from *Cs. melanura* mosquitoes and several other mosquito species.

Our findings highlight the need to consider virus titer when interpreting virus isolation or PCR detection data for field-collected mosquitoes. Although we isolated EEEV from several mosquito species, *Cs. melanura* was the only species that had consistently high titers of EEEV sufficient for transmission. This finding may help reconcile the paucity of symptomatic human and equine cases, despite frequent detection or isolation of virus from mammalophilic mosquitoes during episodes of virus amplification. These results should be verified in other regions, where involvement of other locally abundant mosquitoes is suspected during disease outbreaks. When information on virus titers in mosquitoes is considered, the number of candidate vectors may be reduced to a few key species that are capable of supporting virus transmission in nature.

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Bartonella spp. in Bats, Kenya

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We report the presence and diversity of *Bartonella* spp. in bats of 13 insectivorous and frugivorous species collected from various locations across Kenya. *Bartonella* isolates were obtained from 23 *Eidolon helvum,* 22 *Rousettus aegyptiacus,* 4 *Coleura afra*, 7 *Triaenops persicus*, 1 *Hipposideros commersoni*, and 49 *Miniopterus* spp. bats. Sequence analysis of the citrate synthase gene from the obtained isolates showed a wide assortment of *Bartonella* strains. Phylogenetically, isolates clustered in specific host bat species. All isolates from *R. aegyptiacus, C. afra,* and *T. persicus* bats clustered in separate monophyletic groups. In contrast, *E. helvum* and *Miniopterus* spp. bats harbored strains that clustered in several groups. Further investigation is needed to determine whether these agents are responsible for human illnesses in the region.

An unprecedented, increasing interest in bats as reservoirs of infectious diseases occurred during the past decade. Mounting evidence indicates an association of bats with various emerging infections, some with high mortality rates, including lyssaviruses, severe acute respiratory syndrome and other coronaviruses, and henipa, Ebola, and Marburg viruses (*1*–*6*). However, the list of pathogens discovered in bats is even more extensive and includes other representatives from various taxonomic groups (*6*–*8*). Most infectious agents in bats are viruses; bacterial species have been rarely reported (*9*). Excluding reports resulting from serologic and microscopic observations before the 1990s, only a few recent publications describe bacterial species in bats. One publication reports fatal borreliosis in a bat caused by a relapsing fever spirochete in the United Kingdom (*10*). Another study on the molecular detection of hemoparasites infecting bats in southwest England showed the presence of *Bartonella* spp. DNA in the blood of 5 of 60 tested bats (*11*). This report on bat infection potentially caused by bacteria of the genus *Bartonella* is consistent with studies showing detection of *Bartonella* spp.–specific DNA in ectoparasites collected from bats in Egypt and the United States (*12*–*14*).

Bartonella spp. are mainly hemotropic, facultative intracellular parasites associated with erythrocytes and endothelial cells of mammals (*15*,*16*). *Bartonella* spp. organisms are highly adapted to a wide variety of mammals, including rodents, insectivores, carnivores, ungulates, and marine mammals such as dolphins. New insights into the natural history of various *Bartonella* spp. suggest that these bacteria have adapted to their mammalian reservoir hosts in unique ways with frequently restricted host species ranges (*17*). The bacteria can cause chronic intraerythrocyte infections that sometimes result in a large proportion of the reservoir host population being bacteremic simultaneously (*18*). Infections usually cause few or no clinical signs in the reservoir hosts. Host adaptation is evident as some *Bartonella* species and genotypes are found in very specific mammalian species.

Available data on *Bartonella* spp. have expanded rapidly during recent years, as this group of organisms has been found to be associated with a growing spectrum of emerging and reemerging diseases. In addition to cat-scratch disease, trench fever, and Carrión disease, other illnesses linked to *Bartonella* spp. infection range from a self-limiting, short-term fever to potentially fatal systemic diseases with cardiovascular, nervous system, or hepatos-

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plenic involvement (*19*). Some *Bartonella* spp. that have been implicated as human pathogens are linked to rodent species; these species include *B. elizabethae*, *B. grahamii*, *B. washoensis*, and *B. vinsonii* subsp. *arupensis* (*20*–*23*). Other *Bartonella* spp. are linked to wild and pet carnivores. For example, *B. henselae*, carried by cats, causes cat-scratch disease in immunocompetent persons and bacillary angiomatosis in immunocompromised persons (*19*,*24*); *B. vinsonii* subsp. *berkhoffii* is carried by dogs and has been responsible for endocarditis in a human patient (*24*,*25*). For some *Bartonella* spp. recently implicated as human pathogens (such as *B. rochalimae*, which was isolated from an American tourist traveling to Peru, or *B. tamiae*, isolated from 3 patients in Thailand), a mammalian reservoir has not been determined despite a wide range of tested animals collected in these countries (*26*,*27*). These unidentified reservoirs indicate a need for extensive surveillance among diverse groups of animals for *Bartonella* strains, especially among bats, which represent around 20% of all mammalian species *(6)*.

This study was conducted within the framework of the Centers for Disease Control and Prevention (CDC) Global Disease Detection program, which is designed to estimate the health and financial effects and transmission patterns of emerging infectious pathogens associated with humans and animals (including bats, rodents, and other likely reservoirs for human infection) in Kenya and other locations. This study had 5 objectives: 1) to estimate prevalence of *Bartonella* spp. infections among diverse chiropteran species in Kenya; 2) to isolate and identify detected *Bartonella* spp. and create a reference collection of *Bartonella* isolates from East Africa with further characterization and diagnostic investigation; 3) to evaluate genetic heterogeneity of circulating *Bartonella* strains by using the partial sequence variability of the citrate synthase gene (*gltA*), proven to be an excellent genetic marker for analysis of *Bartonella* spp. (*28*); 4) to compare *Bartonella* strains obtained from bats in this study with strains obtained from other animal reservoirs and available from the public domain; and 5) potentially to identify new species of *Bartonella*.

Material and Methods

Blood Samples Collection

More than 500 bats were collected from 25 locations across Kenya (Figure 1). Sampling sites were chosen on the basis of available information about bat roosts and by using field observations of flying and foraging bats. The number of samples and the collection protocol were justified and approved by the National Museums of Kenya and the Kenyan Wildlife Service. Detailed information on the collection procedure has been described elsewhere (*5*). Briefly, bats were collected by using hand nets or collected manually in caves and human dwellings and were captured in mist nets around roosts or in locations of nocturnal foraging. Captured bats were anesthetized by an intramuscular injection of ketamine hydrochloride (0.05–0.1 mg/g body-weight) and euthanized under sedation in compliance with the field protocol approved by CDC's Institutional Animal Care and Use Committee. The bats were measured, and their sex and species were identified.

Species were identified phenotypically by using available field keys. Additionally, representative tissues of each collected species were submitted for confirmation to Guelph University (Guelph, Ontario, Canada), where partial sequences of the cytochrome oxidase gene were generated and compared with those available from the database of the Barcode of Life Data Systems (BOLD; www.barcodinglife.org). Because the taxonomy of African bats is under development and reference sequences for several species were unavailable in the BOLD database, the examined sequences were identified to the genus level only.

For microbiologic studies, selected organs and swabs were collected by using sterile plastic tubes. Serum was separated from the blood clots by centrifugation. All samples were transported on dry ice and stored at –80°C until testing.

Culture

Preliminary attempts to culture *Bartonella* spp. from bat blood showed that the technique developed for isolation of these bacteria from rodent blood (*18*) is appropriate, with some minor modifications, for processing bat samples. Specifically, we used agar plates supplemented by a 10% addition of rabbit blood. Blood from bats was resuspended in brain–heart infusion (BHI) broth supplemented with 5% amphotericin B. The ratio between blood and BHI was determined to be 1:4. However, this ratio was difficult to adhere to for samples with a limited amount of blood (obtained from small bats). Although dilution of tested blood reduces our ability to detect bacteria in cases with a low level of bacteremia, this approach allowed us to reduce the likelihood that bacterial and fungal contaminants would overgrow the fastidious and slow-growing *Bartonella* spp. bacteria. Bacterial colonies were presumptively identified as *Bartonella* spp. on the basis of their morphology and later were confirmed by PCR amplification and sequencing of a specific fragment of the *gltA*. Subcultures of *Bartonella* spp. colonies from the original agar plate were streaked onto secondary agar plates, also supplemented by a 10% addition of rabbit blood, and, in case of confluent and pure cultures, harvested and stored in 10% glycerol. Agar plates inoculated with bat blood were incubated at 35°C in an aerobic atmosphere of 5% carbon dioxide for ≤30 days postinoculation, and plates with subcultures were incubated with

the same conditions until sufficient growth was observed, usually 5–7 days.

Amplification of the *gltA* Fragment

A heavy suspension of the microorganisms was heated for 10 min at 95°C followed by 1-min centrifugation at 8,000 rpm to precipitate the lysed cells. The supernant containing the genomic DNA was then moved to a clean centrifuge tube to be examined. PCR amplifications were performed in a 25-μL reaction mixture containing 5 μL 5× Green GoTaq PCR buffer (Promega, Madison, WI, USA), 0.4 μmol of each primer, 200 μM each dNTP, 1 U Taq DNA polymerase (Promega), and ≈20 ng of template DNA. Two oligonucleotides, BhCS781.p (5′-GGGGACCAGCTCATGGTGG-3′) and BhCS1137.n (5′-AATGCAAAAAGAACAGTAAACA-3′) were used as PCR primers to generate a 379-bp amplicon of the *Bartonella gltA* gene. Positive and negative controls were included with each PCR to evaluate the presence of appropriately sized amplicons and contamination, respectively. Each PCR was performed in a PTC 200 Peltier thermal cycler (MJ Research, Inc., Waltham, MA, USA) or in an Eppendorf Mastercycler Gradient (Eppendorf, Westbury, NY, USA). PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Sequencing and Analysis of DNA

PCR products of correct size were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions and sequenced in both directions by using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed in a PTC 200 Peltier Thermal cycler by using the same primers as the initial PCR with a concentration of 1.6 μM. Sequences were analyzed by using Lasergene (DNASTAR, Madison, WI, USA) sequence analysis software to determine consensus of sequences for the amplified region of the *gltA* gene. The Clustal V program within MegAlign (DNASTAR) was used to align and compare homologous *Bartonella* spp. *gltA* sequences obtained from bat samples and from the GenBank database.

Results

Bartonella spp. Cultures

Culturing from bat blood pellets, especially from small-sized bats, presented a challenge because of the limited sample volume and the potential for contamination with other bacteria and fungi, problems hard to avoid during field sampling. Consequently, of >500 processed blood samples, the presence or absence of *Bartonella* spp. cultures have been conclusively evaluated in samples from 331 bats of 13 species from 9 genera. Further estimation of the infection rate was determined exclusively on the basis of the specimens from these 331 animals. The time required for growth of *Bartonella* spp. colonies on agar greatly varied from 3 days, observed for several samples from *Triaenops persicus* bats to 28 days in one of the samples from *Rousettus aegyptiacus* bats.

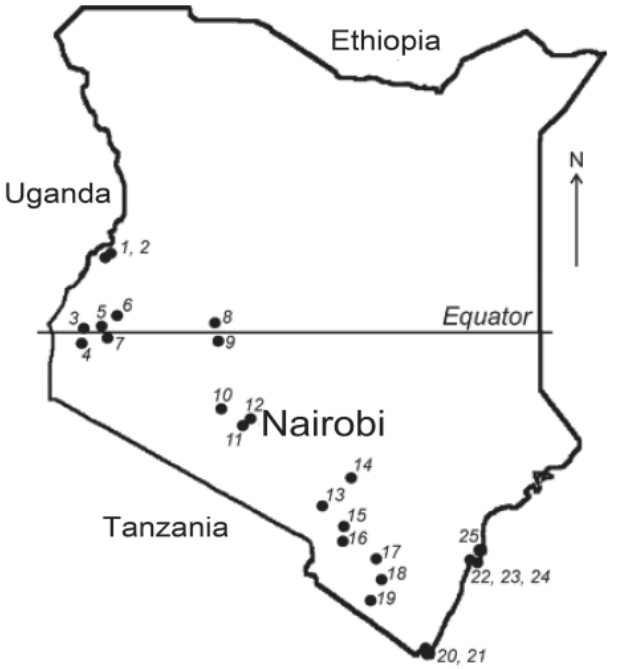

Figure 1. Field sites where bats were collected in Kenya. Numbers identify collection sites (*5*).

Bartonella spp. Prevalence

Bartonella isolates were cultured from the blood of 30.2% (106/331) of the bats tested. All isolates were confirmed genetically by amplification and sequence of the *gltA*. Prevalence of bats positive for *Bartonella* spp. by culture was as follows: *Eidolon helvum* (straw-colored fruit bat), 23/88 (26.1%); *R. aegyptiacus* (Egyptian fruit bat), 22/105 (21.0%); *Coleura afra* (African sheath-tailed bat), 4/9 (44.4%); *T. persicus* (Persian trident bat), 7/8 (87.5%); *Hipposideros commersoni* (giant leaf-nosed bat), 1/4 (25.0%); and *Miniopterus* spp. (long-fingered bats), 49/87 (56.3%) (Table). *Miniopterus* spp. and *T. persicus* bats were at significantly higher risk ($p<0.0001$ and $p<0.001$, respectively) for being infected with *Bartonella* spp. when each was compared with all other bats, and *R. aegyptiacus* and *Epomophorus* spp. bats were at significantly lower risk for infection ($p<0.01$ for both) (Table).

Table. Prevalence of culture-positive test results for *Bartonella* spp. among bat species, Kenya*

Bat species	No. tested	No. (%) positive	Relative risk	p value
Miniopterus spp.	87	49 (56)	2.41	<0.0001
Eidolon helvum	88	23 (26)	NS	
Rousettus aegyptiacus	105	22 (21)	0.6	<0.01
Coleura afra	9	4 (44)	NS	
Triaenops persicus	8	7 (88)	2.85	<0.001
Hipposideros commersoni	4	1 (25)	NS	
Epomophorus spp.	23	0	Not defined†	<0.01
Rhinolophus spp.	6	0	Not defined†	
Chaerephon sp.	1	0	Not defined†	

*Relative risk was calculated by comparing proportions positive for each bat species compared with that for all other bats. NS, not statistically significant.
†Relative risk is <1 but is not defined because of 0 in 1 of the cells.

Genetic Heterogeneity and Sequence Clustering

Sequence analyses of DNA from the 94 *Bartonella* isolates obtained from bats revealed 58 *gltA* genotypes (unique sequence variants with ≥1 nucleotide difference) that represented 11 genogroups with a sequence identity value of >96% between genotypes within each group, as proposed by La Scola et al. (*28*), as a cutoff for this specific gene fragment for species definition within the *Bartonella* genus. The 11 novel *gltA* sequences representing each genogroup were submitted to GenBank and assigned accession nos. HM363764–363768 and HM545136–545141.

Association of *Bartonella* Genotypes with Particular Bat Species

All 15 *Bartonella* spp. *gltA* sequences obtained from *R. aegyptiacus* bats were similar to each other (>96%) and distant from all sequences found in other bat species (<91% identity) and previously described *Bartonella* species (<84% identity). Six unique genetic variants identified in *R. aegyptiacus* bats were clustered in a monophyletic genogroup, marked as R in Figure 2.

The level of *gltA* sequence identity between 16 *Bartonella* genotypes identified in *E. helvum* bats varied greatly, ranging from 78.6%–100%. Eight genotypes (sequence variants) were clustered around 4 clades (genogroups), which are marked as E1, E2, E3, and EW in Figure 2. Intergroup identity values ranged from 78.6%–87.2%, much higher values than the sufficient criteria proposed for species demarcation within the *Bartonella* genus (*28*).

All 4 *gltA* genotypes of *Bartonella* spp. identified in *C. afra* bats differed slightly from one another but clustered tightly within genogroup C with a sequence identity range of 98.2%–99.7%, compared with a <90% identity between these sequences and *Bartonella* strains found in other bat species or other sources. Similarly, all 7 *gltA* sequences identified in *T. persicus* bats formed a monophyletic genogroup, marked as T in Figure 2, with the identity range of 98.2%–99.7% within the group and <90% identity to any *Bartonella* sequence outside the group. The only isolate obtained from *H. commersoni* bats also differed from all described *Bartonella* strains or sequences (identity <85%).

More complex phylogeny was observed when isolates obtained from bats of the genus *Miniopterus* were compared by using a similarity between the *gltA* sequences. In total, 51 *Bartonella* spp. *gltA* sequences, obtained from 3 identified *Miniopterus* spp. (*M. africanus*, *M. minor*, and *M. natalensis*) and from *Miniopterus* spp. bats that have not been identified at the species level, were analyzed. Among the 51 bats, 24 unique *gltA* genotypes represented at least 3 genogroups with a level of divergence ranging from 6.6%–18.5%, higher than has been recommended previously for differentiation of *Bartonella* spp. (*28*). The first genogroup, M1, was composed of 30 sequences obtained from *M. minor* bats, 1 sequence from *M. natalensis* bats, and 1 sequence from an unidentified species within the genus *Miniopterus.* The second genogroup, M2, consisted of 5 sequences from *M. minor* bats, 2 sequences from *M. africanus* bats, 1 sequence from *M. natalensis* bats, and 2 sequences from an unidentified species of *Miniopterus* spp. bats. Although the second genogroup looked more diverse, an insufficient number of isolates were available to describe an association of specific *Bartonella* spp. lineages to certain *Miniopterus* spp. bats. In addition, 1 genotype, M3, from *M. natalensis* bats, was distant from both groups (identity <90%), but was relatively close (identity 94.2%) to the genotype identified in a whiskered bat (*Myotis mystacinus*) from the United Kingdom (*11*).

Discussion

This investigation has resulted in the identification and characterization of *Bartonella* strains in bats and describes the prevalence and genetic characteristics of *Bartonella* spp. in bats in Africa. Detection of viable bacteria in a high proportion of bats in ≥6 bat species suggests that *Bartonella* spp. infection is highly prevalent in bat communities in eastern Africa. Some bat species tested negative for the bacteria; however, we had few samples from these species, making speculation difficult concerning whether those species are truly free from *Bartonella* spp. infection. However, we identified some bat species that were statistically more likely to be infected with *Bartonella* spp.

A high prevalence of *Bartonella* spp. in bats, ranging from 21%–88% in various species, is especially surprising considering the life spans of bats. Numerous investigations have shown that prevalence of *Bartonella* spp. infection can reach high rates in rodent populations, but rodents usually live no longer than 1–2 years, whereas bats can live for >20 years (*6*). Explaining such high prevalence is difficult without assuming persistent infection. This hypothesis was not confirmed for some rodent species, specifically for cotton rats; a high prevalence of *Bartonella* spp. in that population could be explained by replacement of diverse *Bartonella* strains sequentially colonizing an individual rat rather than by a long-term bacteremia (29). This scenario is unlikely in bats because they have much longer lives than rodents.

The lifestyle of bats, such as the colonial structure of their populations, close physical contact, aggressive interactions, and typically heavy ectoparasite infestations also might contribute to the frequent transmission of *Bartonella* spp. among individual animals. All *Bartonella* spp. are widely regarded as vector-transmitted agents, and diverse arthropods, such as sandflies, lice, fleas, ticks, and mites, have been implicated as potential vectors (*30*). Bats carry a wide range of ectoparasites, including bat flies, fleas, soft ticks, and mites, some of which are highly specific to bats (*31*). Limited information is available to suggest that alternative mechanisms beyond vector transmission may be responsible for the spread of *Bartonella* spp. infections among animals. For example, identification of viable *Bartonella* spp. bacteria in the blood of cotton rat embryos and neonates indicated a possibility of vertical transmission from a pregnant female to offspring (*32*). Detection of *Bartonella* spp. DNA in the saliva of dogs suggests a potential possibility of transmission through biting (*33*), and transmission of *Bartonella henselae* from cats to humans by cat scratch is well documented (*24*).

Comparative analyses of the *gltA* sequences obtained from *Bartonella* spp. cultures showed that bats in Kenya harbor a diverse assemblage of *Bartonella* strains, some of which appear to represent distinct species. Although we used only a portion of the citrate synthase gene for phylogenetic analysis, this gene has been shown to be a reliable tool for distinguishing between closely related *Bartonella* genotypes (*28*,*34*). By using this gene, we were able to compare the variety of *Bartonella* genotypes isolated from bats with homologous sequences of *Bartonella* strains found in other mammals. Finding considerable sequence diversity is typical for different species of Bartonella, although more characteristics are needed to describe novel *Bartonella* species.

Evidence for cospeciation of *Bartonella* spp. with natural hosts varies among studies and around the world. A strong association exists between specific *Bartonella* species and their mammalian hosts, whereas some observations have indicated that 1 species of *Bartonella* can infect a variety of rodent species at a given site in Europe (*35*). Our investigation indicated a definite host-specificity for *Bartonella* strains in bat species. All isolates obtained from *R. aegyptiacus, C. afra,* and *T. persicus* bats clearly belonged to the specific *Bartonella* spp. group found exclusively in the particular bat species. By contrast, straw-colored fruit bats (*E. helvum*) and long-fingered bats (*Miniopterus* spp.) harbored strains clustered around 3 and 4 different groups of *Bartonella* spp., respectively, based on their *gltA* identity. Nevertheless, all strains of *Bartonella* spp. recovered from *E. helvum* bats were typical for this species of bats only. This pattern of cospeciation resembles the *Bartonella* spp.–host relations observed in cotton rats (*18*). Similarly, the *gltA* sequences from all strains obtained from *Miniopterus* spp. bats have not been found in bats of other bat genera. More investigations of *Bartonella* spp. in diverse bat species are required to test this hypothesis.

No evidence is available to suggest whether novel strains of *Bartonella* spp. found in bats from Kenya cause

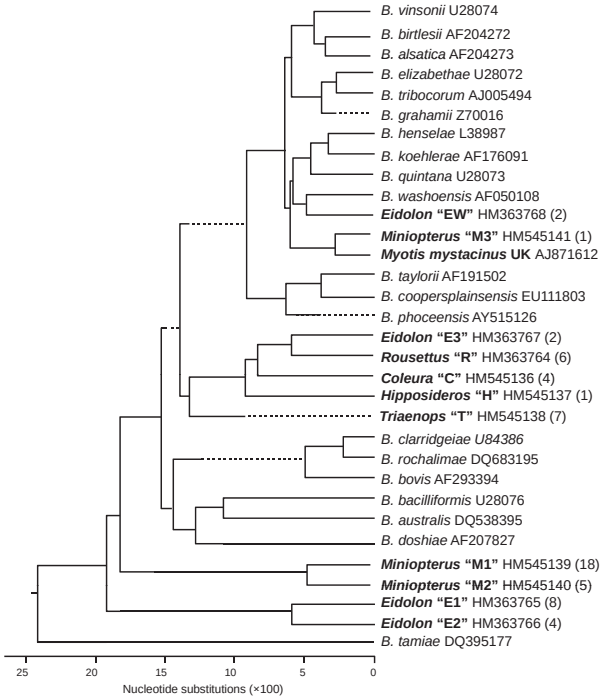

Figure 2. Phylogenetic relations among the citrate synthase sequences of *Bartonella* spp. genotypes detected in bats from Kenya and previously described *Bartonella* spp. The phylogenetic tree was constructed by the neighbor-joining method. Each *Bartonella* spp. genogroup detected in bats was provided with the Latin name of the bat genus from which the *Bartonella* strains were obtained (**boldface**), the proposed name of genogroup (quotation marks), the GenBank accession number, and the number of genotypes assigned to the genogroup (parentheses).

human illness. However, relevant surveillance in Kenya and other African countries has not been implemented. The significance of African bats in public and veterinary health is not understood because of a lack of surveillance. Bats are known as principal reservoir hosts of lyssaviruses. In Africa, these include Lagos bat virus, which circulates in pteropid bats (including *E. helvum* and *R. aegyptiacus*), and Duvenhage virus, which circulates in insectivorous bats (although specific reservoirs have not been established). In addition, Shimoni bat virus was identified recently in an insectivorous bat *Hipposideros commersoni* (*32*). Furthermore, multiple species of African bats have been shown to harbor coronaviruses (*33*). Nipah virus was identified in straw-colored fruit bats *E. helvum* (*34*), and Marburg virus was identified in tomb bats *R. aegyptiacus* (*35*). Circulation patterns of these agents in bat populations have not been sufficiently studied.

Characterization of the isolates obtained from bats and comparison with those obtained from human cases associated with *Bartonella* spp. of unknown origin (e.g., *B. tamiae*) can be helpful in the search for potential reservoirs. The reagents prepared from bat isolates of *Bartonella* spp. can be used for serologic surveys conducted in high-risk areas of the world. Application of antigens produced from bat-derived strains is especially relevant to serologic investigations of cases of infectious disease of unknown origin among persons occupationally exposed to bats. In addition, cocirculation of *Bartonella* spp. with other pathogens in a bat population can affect the bat's ecology and pathobiology. These aspects need further investigation.

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Alkhurma Hemorrhagic Fever in Humans, Najran, Saudi Arabia

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Alkhurma virus is a flavivirus, discovered in 1994 in a person who died of hemorrhagic fever after slaughtering a sheep from the city of Alkhurma, Saudi Arabia. Since then, several cases of Alkhurma hemorrhagic fever (ALKHF), with fatality rates up to 25%, have been documented. From January 1, 2006, through April 1, 2009, active disease surveillance and serologic testing of household contacts identified ALKHF in 28 persons in Najran, Saudi Arabia. For epidemiologic comparison, serologic testing of household and neighborhood controls identified 65 serologically negative persons. Among ALKHF patients, 11 were hospitalized and 17 had subclinical infection. Univariate analysis indicated that the following were associated with Alkhurma virus infection: contact with domestic animals, feeding and slaughtering animals, handling raw meat products, drinking unpasteurized milk, and being bitten by a tick. After multivariate modeling, the following associations remained significant: animal contact, neighboring farms, and tick bites.

Alkhurma virus (ALKV) was discovered in Saudi Arabia in 1995 in a butcher with suspected Crimean-Congo hemorrhagic fever. His fever developed after he had slaughtered a sheep from the city of Alkhurma. Diagnostic testing identified a flavivirus as the etiologic agent (*1*,*2*). Subsequently, ALKV was isolated from the blood of 6 male butchers in Jeddah, and another 4 cases were diagnosed serologically. This disease was named Alkhurma hemorrhagic fever (ALKHF) because the first case was reported from the Alkhurma governorate (*1*).

After initial virus identification, from 2001 through 2003, another 37 suspected ALKHF cases, of which 20 were laboratory confirmed, were reported in Alkhumra district, south of Jeddah (*3*). Among the 20 patients with confirmed cases, 11 had hemorrhagic manifestations and 5 died.

Full genome sequencing has indicated that ALKV is a distinct variant of Kyasanur Forest disease virus, a virus endemic to the state of Karnataka, India (*4*). Recently, ALKV was found by reverse transcription–PCR in *Ornithodoros savignyi* ticks collected from camels and camel resting places in 3 locations in western Saudi Arabia (*5*). ALKHF is thought to be a zoonotic disease, and reservoir hosts may include camels and sheep. Suggested routes of transmission are contamination of a skin wound with blood of an infected vertebrate, bite of an infected tick, or drinking of unpasteurized, contaminated milk (*6*).

Several studies have been conducted to describe the characteristics and determinants of ALKHF (*1*,*3*,*5*,*6*). We conducted a case–control study to assess associated risk factors.

Materials and Methods

Study Area

The study was conducted in the city of Najran, which is in the southern part of Saudi Arabia on the border with Yemen. It is the capital of Najran region and has a population of ≈250,000. It is an agricultural city in which residents commonly raise domestic animals in their backyards. Cases of ALKHF were found in 6 districts, which were close to each other (within ≈30 km) and rural and in which hygiene was poor.

Case Identification

From 2006 through 2009, laboratory testing for ALKV was performed for Najran residents who sought medical care and whose illnesses met the case definition for suspected ALKHF. Infection with ALKV was suspected if a patient had acute febrile illness for at least 2 days; nega-

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tive Rift Valley fever, Crimean-Congo hemorrhagic fever, and dengue confirmatory test results; and ≥2 of the following: 1) at least 3-fold elevation of alanine transferase or aspartate transferase or clinical jaundice; 2) signs of encephalitis such as confusion, disorientation, drowsiness, coma, neck stiffness, hemiparesis, paraparesis, or convulsions; 3) signs of hemorrhage such as ecchymosis, purpura, petechiae, gastrointestinal bleeding (hematemesis, melena, hematochesia), epistaxis, bleeding from puncture sites, or menorrhagia; and 4) platelet count <100 × 10^9/L, or lactate dehydrogenase or creatine phosphokinase 2× upper reference level.

In addition, as part of public health surveillance, blood samples were collected from household contacts of patients with laboratory-confirmed ALKHF. Samples from persons seeking medical care were tested by ELISA for ALKV-specific immunoglobulin (Ig) M and IgG by using ALKV antigen as described (*7*,*8*) and for viral-specific sequence by reverse transcription–PCR (TiBMolbiol, LightMix kit; Roche Applied Science, Basel, Switzerland). Samples from follow-up testing of household contacts were tested by ELISA for ALKV-specific IgG. A total of 11 cases were identified through persons seeking medical care whose illnesses met the case definition for ALKHF, and another 17 cases were identified through follow-up testing of household contacts.

Case–Control Study

A case of ALKHF was defined as illness in any person who lived in the catchment area of Najran General Directorate of Health Affairs and who had serologic evidence of ALKV infection during January 1, 2006–April 30, 2009. All 28 case-patients identified were included in the study. For each case-patient, 2 controls selected from the same house or the nearest neighboring house. A total of 65 controls were enrolled, each of whom was serologically negative for ALKV-specific antibodies.

A structured questionnaire was based on information collected during the initial review of the outbreak and asked for demographic data (name, age, gender, nationality, educational status, occupation, marital status, and place of residency), clinical features, and possible risk factors such as exposure to domestic animals. Exposure history for case-patients was limited to the 30 days before onset of illness and for controls to the past 30 days. If the case-patient or control was a child and unable to respond, the child's mother, father, or an older family member helped answer the questions.

Data Analysis

After the samples (frequency and percentage distributions, means, and standard deviations) were described, cross-tabulations were constructed to compare risk factors. To estimate the strength of association, we calculated odds ratios (ORs) with 95% confidence intervals (CIs). For the multivariate analysis, the most significant variables from the bivariate analysis (at $p<0.05$) and relevant variables thought to be associated with ALKHF from other studies were selected for backward stepwise inclusion in the model.

Results

Outbreak Description

Of 28 case-patients, 11 (39%) were seeking medical advice and hospitalized for ALKHF, and the others were identified through surveillance among household contacts. Among the 11 hospitalized case-patients, 7 diagnoses of ALKHF were made by PCR and 4 by serologic testing. Among the hospitalized case-patients, only 1 had severe symptoms and was admitted to the intensive care unit. The remaining 10 stayed in the hospital for 5–15 days (mean duration 9.3 ± 3.3 days) and received supportive care, including intravenous fluid administration and antimicrobial drugs when indicated.

Although only 11 case-patients sought medical care, 4 reported having had an illness during the study period with symptoms consistent with those of ALKV infection, and 13 seropositive persons reported having had no illness. Among all seropositive persons, fever was found for 15 (54%), bleeding (epistaxis) for 8 (29%), rash for 7 (25%), change in urine color for 6 (21%), gum bleeding for 5 (18%), neurologic signs (e.g., neck rigidity) for 3 (11%), and change in feces color for 1 (4%) (Table 1). No case-patient died. Timing of seeking care for the 11 case-patients was December 2006 (n = 1), January 2007 (n = 1), 2008 (n = 4), and the first 4 months of 2009 (n = 5) (Figure); most cases occurred during March–July. Among the 28 persons with positive serologic test results, 5 clusters were identified in which multiple persons within a single family were infected (these family clusters involved 7, 5, 3, 3, and 2 persons in the respective families).

The 28 case-patients were 4–55 years of age (mean 22 ± 11.2 years), and most (14 [50%]) were 20–39 years of age. Most case-patients were male (18 [64%]) and single (15 [54%]). Case-patients were of 3 nationalities: Yemeni (24 [86%]), Saudi (3 [11%]), and Bangladeshi (1 [4%]). Regarding educational level, 1 (4%) was in preschool, 4 (14%) were illiterate, 13 (46%) had primary or intermediate degree, 8 (29%) had a high school degree, and 2 (7%) had a university degree. Most (12 [43%]) case-patients were students, 4 (14%) had livestock-related jobs, 5 (18%) were housewives, and 7 (25%) had other jobs. Most (20 [71%]) case-patients lived in modern houses (Table 2).

Table 1. Clinical signs of Alkhurma hemorrhagic fever virus among 28 patients, Najran, Saudi Arabia, 2006–2009

	No. (%) patients		
Clinical feature	Hospitalized, n = 11	Not hospitalized, n = 17	Total
Fever	11 (100)	4 (24)	15 (54)
Nosebleed	5 (46)	3 (18)	8 (29)
Rash	5 (46)	2 (12)	7 (25)
Jaundice	5 (46)	1 (6)	6 (21)
Change in urine color	5 (46)	1 (6)	6 (21)
Gum bleeding	5 (46)	0	5 (18)
Neck rigidity	3 (27)	0	3 (11)
Numbness of extremities	2 (18)	0	2 (7)
Seizures	2 (18)	0	2 (7)
Change in feces color	1 (9)	0	1 (4)

Outbreak Setting

Cases were identified within 6 different districts; most were in the city of Najran. They were in agricultural areas with livestock, some of which lived with the people in the houses.

Patient Demographics and Risk Factors

The mean age of case-patients was 22.3 years ± 11.2 years, and mean age of controls was 25.2 years ± 15.4 years; the difference was not significant (*t* test, p = 0.657). The age group 20–39 years contained 14 (50%) case-patients and 31 (48%) controls; the age group <20 years contained 13 (46%) case-patients and 24 (37%) controls. No significant difference was noted between case-patients and controls in terms of gender, nationality, education level, marital status, type of home (modern or not), occupation, or district of residence (Table 2).

Among the case-patients, 14 (50%) gave a history of owning or raising domestic animals, compared with 17 (26%) controls (OR 2.82, 95% CI 1.02–7.91). Among those who reported owning or raising domestic animals, all case-patients and controls had sheep on their farms; thus, owning sheep was a significant risk factor (OR 2.82, 95% CI 1.02–7.91). Owning cows and camels was less common; no significant association was found (OR 1.37, 95% CI 0.30–5.86 and OR 10.29, 0.84–279.2, respectively). Risk for infection was significantly higher for those who lived <100 meters from farms (OR 4.00, 95% CI 1.40–11.75) than for those who lived farther from farms (Table 3).

A significantly higher proportion of case-patients (46%) than controls (14%) had direct contact with animals (OR 5.39, 95% CI 1.74–17.3). Furthermore, 9 (32%) case-patients and 3 (5%) controls fed animals (OR 9.79, 95% CI 21.1–51.48); 10 (36%) case-patients and 6 (9%) controls slaughtered animals (OR 5.46, 95% CI 1.54–20.02); and 9 (32%) case-patients and 7 (11%) controls handled raw meat products (OR 3.92, 95% CI 1.14–13.84). A borderline significant association with disease was found for milking animals (OR 4.00, 95% CI 0.99–16.64). Risk was higher for those who dealt with animals in multiple ways (e.g., feeding, slaughtering, milking, handling raw meat products) (χ^2 for trend 15.53; p<0.001). Unpasteurized milk was consumed by 8 (29%) case-patients and 6 (9%) controls (OR 3.93, 95% CI 1.06–14.88). A statistically significant association was found for tick bites and disease; a higher proportion of case-patients (36%) than controls (5%) reported a history of tick bites (OR 11.48, 95% CI 2.51–59.73). No statistically significant difference between case-patients and controls was found for exposure to mosquitoes (OR 2.72, 95% CI 0.65–13.03) (Table 2).

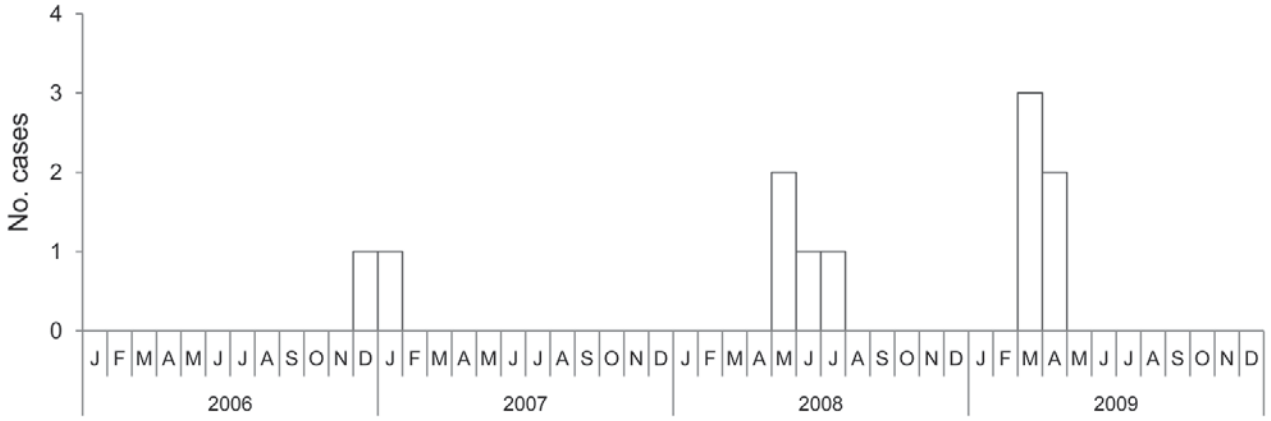

Figure. Annual distribution of Alkhurma hemorrhagic fever case-patients admitted to hospitals in Najran, Saudi Arabia, 2006–2009.

Multivariate analysis, conducted with variables that were significant ($p<0.05$) in bivariate analysis and with variables previously reported to be associated with ALKHF (contact with animals, tick bites, close proximity of neighboring farms, consumption of unpasteurized milk, and mosquitoes bites) was conducted by backward stepwise regression analysis. Among these variables, contact with animals, tick bites, and neighboring farms remained predictors for ALKHF (adjusted ORs 3.17, 95% CI 0.96–10.43; 6.20, 95% CI 1.34–28.70; and 3.63, 95% CI 1.25–10.4, respectively) (Table 4).

Discussion

Our findings that some patients had subclinical illness and that no deaths were documented among the 28 case-patients suggest that previous studies may not have characterized the full spectrum of ALKV-associated illness and that case-fatality rates as high as 25% may have resulted from detecting only severe cases of ALKHF. In addition, our identification of multiple seropositive members within families suggests the occurrence of family-based clusters of ALKV infection.

ALKV-positive persons with subclinical disease, identified by the active surveillance system, did not undergo thorough clinical and laboratory investigations and were not directly observed by a clinician. Because of this lack of observation and because of the inability of some case-patients to recall and report low-grade fever within 1 month before onset of illness, only 53% of case-patients reported fever, but all case-patients who were hospitalized had fever. Our findings underscore the role of the high percentage of case-patients with subclinical infection in the epidemiology of this disease; seroprevalence studies should be encouraged.

Table 2. Demographic characteristics of persons with and without Alkhurma hemorrhagic fever, Najran, Saudi Arabia, 2006–2009*

Characteristic	Case-patients, no. (%), n = 28	Controls, no. (%), n = 65	Total, no. (%), n = 93	OR (95% CI)
Age, y				
<20	13 (46)	24 (37)	37 (40)	5.42 (0.58–125.74)
20–39	14 (50)	31 (48)	45 (48)	4.52 (0.50–10.43)
≥40	1 (4)	10 (15)	11 (12)	Reference
Gender				
M	18 (64)	31 (48)	49 (53)	1.97 (0.72–5.45)
F	10 (36)	34 (52)	44 (47)	Reference
Marital status				
Single	15 (54)	30 (46)	45 (48)	1.35 (0.51—3.63)
Married	13 (46)	35 (54)	48 (52)	Reference
Nationality				
Saudi	3 (11)	14 (22)	17 (18)	0.86 (0.05–28.00)
Yemeni	24 (86)	47 (72)	71 (76)	2.04 (0.19–50.75)
Bangladeshi	1 (4)	4 (6)	5 (5)	Reference
Education				
Preschool	1 (4)	6 (9)	7 (8)	0.25 (0.01–6.51)
None (illiterate)	4 (14)	7 (11)	11 (12)	0.86 (0.06–12.21)
Primary	9 (32)	29 (45)	38 (41)	0.85 (0.18–4.15)
Intermediate	4 (14)	11 (17)	15 (16)	0.55 (0.04–7.15)
Secondary	8 (29)	9 (14)	17 (18)	1.33 (0.12–15.74)
University	2 (7)	3 (5)	5 (5)	Reference
Occupation				
Livestock related†	4 (14)	9 (14)	13 (14)	1.27 (0.23–6.79)
Student	12 (43)	17 (26)	29 (31)	2.02 (0.57–7.34)
Housewife	5 (18)	19 (29)	24 (26)	0.75 (0.17–3.30)
Other‡	7 (25)	20 (31)	27 (29)	Reference
District				
Al Hadhan	4 (14)	5 (8)	9 (10)	2.80 (0.25–36.19)
Al Balad	7 (25)	15 (23)	22 (24)	1.63 (0.21–15.05)
Al Jarbah	12 (43)	29 (45)	41 (44)	1.45 (0.22–11.83)
Al Mashaliah	3 (11)	9 (14)	12 (13)	1.17 (0.10–14.06)
Al Ghwaila	2 (7)	7 (11)	9 (10)	Reference
House				
Modern	20 (71)	50 (77)	70 (75)	0.75 (0.25–2.30)
Traditional	8 (29)	15 (23)	23 (25)	Reference

*OR, odds ratio; CI, confidence interval.
†Shepherd, butcher.
‡Teacher, driver, military, none.

Table 3. Risk factors for Alkhurma hemorrhagic fever virus, Najran, Saudi Arabia, 2006–2009*

Risk factor	Case-patients, no. (%), n = 28	Controls, no. (%), n = 65	Total, no. (%), n = 93	OR (95% CI)
Owning or raising domestic animals				
Yes	14 (50)	17 (26)	31 (33)	2.82 (1.02–7.91)
No	14 (50)	48 (74)	62 (67)	Reference
Owning the following animals				
Sheep	14 (50)	17 (26)	31 (33)	2.82 (1.02–7.91)
Cows	4 (14)	10 (15)	14 (18)	1.37 (0.30–5.86)
Camels	3 (11)	1 (2)	4 (4)	10.29 (0.84–279.20)
None	14 (50)	48 (74)	62 (67)	Reference
Owning animal with abnormalities				
Yes	5 (36)	7 (41)	12 (39)	0.79 (0.14–4.31)
No	9 (64)	10 (59)	19 (61)	Reference
Owning animals with the following type of abnormality				
Sudden death	2 (14)	5 (29)	7 (23)	0.44 (0.04–3.78)
Recurrent abortion	1 (7)	1 (6)	2 (7)	1.11 (0.00–49.10)
Weakness	2 (14)	1 (6)	3 (10)	2.22 (0.12–74.91)
None	9 (64)	10 (59)	19(61)	Reference
Living how far from farm, meters				
<100	20 (71)	25 (39)	45 (48)	4.00 (1.40–11.75)
≥100	8 (29)	40 (62)	48 (52)	Reference
Contact with domestic animals				
Yes	13 (46)	9 (14)	22 (24)	5.39 (1.74–17.30)
No	15 (54)	56 (86)	71 (76)	Reference
Feeding animals				
Yes	9 (32)	3 (5)	12 (13)	9.79 (2.11–51.48)
No	19 (68)	62 (95)	81 (87)	Reference
Milking animals				
Yes	7 (25)	5 (8)	12 (13)	4.00 (0.99–16.64)
No	21 (75)	60 (92)	81 (87)	Reference
Slaughtering animals				
Yes	10 (36)	6 (9)	16 (17)	5.46 (1.54–20.02)
No	18 (64)	59 (91)	77 (83)	Reference
Handling raw meat products				
Yes	9 (32)	7 (11)	16 (17)	3.92 (1.14–13.84)
No	19 (68)	58 (89)	77 (83)	Reference
Drinking unpasteurized milk				
Yes	8 (29)	6 (9)	14 (15)	3.93 (1.06–14.88)
No	20 (71)	59 (91)	79 (85)	Reference
Being bitten by tick				
Yes	10 (36)	3 (5)	13 (14)	11.48 (2.51–59.73)
No	18 (64)	62 (95)	80 (86)	Reference
Being bitten by mosquito				
Yes	25 (89)	49 (75)	74 (80)	2.72 (0.65–13.03)
No	3 (11)	16 (25)	19 (20)	Reference

*OR, odds ratio; CI, confidence interval.

A similar seasonal pattern of disease (March–July) was found in western provinces (Jeddah and Makkah) among 11 case-patients who recovered during 1994–1999. This finding might support evidence of disease association with the peak activity of ticks in the beginning of March (*9*,*10*).

Similar to other hemorrhagic fevers, ALKHF showed no predilection for patient age, gender, or nationality (*11*). Risk factors identified by this study included a broad array of activities associated with animal exposures but most significantly with direct contact with animals. Similarly, a higher proportion of case-patients than controls owned or raised animals; however, no difference was noted in the proportion reporting abnormalities in their animals. This finding might suggest the low virulence of the virus in animals and highlights the need for animal studies. Among the animals raised, sheep were significantly associated with the disease.

Although we found no significant association between ALKHF infection and livestock-related occupations such as butchering, we found a high association for history of slaughtering livestock. These findings agree with those of

Table 4. Multivariate logistic regression results of risk factors for Alkhurma hemorrhagic, Najran, Saudi Arabia, 2006–2009*

Risk factor	Crude OR (95% CI)	Model aOR† (95% CI)
Contact with domestic animals	5.39 (1.74–17.3)	3.17 (0.96–10.43)
Tick bites	11.48 (2.51–59.73)	6.20 (1.34–28.70)
Adjacent farm distance	4.00 (1.40–11.75)	3.63 (1.25–10.49)

*OR, odds ratio; CI, confidence interval; aOR, adjusted OR.
†aOR for risk factors (contact with domestic animals, tick bites, adjacent farm distance) after elimination of nonsignificant variables (drinking unpasteurized milk and owning or raising domestic animals) calculated by using backward stepwise strategy.

other studies conducted in the cities of Jeddah and Makkah (*1*,*3*). Furthermore, we found that another major risk factor for human infection was direct contact with blood or body fluids from animals.

Ingestion of unpasteurized milk has been noted as a risk factor in previous studies; the mode of transmission is not yet clear but has been suggested to result from contamination of the milk (*6*,*12*). However, our multivariate analysis found no association.

Only 10 of the 28 interviewed case-patients had a history of a tick bite within 1 month before symptom onset; however, tick exposure was significantly more common among case-patients than among controls. The association of tick bites and ALKV was supported by Charrel et al., who detected ALKV RNA in ticks (*Ornithodoros* spp.) collected from camels and camel resting places in western Saudi Arabia (*5*).

ALKV has been identified only in the southern and western parts of Saudi Arabia. Given our current evidence that subclinical ALKV infections occur in humans, the virus may be more widespread in Saudi Arabia than previously realized. We are conducting studies to further characterize the distribution of ALKV in Saudi Arabia. In addition, the history of the reported disease in Makkah during the Hajj, when thousands of livestock are imported to Saudi Arabia, and the existence of the outbreak in Najran, which is at the border with Yemen, necessitate further studies in adjacent countries (*3*,*5*). Additionally, ALKV is closely related to Kyasanur Forest disease virus, which has been well characterized in India. The possibility remains that this virus has a wider geographic range in the Middle East and central Asia than previously realized.

Because our investigations were retrospective, we cannot exclude recall bias about exposure, but as long as the controls were from the same households, they were exposed to some of the questions during the surveillance done by the preventive department. Furthermore, the investigators who administered the questionnaires based the questions on the month before the interview, which should minimize recall bias.

ALKHF is a zoonotic disease with clinical features ranging from subclinical asymptomatic to severe complications. This study highlights the different activities related to exposure to animals and tick bites in the transmission of ALKV to humans; it found no significant association with mosquitoes. Further studies are needed to understand the role of livestock, wildlife, and ticks in the maintenance of the virus and the risk factors so that public health measures can be developed to reduce the extent of the disease in humans.

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Environmental Sampling for Avian Influenza Virus A (H5N1) in Live-Bird Markets, Indonesia

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To identify environmental sites commonly contaminated by avian influenza virus A (H5N1) in live-bird markets in Indonesia, we investigated 83 markets in 3 provinces in Indonesia. At each market, samples were collected from up to 27 poultry-related sites to assess the extent of contamination. Samples were tested by using real-time reverse transcription–PCR and virus isolation. A questionnaire was used to ascertain types of birds in the market, general infrastructure, and work practices. Thirty-nine (47%) markets showed contamination with avian influenza virus in ≥1 of the sites sampled. Risk factors were slaughtering birds in the market and being located in West Java province. Protective factors included daily removal of waste and zoning that segregated poultry-related work flow areas. These results can aid in the design of evidence-based programs concerning environmental sanitation, food safety, and surveillance to reduce the risk for avian influenza virus A (H5N1) transmission in live-bird markets.

Food markets that offer both poultry meat and live birds either for sale or for slaughter are collectively referred to as live-bird markets (LBMs). LBMs are part of the supply chain and are essential for maintaining the health and nutritional status of rural and urban populations, especially in developing countries (*1*,*2*). However, LBMs provide optimal conditions for the zoonotic transfer and evolution of infectious disease pathogens because they provide major contact points between humans and live animals (*3*,*4*).

Studies in Hong Kong Special Administrative Region, People's Republic of China; other areas of China; Indonesia; and the United States have shown that LBMs can harbor avian influenza viruses (AIVs), including highly pathogenic influenza virus A (H5N1), and have been associated with human infection (*4*–*9*). Continual movement of birds into, through, and out of markets provides opportunity for the introduction, entrenchment, and dissemination of AIVs. Most studies have focused on testing live birds rather than environmental sites in the LBMs (*6*,*7*,*10*). However, a study in New York, NY, that tested environmental sites for AIV (H7N2) found that virus could be isolated from samples from floors, walls, and drains from the poultry areas of LBMs (*8*). The study also found that despite the ongoing influx of infected birds into LBMs, the level of environmental contamination decreased with routine cleaning and disinfection. Another study in Hong Kong LBMs showed that AIV (H9N2) could be isolated at higher rates from poultry drinking water than from samples of bird fecal droppings (*11*). Environmental aspects of LBMs are needed for an avian influenza control program for 2 reasons. First, a contaminated environment can provide a continuing source of virus transmission, in which healthy birds coming into the market may become infected and persons working in or visiting the market may also be exposed. Second, ongoing surveillance programs in LBMs based on environmental sampling are more likely than those based on invasive bird testing to be acceptable to traders and stall vendors. Environmental sampling is also safer for public health officers and veterinary health officers than handling and sampling live birds that may be infected with AIV.

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In this study, we aimed to identify the environmental sites commonly contaminated by AIV (H5N1) in LBMs in Indonesia. Identifying these sites is the first step in the design of evidence-based environmental sanitation, food safety, and surveillance programs to reduce the risk for virus transmission and to develop environmental surveillance programs to monitor LBM contamination status.

Methods

Three provinces in the western part of Java Island in Indonesia participated in the study: Jakarta, Banten, and West Java (Figure). Eighteen districts in these provinces were selected on the basis of their proximity to the laboratory, high levels of avian influenza activity in farmed birds (Ministry of Agriculture, unpub. data), and high number of LBMs available for study (n = 300). The required sample size was 73 markets based on an estimated disease prevalence of 50% and a maximum error of 10% at 95% confidence. We based our assumption that 50% of LBMs would be contaminated with AIV (H5N1) on results from a previous study in US LBMs in 2001 (*12*). This study found that 60% of markets tested positive for AIV (H7N2) virus in areas in which the virus was endemic. To account for nonresponse, we increased the total sample size to 83 LBMs. We selected markets for inclusion in the study using systematic sampling. On the basis of a sampling frame of 300 markets, every fourth market (the sampling interval) was selected from a list of all the markets. A random numbers table was used to determine the starting point for selection of the 83 markets from the list. Diagnostic specimens and data were collected during October 2007–March 2008. These months have high rainfall and high AIV transmission according to data gathered during 2005–2007 about AIV (H5N1) outbreaks in farmed birds (Ministry of Agriculture, unpub. data).

A structured questionnaire containing 42 questions to assess risk factors for AIV (H5N1) contamination was developed. Responses to questions were obtained through visual inspection of each LBM and through an interview with the manager of the participating LBM. The questions sought information about volume of poultry in the LBM and the infrastructure in the delivery, holding, slaughter, sale, and waste-disposal zones of the market. These 5 zones reflect general demarcation of work flow and activities relating to poultry in LBMs (*13*). Questions about the sanitation and slaughtering practices were also included.

Questionnaire validation was conducted by members of a study advisory team. The team comprised 2 food safety/environmental health officers from the Ministry of Health, a communicable disease epidemiologist from the World Health Organization, a veterinary epidemiologist from the Food and Agriculture Organization, and 2 virologists from the Ministry of Agriculture in Indonesia. The questionnaire was tested in 3 LBMs in West Java province to ensure coherence, appropriate use of terminology, and high face validity. The same markets were also inspected to ensure that the questionnaire addressed all aspects of the poultry-related work flow in the 5 poultry zones and relevant infrastructure. Members of the study advisory team trained 3 study data collection teams in questionnaire administration and sample collection procedures.

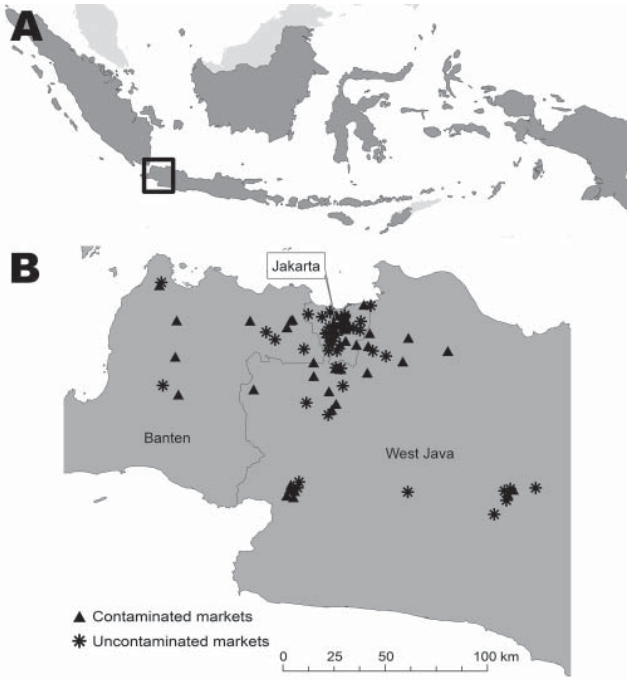

Figure. A) Area of study of avian influenza virus A (H5N1) contamination in live-bird markets (black box), western Java, Indonesia, 2007–2008. B) Distribution of contaminated and uncontaminated markets in the study area.

To select the environmental sites to be sampled in each LBM, the study advisory team visually inspected 3 markets and reviewed the literature to identify LBM sites commonly contaminated with AIVs or similar pathogens. Sites sampled in previous studies for AIV included floors, drains, and water troughs (*8*,*11*,*12*). In this study, 27 sites were selected for environmental sampling (Table 1). The sites represented different poultry-related work activities: 3 sites related to delivery of birds into LBMs, 7 in the bird-holding zone, 9 in the slaughter zone, 6 in the sale zone, and 2 in the waste-disposal zone. Because of variation in LBM infrastructure and processes, each LBM did not necessarily have all 27 sites. Samples were collected from as many of the 27 sites as were available in each LBM.

For each of the 27 sites, 6 swab specimens were collected and pooled. Each pool (vial) consisted of a maximum of 3 swabs. The data collection teams were instructed to increase the representativeness of the samples by swabbing different locations for each environmental site. For

Table 1. Environmental sites in LBMs contaminated by influenza virus A (H5N1) as detected by RT-PCR and virus isolation, Indonesia, 2007–2008*

Poultry zone	Site no.	Environmental site	RT-PCR–positive/markets tested (%), N = 1,862	VI–positive/RT-PCR positive, n = 280	LBMs positive for zone
Delivery	1	Inside cages on truck	6/45 (13.3)	1/6	11
	2	Floor in delivery area	6/49 (12.2)	0/6	
	3	Water run-off in delivery area	4/38 (10.5)	0/4	
Holding	4	Poultry cage floors	6/79 (7.6)	0/6	24
	5	Holding area floor	8/80 (10)	1/8	
	6	Water run-off	11/72 (15.3)	0/11	
	7	Poultry feeding bottle water	8/67 (11.9)	0/8	
	8	Poultry feeding basket food	6/72 (8.3)	0/6	
	9	Handles to poultry cages	9/79 (11.4)	0/9	
	10	Inside of waste bins	10/59 (16.9)	0/10	
Slaughter	11	Handles of knives used for slaughtering	8/75 (10.7)	1/8	29
	12	Basket holding dying chickens	8/71 (11.3)	2/8	
	13	Floor in slaughter area	10/77 (13)	0/10	
	14	Chopping or slaughtering board	14/71 (19.7)	2/14	
	15	Processing table after de-feathering	15/70 (21.4)	0/15	
	16	Baskets holding poultry meat	14/70 (20)	1/14	
	17	Drain path	12/75 (16)	0/12	
	18	Tap handles in slaughter area	7/65 (10.8)	0/7	
	19	Waste bin	13/71 (18.3)	1/13	
Sale	20	Chopping boards	15/80 (18.8)	1/15	30
	21	Scales	12/57 (21.1)	0/12	
	22	Knife handles	12/78 (15.4)	1/12	
	23	Waste bins	10/60 (16.7)	1/10	
	24	Wet cloths for cleaning surfaces	14/78 (17.9)	0/14	
	25	Tables for poultry display	19/80 (23.8)	0/19	
Waste disposal	26	Area waste-disposal bin	15/78 (19.2)	1/15	9
	27	Wet cleaning mops	8/66 (12.1)	0/8	
Total positive			280 (15)	13 (4.6)	

*LBM, live-bird market; RT-PCR, reverse transcription–PCR; VI, virus isolation.

example, if the market had 6 poultry stalls, each with its own scale for weighing poultry, then teams collected 1 swab from each scale and pooled them into 2 pools of 3 swabs each. Swab specimens were pooled in the market, and swabs remained inside the vials until testing. The data collection teams were instructed to focus on visibly dirty, moist, or difficult-to-clean surfaces in an effort to increase the sensitivity of the sampling.

Sample collection, pooling, transportation, and storage were based on techniques used in previous studies (*10*,*12*). Each data collection team comprised 3 persons, 2 of whom collected samples and 1 administered the questionnaire. To reduce the risk for cross-contamination during sample collection, teams changed disposable gloves and shoe covers between each of the 5 LBM poultry zones. Sterile cotton-tipped swabs were used to collect all samples, and samples were placed in viral transport media and transported immediately back to the laboratory on frozen gel packs. The viral transport media consisted of Dulbecco modified Eagle medium (Sigma-Aldrich, St. Louis, MO, USA) with 1,000 IU penicillin and gentamicin, and 1% fetal buffer serum (*14*). Samples were stored in the laboratory at –70°C until tested.

RNA extraction, cDNA synthesis, and real-time reverse transcription–PCR (RT-PCR) were used as described (*15*). Virus isolation methods have also been described (*16*) but in general involved supernatants from a 1,000-μL sample homogenized by vortex and centrifuged at 2,500–3,000 rpm into 9- to 10-day-old specific pathogen–free eggs. Those positive in the hemagglutination assay were tested by hemagglutination-inhibition test with reference antiserum (A/chicken/West Java/Hamd/2006).

The degree of association between AIV (H5N1) positivity in the 5 LBM poultry zones was determined by using Spearman rank correlation. To assess risk factors for environmental virus (H5N1) contamination, we estimated odds ratios (ORs) using multivariable logistic regression analyses, where variables with $p \leq 0.1$ from the univariate analyses were included in the initial model. A backward stepwise variable–selection strategy was used to construct a final model with a significance level of $p<0.05$. The Hosmer and Lemeshow test and the residual χ^2 goodness-of-fit test were used to assess model stability. Microsoft Excel (Microsoft, Redmond, WA, USA), Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA), and

Stata version 10.0 (StataCorp, College Station, TX, USA) were used for the descriptive and statistical analyses.

Approval for the study was obtained from the Health Research Ethics Committee at the Indonesian Ministry of Health and the Australian National University Human Research Ethics Committee. Permission was obtained from LBM managers before participation in the study.

Results

LBM Demographics and Practices

All 83 LBMs selected participated in the study; 62 (75%) were located in urban and 21 in rural areas. LBMs were from 16 districts in 3 provinces: 31 (38%) from Jakarta province, 11 (13%) from Banten province, and 41 (49%) from West Java province (Figure). Most (49 [59%]) LBMs were retail markets, 10 (12%) were wholesale only, and 24 (29%) were a combination of retail and wholesale. Most (82 [99%]) LBMs operated daily, with the same vendors operating in the same stalls.

Most LBMs received their poultry from commercial farms (71 [86%]), and some also sourced poultry from small-scale holders (36 [43%]). Most (42 [51%]) LBMs had medium-sized poultry areas (11–50 poultry cages), and 21 (25%) had large poultry areas (>50 cages). LBMs had village free-ranging chickens (69 [83%]), fighting cocks (13 [16%]), broilers (67 [81%]), spent hens (24 [29%]), Muscovy ducks (48 [58%]), ducks other than Muscovy (32 [39%]), and pigeons (16 [19%]). Most (71 [86%]) LBMs generally kept live poultry in the market for a few days until sold, housing them overnight in cages.

Forty-eight (58%) LBMs reported monthly or more frequent visits from animal/human health personnel to inspect the poultry zones. Eight (10%) LBMs reported that live birds were tested periodically (less frequently than weekly) for AIV infection. For cleaning and sanitation, 80 (96%) LBMs reported washing poultry zones daily, and 55 (66%) applied detergent or disinfectant daily.

Laboratory Findings

Thirty-nine (47%) LBMs had evidence of contamination. For 17 (44%) of these, ≤5 environmental sites were positive for AIV (H5N1) by real-time RT-PCR. For each of 22 (56%) LBMs, ≥6 environmental sites were positive.

The environmental sites most heavily contaminated were in the slaughter and sale zones (Table 1). In the slaughter zone, the most contaminated sites were the poultry-processing tables (21%), baskets holding poultry meat (20%), and chopping boards (20%). In the sale zone, the most contaminated sites were the tables for carcass display (24%) and scales (21%). Another commonly contaminated site was the waste-disposal bin in the waste-disposal zone (19%). In most cases, this bin is not an enclosed bin but rather was a dedicated uncovered floor space where remnants are dumped daily and collected weekly by the local government rubbish collection team.

Thirteen viruses were isolated from LBMs, most frequently from the slaughter zone (7 of 13 viruses isolated, Table 1). All isolated viruses came from 6 LBMs, from which 1–4 viruses were isolated per LBM.

From the zones contaminated in each LBM (Table 1), we calculated correlations between different zones. Contamination in preceding LBM poultry zones correlated with contamination in the subsequent zones (Table 2). Correlations were high between holding and slaughter zones, slaughter and sale zones, and sale and waste-disposal zones.

Risk Factors for Contamination

We assessed risk factors for AIV (H5N1) contamination in LBMs. We compared exposures in 39 LBMs with a minimum of 1 contaminated environmental site to 44 LBMs with no contamination. From the univariate analyses, several exposures predicted AIV (H5N1) contamination in LBMs (Table 3). LBMs with wooden tables, Muscovy ducks, or ≥200 ducks other than Muscovy were at greater risk for AIV (H5N1) contamination, as were LBMs in West Java province.

Six other exposures approached significance, either as protective factors or as risk factors. LBMs that disposed and removed solid waste daily (OR 0.41, 95% confidence interval [CI] 0.16–1.09); had zoning that clearly segregated poultry delivery, holding, slaughter, sale, and waste-disposal areas (OR 0.28, 95% CI 0.07–1.11); or stacked poultry cages vertically rather than side by side (OR 0.38, 95% CI 0.13–1.10) had less risk for avian influenza virus (H5N1) contamination. LBMs with pigeons (OR 3.06, 95% CI 0.96–9.81), mixed bird species in the same cages (OR 2.92, 95% CI 0.98–8.70), or slaughtered birds in the market (OR 3.53, 95% CI 0.89–13.93) were more likely to be contaminated.

None of the 9 other variables considered in the study were associated with AIV (H5N1) contamination in LBMs (data not shown). These included the LBM trading category (wholesale, retail, or combination), days operational per week, chicken population in LBM, source of chickens (small-scale backyard farmers, commercial farms, or com-

Table 2. Correlation coefficient of influenza virus A (H5N1) positivity between 5 poultry zones in live-bird markets, Indonesia, 2007–2008

Site	Delivery	Holding	Slaughter	Sale	Waste disposal
Delivery	1				
Holding	0.84	1			
Slaughter	0.82	0.89	1		
Sale	0.63	0.84	0.87	1	
Waste disposal	0.50	0.26	0.52	1	1

Table 3. Comparison of exposures in LBMs with AIV (H5N1) environmental contamination and in LBMs with no environmental AI (H5N1) contamination, Indonesia, 2007–2008*

Exposure	No. positive markets, n = 39	No. negative markets, n = 44	OR (95% CI)	p value
No. ducks other than Muscovy in LBM				
<11	8	11	Reference group	
11–100	12	16	1.03 (0.32–3.35)	0.959
101–200	2	2	4.13 (0.16–11.95)	0.773
>200	10	2	6.88 (1.17–40.38)	0.033
Muscovy ducks	28	20	3.05 (1.22–7.63)	0.017
Pigeons	11	5	3.06 (0.96–9.81)	0.059
Clear zoning in LBM	3	10	0.28 (0.07–1.11)	0.072
Wooden tables	23	12	3.83 (1.53–9.62)	0.004
Slaughtering in LBM	36	34	3.53 (0.89–13.93)	0.072
Daily solid waste disposal	24	35	0.41 (0.16–1.09)	0.075
Mixing of species in same cage	13	6	2.92 (0.98–8.70)	0.055
Cages stacked vertically	25	33	0.38 (0.13–1.10)	0.069
Province				
Jakarta	23	8	Reference group	
West Java	25	16	4.49 (1.62–12.46)	0.004
Banten	6	5	3.45 (0.82–14.47)	0.090
Multivariable analysis†				
Clear zoning in LBM			0.16 (0.03–0.86)‡	0.030
Slaughtering in LBM			6.43 (1.01–40.82)‡	0.048
Daily solid waste disposal			0.20 (0.06–0.69)‡	0.010
Province				
Jakarta			Reference group	
West Java			6.83 (2.01–23.19)‡	0.002
Banten			2.94 (0.59–14.69)‡	0.190

*LBM, live-bird market; AI, avian influenza; OR, odds ratio; CI, confidence interval.
†Final model with 4 variables, no. observations = 83, goodness-of-fit tests: residual χ^2, p = 0.38; Hosmer and Lemeshow test, p = 0.45.
‡Adjusted OR.

bination), inspection from authorities, use of detergent during cleaning, mixing poultry arriving on different days in the same cages, average length of poultry stay in LBM, and whether poultry were removed from stalls before cleaning.

From the univariate analyses, 10 variables were significant at $p \leq 0.1$. However, the ducks other than Muscovy variable was removed from the multivariate analyses because of its collinearity with another variable (presence of Muscovy ducks, $r > 0.4$). Nine variables were considered for the multivariate analyses. The final multivariable logistic regression model had 4 variables, of which 2 were independent risk factors for subtype H5N1 contamination in LBMs (Table 3). They were location in West Java province (adjusted OR [aOR] 6.83, 95% CI 2.01–23.19) and bird slaughtering in the LBM (aOR 6.43, 95% CI 1.01–40.82). Two variables were independent protective factors: zoning of poultry activities in LBMs (aOR 0.16, 95% CI 0.03–0.86) and daily disposal of solid waste (aOR 0.2, CI 95% 0.06–0.69).

Discussion

We have demonstrated extensive environmental contamination in LBMs with the AIV (H5N1) in Indonesia. Nearly 50% of LBMs in AIV (H5N1)–endemic districts were positive, with all 5 poultry zones affected. The study identified environmental points of contamination and protective and risk factors for contamination. This study provides baseline information for 2 aspects that can aid in control of AIV (H5N1) in LBMs: 1) development of routine monitoring and surveillance programs and 2) structural interventions and work flow modifications to minimize risk for contamination.

Our findings provide further evidence that environmental contamination with AIVs is not uncommon (*8*,*14*). Poultry water, drains, tabletops, cages, tablecloths, utensils, bins, and floors were all contaminated. Environmental sites most commonly contaminated were located in slaughter zones and zones where carcasses were taken after slaughtering, such as the sale and waste-disposal zones. This contamination can be expected because slaughtering generates droplets that may contain viral particles and exposes internal organs with potentially high viral loads. Even if slaughtering is conducted in a separate zone, contamination can spread to the sale and waste-disposal zone through the carcasses and through the process of evisceration usually conducted in both slaughter and sale stalls.

We found rates of contamination in water from poultry feeding bottles similar to those from the study in Hong

Kong on AIV (H9N2) (11% and 7% markets with contamination respectively, p = 0.12) (*11*). Even though AIVs were detected from poultry drinking water, our study suggests that other environmental sites are more efficient for monitoring AIV (H5N1) in markets. Processing tables and baskets holding freshly cut poultry meat in the slaughter area, as well as display tables and scales in the sale area, were positive in 20 (24%) LBMs surveyed.

The risk and protective factors we identified complement findings from previous studies. Daily disposal and removal of waste from the market is part of routine environmental cleaning and sanitation and eliminates AIV reservoirs (*8*). Segregating poultry-related activities into zones limits virus spread (*17*). Vertical stacking of cages can limit transmission because trays between layers of birds prevent the scatter of fecal matter. These results add evidence to the World Health Organization current recommendation that waste trays should be used to segregate stacked cages in markets to prevent cross-contamination (*13*).

LBMs in West Java province had a higher risk for contamination than did other provinces. This risk probably is due to greater AIV (H5N1) disease activity in the province. Surveillance activities during 2006–2008 showed that West Java had a 4.7% outbreak detection rate compared with rates in Banten (4%) and Jakarta (0.2%) (*18*). Furthermore, in West Java province chicken density is high: 14,000 birds/km^2 compared with densities in the neighboring provinces Banten and Jakarta (3,900 birds/km^2 and 400 birds/km^2, respectively) (*19*). Poultry density data are commonly used as a proxy for disease activity where areas of high poultry density have the highest risk for an outbreak (*20*,*21*).

Several issues need to be considered regarding our finding of low virus isolation rates compared with real-time RT-PCR–positive rates. Virus isolation detects viable virus, whereas real-time RT-PCR detects small stretches of nucleic acid, even if the larger genomic RNA is inactivated. This makes real-time RT-PCR a more sensitive detection tool but does not provide information about virus viability. Samples obtained from the environment may be less suitable than animal samples for virus isolation techniques. Organic matter, duration and temperature of exposure, and humidity can all affect virus survival outside the animal host (*22*). Three studies conducted in LBMs tested environmental samples and bird samples by using virus isolation (*8*,*10*,*23*). Only 1 of these studies stratified the avian influenza detection rates by type of sample (bird vs. environment) (*8*); that study found that from 12 LBMs, 11 were positive for avian influenza in bird samples compared with only 5 positive in environmental samples. These results were based on a small sample of LBMs, and real-time RT-PCR was not conducted. Therefore, to determine the suitability of virus isolation for environmental samples, we recommend that future studies compare real-time RT-PCR–positive rates to virus isolation rates in both environmental swab and bird samples.

Risk and protective factors identified in this study, together with findings from other studies, can assist in developing environmental or behavioral interventions to reduce AIV transmission in LBMs. Previous studies have shown that regular cleaning with detergents, including free chlorine concentrations typically used in drinking water treatment, can rapidly decontaminate surfaces from AIVs (*8*,*24*). Previous studies also have shown that periodic market rest days coupled with thorough cleaning can minimize the reservoir of AIV in LBMs (*4*,*12*,*25*). These messages have been disseminated to LBMs throughout Indonesia and formed the basis of the Ministry of Health Decree in 2008 on building healthy food markets (*26*).

For a more systematic food safety monitoring system, this study will be used to develop a risk-based approach for AIV risk reduction in LBMs in Indonesia (*27*). The contamination sites and risk factors will be used to determine critical control points and critical limits for intervention. LBM operators, stall vendors, and other stakeholders (e.g., sanitarians and public health officers) will need to be provided with simple monitoring plans to reduce the risk for contamination. Such monitoring plans are expected to have an impact not only on AIV (H5N1) but also on other viruses and bacteria commonly associated with food safety for poultry products.

In addition to tools for disease control, the study findings can aid AIV (H5N1) surveillance activities in LBMs. Commonly contaminated environmental sites in LBMs can form the basis of an environmental sampling strategy for detection of AIV (H5N1) in LBMs. Environmental sampling is more beneficial than live-bird sampling because it is less time and labor intensive and eliminates the need to handle and restrain live birds. Environmental sampling reduces the potential for virus aerosolization and the risk for infection for persons collecting the samples or standing nearby. Further work is needed to assess the adequacy of environmental sampling for surveillance in LBMs under different conditions, especially because detection sensitivity will vary by AIV (H5N1) prevalence in farms supplying the birds.

A limitation of this study is that the observation of environmental contamination was based on a cross-sectional survey in which LBMs were sampled only once. We recommend that future studies observe persistence of the virus over time in the various environmental sites. Reports from market managers and vendors about inspection and cleaning practices in the LBMs were not verified during the course of the study. These activities may have been overreported because respondents may have wanted to report what they perceived interviewers wanted to hear. Be-

cause of the high cost associated with the field and laboratory work for such studies, studies should focus on a small number of markets and collect in-depth information about contamination trends and associated risk factors, as well as data on other indicator organisms, such as *Escherichia coli* or *Enterobacteriaceae*, that provide information about general market hygiene. Future work also should evaluate the effects of interventions in markets especially in low-resource settings because this would be of most benefit to low-income and middle-income countries.

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Surveillance and Analysis of Avian Influenza Viruses, Australia

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We investigated carriage of avian influenza viruses by wild birds in Australia, 2005–2008, to assess the risks to poultry industries and human health. We collected 21,858 (7,357 cloacal, 14,501 fecal) samples and detected 300 viruses, representing a detection rate of ≈1.4%. Rates were highest in autumn (March–May) and differed substantially between bird types, areas, and years. We typed 107 avian influenza viruses and identified 19 H5, 8 H7, and 16 H9 (40% of typed viruses). All were of low pathogenicity. These viruses formed clearly different phylogenetic clades to lineages from Eurasia or North America, suggesting the potential existence of Australian lineages. H7 viruses were similar to highly pathogenic H7 strains that caused outbreaks in poultry in Australia. Several periods of increased detection rates (numbers or subtypes of viruses) were identified. This study demonstrates the need for ongoing surveillance to detect emerging pathogenic strains and facilitate prevention of outbreaks.

Shorebirds (Charadriiformes) and wild waterfowl (Anseriformes) represent the major natural reservoirs of avian influenza viruses (AIVs). These birds can carry all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes (*1*); the viruses typically cause asymptomatic infections in these hosts. Studies in Europe and North America demonstrated the following: AIV carriage is highest in autumn but may also be high in spring; prevalence among shorebirds and ducks is increased during their northward and southward migrations, respectively; and distribution, prevalence, and subtypes involved vary from year to year (*2*,*3*). Interspecies transmission of AIV in several species of wild birds has been documented; however, the most frequent adaptation of these viruses occurs in domestic gallinaceous poultry.

The respiratory tract of poultry and gastrointestinal tract of waterfowl are replication sites for AIVs, and poultry are incubators for the progression of low-pathogenicity avian influenza (LPAI) virus into highly pathogenic avian influenza (HPAI) virus (*4*–*6*), usually through the acquisition of polybasic amino acids at the HA cleavage site. HPAI, particularly HPAI (H5N1), may induce up to 100% deaths in poultry and cause substantial economic losses (*4*,*7*–*9*). Strains that are highly pathogenic in gallinaceous species may cause a range of clinical signs in other avian species, from mild illness to highly contagious and fatal disease. H5 and H7 AIVs have the propensity to become HPAI and thus are a significant risk to the poultry industry. These subtypes and H9 have also caused disease and death in humans. Subtype H5N1 first caused outbreaks in wild migratory waterfowl in the People's Republic of China in 2002 and in domestic poultry in Hong Kong Special Administrative Region, China, in 2003 (*10*). The World Health Organization has since confirmed 433 human cases of avian influenza (H5N1) with 262 deaths (*11*).

AIVs may be transported by infected migratory birds (*12*–*14*). Shorebirds and waterfowl usually survive infection, and transmission by migratory waterfowl over long distances within Asia and between continents has been documented (*15*–*17*). Nevertheless, the role of migratory

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birds in the distribution and transmission of AIVs remains controversial. Managing the potential threat of transport of AIVs by wild birds requires appropriate surveillance programs that assess the occurrence, subtypes, and pathogenicity of isolates that the birds carry.

Australia is isolated by sea, and shorebirds make up the majority of long-distance migratory birds that visit the continent (3 million/year [*12*]). These birds breed in Siberia (May–July) and stop off throughout Asia (April–May, July–September) in areas where HPAI (H5N1) epizootics have recently occurred (e.g., Vietnam, Thailand, Hong Kong, China, Indonesia) (*18*). Most arrive in Australia in spring (August–September) and depart in autumn (March). Shorebirds are known to carry a variety of AIVs, including subtype H5N1 (*1*). Wild waterfowl, such as ducks, geese and swans are common in Australia. However, they do not migrate out of Australia in large numbers, although they do undertake intracontinental movements and occupy the same habitats as migratory shorebirds. Collectively, these factors provide an environment that allows the assessment of the import of AIVs by migratory birds and transmission to, and distribution by, local waterfowl.

Until recently, only small and historical studies of AIVs have been undertaken in Australia (*14*,*19*–*22*). No outbreaks of HPAI H5 viruses have been identified, despite the close proximity of Indonesia, where AIV (H5N1) is endemic and outbreaks frequently occur. Five outbreaks of HPAI have occurred in Australia; all outbreaks were caused by H7 viruses. In all cases of disease, transmission of LPAI H7 from wild birds and subsequent mutation to HPAI after serial passage in chickens was considered the probable source (*13*,*23*,*24*). Nevertheless, the source of infection in wild birds has not been identified. Therefore, surveillance for AIVs is needed in Australia in localities where large numbers of migratory shorebirds and waterfowl occur in close proximity to poultry operations (*13*). We examined the occurrence and subtypes of AIVs carried by migratory shorebirds and waterfowl in southeast Australia over a 4-year period.

Methods

Sample Collection

Sampling site selection (2005–2008) was based on abundance of migratory shorebirds, risk for transmission to waterfowl inhabiting the same area, proximity of commercial poultry, and human population density. Areas around Newcastle and Orange, New South Wales (*13*), and Melbourne, Victoria, were selected. Samples were also collected opportunistically from other locations (Figure 1). Sites were generally inland swamps or coastal wetlands.

Most samples were collected from coastal wetlands or inland swamps around Newcastle and Orange, New South Wales, and Melbourne, Victoria, with small numbers from other sites (Figure 1). Samples from Tasmania were included with samples from Victoria for analysis. Coastal New South Wales and Victoria samples were from sites co-inhabited by large numbers of migratory shorebirds and waterfowl. A total of 21,858 samples were collected and tested during 2005–2008 (Table 1). Of these, 10,003 were from migratory shorebirds, 10,231 from waterfowl, and 1,624 from other bird species. Samples from other bird species were from birds trapped incidentally or were collected opportunistically. In most instances, the species, or pairs of sister species (e.g., grey/chestnut teal, bar-tailed/black-tailed godwit), that produced the feces collected were known. Identification of species sampled was identified by observing the bird, the bird's footprints, and the size and shape of feces.

Samples were fresh feces or cloacal swabs (*14*). Fecal samples were collected from roosting or feeding flocks, and the species involved was recorded. Cloacal samples were collected from birds captured by cannon netting, funnel traps, and hand-held nets, and from ducks shot for recreation,

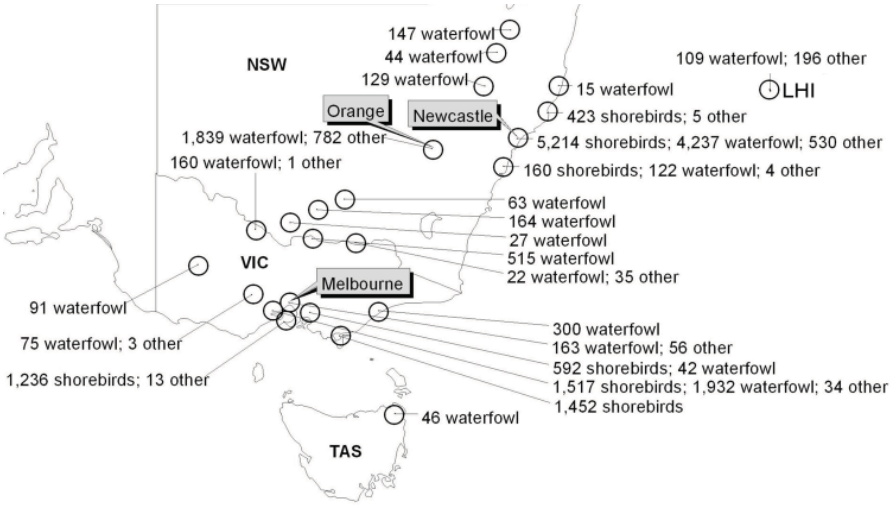

Figure 1. Sampling sites for avian influenza in Australia. Most avian fecal and cloacal samples were collected from wetlands in coastal and inland New South Wales (NSW) or around Melbourne, Victoria (VIC), with minor sampling sites around Old Bar, Sydney, and Albury, NSW; Lord Howe Island (LHI); and northeastern Tasmania (TAS). Shorebirds refers to migratory shorebirds only.

damage mitigation, or conservation. Samples were placed in phosphate-buffered gelatin saline or brain-heart-infusion broth base, each containing penicillin (2×10^6 IU/L), streptomycin (0.2 mg/mL), gentamicin (0.5 mg/mL), and amphotericin B (500 U/mL), and transported chilled to our laboratories at the University of Newcastle, the Department of Primary Industries or the Orange Agricultural Institute for storage at –80°C until analysis (Figure 2).

PCR Detection of AIVs

Viral RNA was extracted according to manufacturers' instructions by using MagMax96 viral RNA (Life Technologies, Scoresby, Victoria, Australia), or RNeasy isolation kits (QIAGEN, Doncaster, Victoria, Australia). AIVs were detected by real-time quantitative reverse transcription–PCR (qRT-PCR) by using the conserved matrix gene as the amplification target (*25*,*26*). Influenza A–positive samples

Table 1. Summary of numbers and bird types sampled and PCR-positive rates of avian influenza viruses detected, by month and state, New South Wales and Victoria, Australia, 2005–2008*

Bird type and month	New South Wales			Victoria			Total		
	No. samples†	Pos	PPR, %	No. samples†	Pos	PPR, %	No. samples†	Pos	PPR, %
Shorebirds									
Jan	704 (5)	1	0.14	206 (206)			910	1	0.11
Feb	1,087 (8)	2	0.18	901 (901)	7	0.78	1,988	9	0.45
Mar	939 (2)	2	0.21				939	2	0.21
Apr	328	6	1.80				328	6	1.80
May	163	5	3.10				163	5	3.10
Jun	217	4	1.80				217	4	1.80
Jul	101	1	0.99				101	1	0.99
Aug	126						126		
Sep	529	1	0.19				529	1	0.19
Oct	586			404 (404)	7	1.70	990	7	0.71
Nov	694 (7)	10	0.76	627 (627)			1,321	10	0.76
Dec	323			2,068 (2,068)	5	0.24	2,391	5	0.21
Total	5,797	32	0.55	4,206	19	0.45	10,003	51	0.51
Waterfowl									
Jan	265	4	1.50	406	23	5.70	671	27	4.00
Feb	393	9	2.30	265	2	0.75	658	11	1.70
Mar	591 (51)	14	2.40	836 (836)	44	5.30	1,427	58	4.10
Apr	836 (80)	25	3.00	241	5	2.10	1,077	30	2.80
May	1,262 (232)	18	1.40	175	10	5.70	1,437	28	1.90
Jun	740 (241)	10	1.40	14			754	10	1.30
Jul	525 (127)	13	2.50	4			529	13	2.50
Aug	496 (69)	10	2.00				496	10	2.00
Sep	596 (111)	25	4.20	5	1	20.0	601	26	4.30
Oct	381 (97)	2	0.50	329	9	2.70	710	11	1.50
Nov	770 (513)	6	0.80	366	6	1.60	1,136	12	1.00
Dec	578 (89)	10	1.70	157	1	0.64	735	11	1.50
Total	7,433	146	2.00	2,798	101	3.60	10,231	247	2.40
Other									
Jan	62 (12)			1 (1)			63		
Feb	198 (1)			10 (10)			208		
Mar	35 (27)			1 (1)			36		
Apr	314 (12)	1	0.40				314	1	0.30
May	159 (60)						159		
Jun	72 (44)						72		
Jul	17 (6)			3 (3)			20		
Aug	107 (28)						107		
Sep	133 (118)						133		
Oct	196 (149)			1 (1)			197		
Nov	259 (154)			23 (23)			282		
Dec				33 (33)	1	3.00	33	1	3.00
Total	1,552	1		72	1	1.40	1,624	2	0.10
Total for all birds	14,782	182	1.32%	7,076	121	1.70	21,858	300	1.40

*Shorebirds refers to migratory shorebirds only. Sampling periods were defined as calendar months. Pos, number of PCR-positive results; PPR, PCR-positive rate.
†Numbers in parenthesis indicate the numbers of cloacal samples; the remainder were fecal samples.

were tested by using specific primers targeting H5 and H7 subtypes (*26*). Proportion tests (Pearson χ^2 statistics in R [*27*]) were used to test differences in influenza A–positive by PCR rates according to season.

AIV Subtype Determination

To determine AIV subtypes, HA2 and NA genes were amplified by conventional PCR and sequenced (*14*). Sequences were compared with known sequences by BLAST search (www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) to determine subtype and relatedness to other viruses. For H5, H7, and H9 subtypes, the full HA genes were sequenced and HA cleavage sites were assessed to determine potential pathogenicity (*14*).

Phylogenetic Analysis

Phylogenetic trees were constructed for H5, H7, and H9 viruses by comparison of the relatedness of the subtypes isolated in this study with those from other geographic locations (Figure 3). HA genes of 10 H5, 3 H7, and 8 H9 viruses from this study were compared with those of representative subtypes of major AIV lineages from GenBank. Sequences were assembled and edited with SeqMan, DNASTAR Lasergene 8. Geneious (Biomatters Ltd, Auckland, New Zealand) and Se-Al (http://evolve.zoo.ox.ac.uk/) were used for alignment. MRMODELTEST 2.2 (www.abc.se/~nylander/) was used to determine the appropriate DNA substitution model and γ-rate heterogeneity. The best-fit model was used to generate neighbor-joining trees by using PAUP* 4.0 (*28*). Only strains from which a full HA gene sequence was obtained were included. Estimates of phylogenies were calculated from 1,000 neighbor-joining bootstrap replicates.

Results

AIVs Detected

Three hundred AIVs were detected by qRT-PCR, representing a total PCR-positive detection rate of 1.4% ,of which 51 (17%) were detected in migratory shorebirds (including 16 bar-tailed godwits, 14 red-necked stints, 11 eastern curlews, and 7 red knots) and 247 in waterfowl (including 224 dabbling ducks), corresponding to rates of 0.51% and 2.4%, respectively (Table 1). Two viruses were detected in other birds (Eurasian coot and whiskered tern). *Numenius* spp. waders (predominantly eastern curlew, 11/690, 1.6%) were the most common shorebird carriers. Dabbling ducks had slightly higher detection rates (224/7,607, 2.9%) compared with all waterfowl.

PCR-positive Samples

PCR-positive detection rates were similar for migratory shorebirds (0.55% vs. 0.45%) and waterfowl (2.0% vs. 3.6%) between New South Wales and Victoria (Table 1). Rates were highest in autumn and early winter (April–June, $\chi^2 = 18.0$, degrees of freedom [df] = 3, p = 0.0004) in migratory shorebirds, and in autumn (April–May, $\chi^2 = 11.2$, df = 3, p = 0.01) in waterfowl (Figure 4). Rates were similar for different years: migratory shorebirds 0.65% in 2005, 0.50% in 2006, 0.46% in 2007, and 0.72% in 2008; and waterfowl 2.7% in 2006, 2.6% in 2007, and 3.7% in 2008. However, rates differed substantially for different bird types, areas, and years, which could explain the high variability observed in seasonal trends (Figure 4). For example, the rate for migratory shorebirds in coastal New South Wales in 2008 (0.72%, mostly bar-tailed godwit and eastern curlew) was double that in Victoria in 2006 (0.38%), rates for waterfowl in Victoria (4.8%, mostly in Pacific black duck) in 2008 were almost double those in 2007 (2.8%), and rates for dabbling ducks in Victoria in 2008 (6.3%) were 3-fold greater than in coastal New South Wales in 2006 (1.9%). Rates were generally similar for different years for migratory shorebirds sampled in New South Wales and Victoria; however, rates reached 5.2% for waterfowl (mostly grey and chestnut teal) in March 2006 in Victoria, compared with 3.6% at other times.

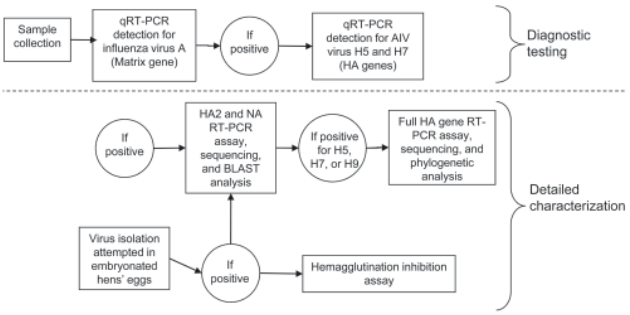

Figure 2. Procedures followed in avian influence surveillance and analysis, Australia, 2005–2009. qRT-PCR, real-time quantitative reverse transcription–PCR; AIV, avian influenza virus; HA, hemagglutinin; NA, neuraminidase; BLAST, BLAST analysis (http://blast.ncbi.nlm.nih.gov).

AIV Ecology

It was possible to subtype 107/300 (36%) AIVs detected by qRT-PCR (Table 2; online Technical Appendix Tables 1–3, www.cdc.gov/EID/content/16/12/1876-Techapp.pdf). It was not possible to subtype all AIVs because the conventional PCR used for subtyping was not as sensitive as the surveillance qRT-PCR. Notably, 19 H5, 8 H7, and 16 H9 AIVs were identified. No H5 or H7 AIVs contained multiple basic cleavage sites, a known molecular determinant for HPAI; therefore, all were classified as LPAI. H5, H7, and H9 subtypes represented a high proportion (43/107, 40%) of all viruses subtyped. H9 subtypes were the most common viruses identified in migratory shorebirds (5/11,

45%). H3 and H5 viruses were the most common subtypes identified in waterfowl (21/96, 22%, and 18/96, 19%, respectively). One H5 and 5 H9 AIVs were detected in migratory shorebirds, 1 H9 was from a black swan, and the remainder (18 H5, 8 H7, and 10 H9) were from dabbling ducks. In PCR-positive samples for which NA subtype was determined, we detected 1 N1, 4 N3, 1 N5, 5 N6, 4 N7, and 1 N9 (online Technical Appendix Table 1). The NAs associated with H5, H7, and H9 viruses were of the following subtypes: H5N3 (1), H5N7 (2), H7N1 (1), H7N6 (2), and H7N7 (1).

The detection of PCR-positive samples was sporadic and was increased in some periods, particularly in ducks, in which larger numbers of AIVs were identified at the same time and location (online Technical Appendix Table 4). During periods of increased detection, rates of up to 6.2% and 12.3% were found for migratory shorebirds and waterfowl, respectively. These events occurred throughout the year but were more common in autumn (March–May) and early spring (September). Some evidence showed different seasonal increases in rates for particular subtypes of AIVs. Twenty of 23 H3 subtypes were primarily detected from

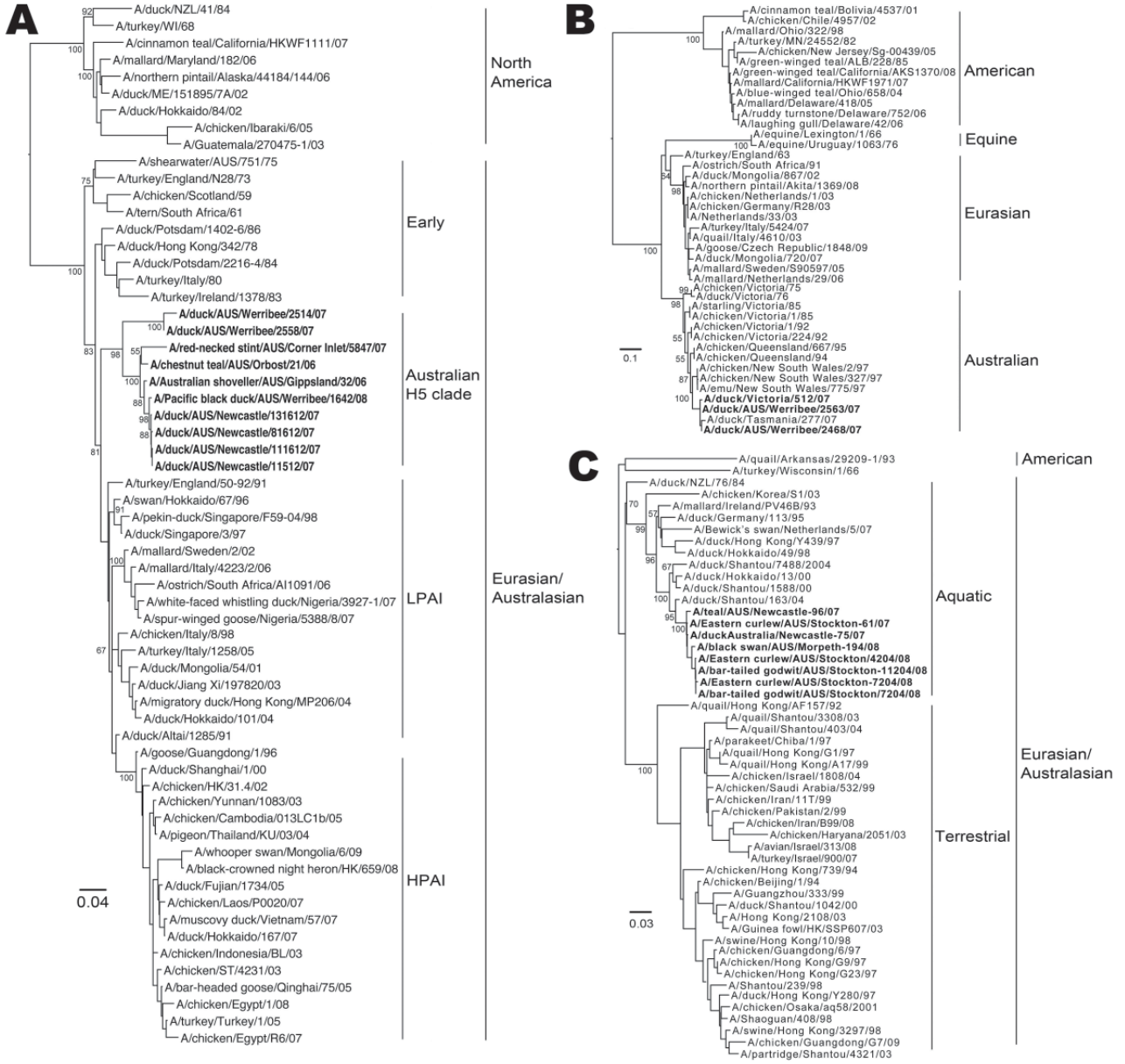

Figure 3. Phylogenetic analysis of avian influenza viruses from Australia. Viruses were subtyped, and hemagglutinin genes from subtypes H5 (A), H7 (B), and H9 (C) were sequenced (**boldface**) and compared with isolates from other geographic locations. Only bootstrap values >50 are shown. Scale bar indicates nucleotide substitutions per site. All sequences were submitted to GenBank (accession numbers pending). LPAI, low-pathogenicity avian influenza; HPAI, highly pathogenic avian influenza.

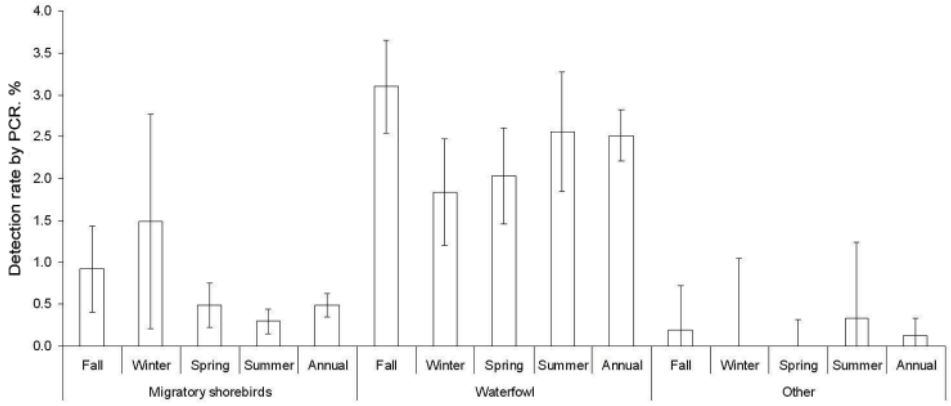

Figure 4. PCR-positive rates of avian influenza virus by season and species. Error bars indicate 95% confidence intervals.

autumn to early spring (March–September), whereas 16/19 H5 and 7/8 H7 subtypes were detected from late spring to early autumn (November–March), and all 16 H9 subtypes were detected in autumn (March–May). Most H3 (13/23), H5 (12/19), and H7 (5/8) strains were detected in 2007, whereas half (8/16) of the H9 strains were from 2007 and half were from 2008. Notably, 8 H5 viruses were identified in summer (December–February) in New South Wales in 2007–2008, and only 1 strain of a different subtype (H4) was identified during this period. Also, notable increases in detection rates of H9 subtypes occurred in New South Wales in autumn in 2007 (April–May) and 2008 (March–April). In addition, rates involving numerous different subtypes increased on 3 occasions: 2 H3, 3 H5, 1 H11, 1 H12, from dabbling ducks, Victoria, March 2006; 1 H2, 1 H4, 2 H5, 2 H7, 1 H8, from ducks, Victoria, January 2007, and 3 H2, 6 H3, 1 H4, 1 H5, 1 H10, mostly from teal, New South Wales, August–September 2007. However, these increases may be the result of the large numbers of samples collected on these dates (365, 341, and 504, respectively).

Increased detection rates of individual AIV subtypes were generally localized because the same subtypes were only identified at the same time from different sites on 3 occasions (online Technical Appendix Table 1): 1 H12, New South Wales, and 1 H12, Victoria, in March 2006; 2 H9, coastal New South Wales, and 3 H9 inland in May 2007; and 2 H11, New South Wales, and 2 H11, Victoria, in May 2008. Some evidence for cross-species infection was found with the same subtypes of virus identified in different species at the same time and location: 2 H9 in both bar-tailed godwits and eastern curlews in April 2008 in New South Wales. Full HA sequences for the 4 strains demonstrated >99.6% nt similarity; the 2 strains from the bar-tailed godwits showed 100% nt homology (Figure 3). Limited evidence was shown for the cross-species infection of migratory shorebirds and waterfowl, with the same subtype isolated from each group on just 2 occasions, both involving H9 viruses in New South Wales: 1 H9 from an eastern curlew and 1 H9 from a duck in May 2007 and H9s from 2 bar-tailed godwits, 2 eastern curlews, 1 black swan, and 1 chestnut teal, all in April 2008. The H9 strains from the bar-tailed godwit and eastern curlew had a >98.8% nt similarity to the H9 strain from the black swan (Figure 3). Sequence data were not available for the duck or chestnut teal AIVs.

Phylogenetic Analysis

All H5 viruses detected in this study clustered closely together and were clearly divergent from other LPAI H5 viruses from Eurasia and North America (Figure 3, panel A). Both the Australian and the Eurasian lineages appear to have evolved from an early lineage of H5 viruses that includes a range of strains from 1959 through 1986. The H7 strains identified in this study have a close genetic relationship with HPAI H7 viruses previously isolated in Australia during 1975–1997 and as a group are clearly distinct from Eurasian and North American H7 lineages (Figure 4, panel B). Notably, all Australian H7 viruses were closely related to the strains that caused pathogenic outbreaks in poultry in Australia and thus may identify a potential environmental source of these viruses. Eurasian H9 strains have evolved into 2 discrete lineages that are carried by aquatic or terrestrial birds (Figure 3, panel C). The Australian H9 strains detected in this study again grouped closely and, as a lineage, diverged from the Asian aquatic H9 viruses but were distinct from Eurasian and North American lineages. Although the Australian H9 viruses were a less discrete lineage than H5 and H7 viruses, the maximum bootstrapping value (100) confirmed that they formed their own distinct lineage. Taken together, these results indicate that the viruses within each subtype in Australia are closely related and form Australian-specific lineages that are distinct from other lineages.

Discussion

This large surveillance effort for AIVs in Australia longitudinally and geographically characterized the extent and profile of AIVs in wild birds. We detected 300 AIVs from ≈22,000 samples tested and subtyped 107 of these.

Table 2. Hemagglutinin subtypes of avian influenza viruses detected, by month and state, New South Wales and Victoria, Australia, 2005–2008

Species and month	New South Wales	Victoria	Total
Shorebirds*			
Feb		1 H5	1 H5
Apr	4 H9		4 H9
May	2 H3, 1 H9		2 H3, 1 H9
Nov	1 H12	1 H6	1 H6, 1 H12
Dec		1 H4	1 H4
Total	2 H3, 5 H9, 1 H12	1 H4, 1 H5, 1 H6	2 H3, 1 H4, 1 H5, 1 H6, 5 H9, 1 H12
Waterfowl			
Jan	1 H5	1 H2, 2 H4, 2 H5, 2 H7, 1 H8	1 H2, 2 H4, 3 H5, 2 H7, 1 H8
Feb	1 H5, 3 H7		1 H5, 3 H7
Mar	1 H3, 2 H9, 1 H12	3 H3, 5 H5, 1 H11, 2 H12	4 H3, 5 H5, 2 H9, 1 H11, 3 H12
Apr	5 H3, 1 H4, 1 H6, 5 H9		5 H3, 1 H4, 1 H6, 5 H9
May	1 H3, 1 H5, 1 H6, 4 H9, 2 H11	2 H11	1 H3, 1 H5, 1 H6, 4 H9, 4 H11
Jun	1 H1, 2 H4, 1 H5,		1 H1,2 H4, 1 H5
Jul	3 H1, 1 H4, 3 H8, 1 H11, 1 H12		3 H1,1 H4, 3 H8, 1 H11, 1 H12
Aug	3 H2, 3 H3		3 H2, 3 H3
Sep	5 H3, 1 H4, 1 H5, 1 H7, 3 H8, 1 H10		5 H3, 1 H4, 1 H5, 1 H7, 3 H8, 1 H10
Oct		1 H3, 1 H8	1 H3, 1 H8
Nov	1 H3	2 H7	1 H3, 2 H7
Dec	1 H3 6 H5, 1 H8	1 H1	1 H1, 1 H3, 6 H5, 1 H8
Total	4 H1, 3 H2, 17 H3, 5 H4, 11 H5, 2 H6, 4 H7, 7 H8, 11xH9, 1 H10, 3 H11, 2 H12	1 H1, 1 H2, 4 H3, 2 H4, 7 H5, 4 H7, 2 H8, 3 H11, 2 H12	5 H1, 4 H2, 21 H3, 7 H4, 18 H5, 2 H6, 8 H7, 9 H8, 11 H9, 1 H10, 6 H11, 4 H12
Total for all birds	4 H1, 3 H2, 19 H3, 5 H4, 11 H5, 2 H6, 4 H7, 7 H8, 16 H9, 1 H10, 3 H11, 3 H12	1 H1, 1 H2, 4 H3, 3 H4, 8 H5, 1 H6, 4 H7, 2 H8, 3 H11, 2 H12	5 H1, 4 H2, 23 H3, 8 H4, 19 H5, 3 H6, 8 H7, 9 H8, 16 H9, 1 H10, 6 H11, 5 H12

*Shorebirds refers to migratory shorebirds only.

Anas species ducks were the predominant carriers, and the peak of detection occurred in autumn. Detection rates varied among different locations and times. Numerous H5, H7, and H9 AIVs were detected, although no HPAI strains were identified. The Australian viruses within each subtype were closely related and formed separate clades from Eurasian or North American lineages, indicating that separate lineages of H5, H7, and H9 AIVs are circulating in Australia.

PCR-positive Rates

Most AIV ecology studies have been conducted in Europe and the United States (*2*). In Australia, the overall PCR-positive detection rate of 1.4% for all bird species is similar. However, rates of 0.51% for shorebirds and 2.4% for waterfowl are similar to, or less than, those detected in other geographic regions where 0.2%–20% of shorebirds and 7%–37% of waterfowl were carriers (*2*,*29*–*32*). Our results agree with those of historical Australian studies in which rates of 0.6% for all birds and 1%–5% for ducks were found (*19*–*21*). Rates for AIVs in shorebirds in Australia were previously unknown. We found rates were highest for dabbling ducks, which is consistent with findings of other studies (*2*,*29*). These higher rates may be a result of the ducks' feeding technique of filtering soft mud, which may be an environment conducive to the persistence of AIVs.

Autumn and Winter Detection Rates

Detection rates for migratory shorebirds were highest during April–June and were highest in overwintering eastern curlews. This finding contrasts with results of studies from North America that show a low prevalence in winter (*29*). The Australian winter is considerably milder than winter in areas studied in North America, and differences among winter rates may result from these climatic differences.

Shorebirds migrate to Australia down the East Asian–Australian flyway and then subsequently disperse throughout Australia. Our results suggest that migratory shorebirds are not commonly carrying AIVs into Australia, which would be indicated by peak detections in newly arrived birds in September, but that they become infected during autumn and winter in Australia. This provides further evidence that AIV infection is not maintained during migration (*33*), although studies in Europe have shown that ducks can carry AIVs during migration (*2*).

Rates in waterfowl were high in early spring (September), which corresponds with the period when young birds arrive in coastal Australia (*34*). This finding agrees with those of studies from other locations, which show that immunologically naive juvenile birds carry more viruses, which may have been transmitted from adult birds or the environment (*29*,*32*).

Variability and Increases in Detection Rates

AIV detection rates were variable and seasonal, and periods of increased rates occurred. Large numbers of viruses were detected during some sampling periods but not others, and some subtypes were often identified at the same time and location. Detection of H5 viruses increased in the summer of 2007–08 in New South Wales; all H9 viruses were detected in New South Wales, and most H5, H7, and H9 viruses were identified in different years. These increases in detection rates were generally localized to particular times and places. These results are supported by findings of our smaller previous study that found that subtypes H11N9 and H4N8 were common in migratory shorebirds in November 2004 but not since then (*14*). Detection of the same subtypes at the same times indicated limited evidence of cross-infection, which suggests that occasionally viruses may be passed between bird species (e.g., shorebird to shorebird) and families (e.g., between shorebirds and waterfowl).

Phylogenetics

In this study, H1–H12 and all NA subtypes, excluding N2, N4, and N8, were detected, a similar level of diversity as that observed in other studies (*2*). The AIVs of particular concern are H5, H7, and H9 because they have been associated with outbreaks in poultry and disease in humans. Notably, in this study, we found that these were the most common subtypes, representing 40% of all AIVs identified; however, no HPAI (H5N1) strains were detected. This pattern is different from that observed in other locations, e.g., H4, H6, and H7 were most common in Sweden, and H1, H2, H4, and H6 dominated in Germany and North America (*3*,*5*). This difference may indicate variations in host–virus interactions in Australia. Phylogenetic analysis showed that AIVs of the same subtype detected in Australia are closely related and are distinct from viruses isolated from other geographic locations. We attempted to isolate viruses from on all PCR-positive samples, but only 3 viruses were recovered (2 H7, 1 H5, all from fecal samples). Antigenic hemagglutination inhibition assays (*35*) of the 2 H7 viruses showed they were antigenically similar to 5 HPAI H7 strains that caused outbreaks in poultry in Australia during 1976–1992 but were not similar to 2 Asian H7 viruses (data not shown). The genetic and limited antigenic data demonstrate that little genetic evolutionary change has occurred and suggest that no antigenic change has occurred in Australian H7 viruses over >30 years.

Previous studies of AIVs have shown that globally 2 separate HA AIV lineages occur: Eurasian and North American (*2*). However, our study provides clear evidence that Australian AIVs, rather than being part of the Eurasian lineage, have diverged and may be considered as belonging to a different lineage. The genetically discrete Australian lineage suggests that endemic circulation and evolutionary isolation of strains in Australia have occurred and provides little evidence for the importation of exotic strains by migratory birds (*33*).

This and other studies highlight the need for continued longitudinal surveillance, particularly in areas with large numbers of migratory birds and waterfowl located close to commercial poultry, and in northern Australia, which is nearest to areas where HPAI (H5N1) is endemic. Further genetic and antigenic characterization of AIVs in Australian wild bird populations should be performed. Surveillance programs can identify peaks in occurrence and may act as an early warning system. Such measures are essential for maximizing biosecurity for the poultry industry and public health agencies.

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Dr Hansbro is the head of immunology and microbiology and associate director of the Centre for Asthma and Respiratory Disease at the University of Newcastle. His research interests include surveillance and analysis of avian influenza viruses in wild birds and the mechanisms of pathogenesis of avian and human influenza viruses.

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Freshwater Aquaculture Nurseries and Infection of Fish with Zoonotic Trematodes, Vietnam

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Residents of the Red River Delta region of northern Vietnam have a long tradition of eating raw fish. Fish-borne zoonotic trematodes (FZTs) are estimated to infect ≈1 million persons in Vietnam. It remains uncertain at what stages in the aquaculture production cycle fish become infected with FZTs. Newly hatched fish (fry) from 8 hatcheries and juveniles from 27 nurseries were therefore examined for FZT infection. No FZTs were found in fry from hatcheries. In nurseries, FZT prevalence in juveniles was 14.1%, 48.6%, and 57.8% after 1 week, 4 weeks, and when overwintered in ponds, respectively. FZT prevalence was higher in grass carp ($p<0.001$) than in other carp species. Results show that nurseries are hot spots for FZT infections in fish. Thus, sustainable FZT prevention strategies must address aquaculture management practices, particularly in nurseries, to minimize the risk of distributing infected juveniles to grow-out ponds and, subsequently, to markets for human consumption.

Liver and intestinal infections caused by fish-borne zoonotic trematodes (FZTs) are increasingly being recognized as serious public health problems and with FZTs incorporated among causes of neglected tropical diseases (*1*,*2*). FZTs are especially widespread in Southeast Asia, including Vietnam, Lao People's Democratic Republic, Thailand, Cambodia, People's Republic of China, and North and South Korea (*3*–*9*). Liver flukes are associated with high incidence of bile duct cancer (*1*,*10*), and intestinal flukes cause serious pathologic changes in the heart, brain, and spinal cord (*1*,*2*,*11*).

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The epidemiology of FZTs is complex because humans and reservoir hosts, such as dogs, cats, pigs, and fish-eating birds, harbor egg-shedding adult stages (*12*,*13*). These hosts are infected by consumption of raw, inadequately cooked, or pickled fish. For many inhabitants in the Red River Delta provinces of northern Vietnam, the consumption of such fish dishes is a traditional behavior that is difficult to alter (*14*–*16*). In the Nam Dinh and Ninh Binh provinces, the widespread habit of eating raw fish is associated with a high FZT prevalence of 30%–40% in humans (*3*,*4*). Aquaculture fish species commonly used to prepare raw fish dishes, such as carp, frequently also have high a prevalence of FZT metacercariae (*12*,*17*–*19*).

The influence of FZTs on the food safety of aquaculture products can have a noticeable adverse economic and public health effect because fish farming in Asia is expanding rapidly. Farm-raised fish are a main protein source consumed domestically and an essential product for exporting to other countries (*2*,*10*,*20*). Therefore, the production of FZT-free fish for human consumption should be a key objective for the aquaculture industry. Achieving this goal is seriously hampered, however, because the present state of knowledge on FZT infection in the fish production chain is inadequate to devise practical and sustainable prevention strategies, especially for small-scale and integrated freshwater aquaculture. The available knowledge of FZT infection is mainly obtained from studies of fish in grow-out ponds, where fish are harvested for human consumption (*12*,*17*,*19*).

Freshwater fish hatcheries in Nam Dinh, Ninh Binh, and Bac Ninh provinces include facilities such as a water reservoir, water storage facilities, breeding tanks, and incubators for hatching eggs. Depending on the hatchery, the water reservoirs are cement tanks or consist of earthen ponds from which the water is either pumped into cement breeding tanks or supplied from a tower to the breeding

tanks and egg incubators. Water used for the breeding tanks and incubators is filtered through a net to remove different microbiota, e.g., zooplankton. Brood stock are moved from earthen ponds into cement breeding tanks for induced spawning. Fertilized eggs are then incubated in round cement incubators with running water for ≈5 days, depending on fish species and temperature. Newly hatched fish are termed fry. Fry are kept in tanks for 3–5 days, after which they are sold and subsequently stocked in earthen ponds in so-called nurseries.

The fish raised in nursery ponds are called juveniles. The nursery ponds in Nam Dinh and Ninh Binh provinces are mainly backyard earthen ponds located close to households and premises housing livestock and poultry. Juveniles are nursed up to 4 weeks and then sold for further nursing to bigger size or to be stocked to reach market size in grow-out ponds. Juveniles may be kept in ponds during the winter months (overwintered juveniles) for sale in early spring.

Management of nurseries in northern Vietnam often involves the application of livestock manure as fertilizer before stocking fish to increase the density of plankton that serves as a food source for juveniles. Additionally, farmers may also apply night soil (human manure) as fertilizers to the nursing ponds.

We report an investigation that aimed to determine the FZT infection status in integrated small-scale hatcheries and nurseries in Nam Dinh, Ninh Binh, and Bac Ninh provinces, which are major areas endemic for FZTs in Vietnam. By assessing the FZT metacercariae prevalence in fish from the initial stages of production, namely the hatcheries and nurseries, the study provides knowledge needed for a comprehensive assessment of FZT infection during the entire fish production cycle.

Material and Methods

Fish Sampling and Examinations

In a cross-sectional survey, fry were sampled from 3 hatcheries in Nam Dinh Province, 1 hatchery in Ninh Binh Province, and 4 hatcheries in Bac Ninh Province. In each hatchery, 500 fry of individual fish species, including grass carp (*Ctenopharyngodon idellus)*, silver carp (*Hypophthalmichthys molitrix*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), and common carp (*Cyprinus carpio*) were sampled by using a scoop net.

Juveniles were collected from 14 nurseries in Nam Dinh and 13 nurseries in Ninh Binh provinces by cast net (4-week old and overwintered juveniles) or scoop (1-week-old juveniles) nets. Fish were sampled twice from the nursing ponds, initially 1 week after the fry had been stocked and a second time at the end of the 1-month nursing period. In addition, overwintered juveniles were sampled from the same nurseries to compare their infection status with juveniles sampled before the winter period. On the basis of sampling size calculations, 15 juveniles were collected for each species at each time of sampling in each nursery.

The sample fish were transported to the laboratory for metacercariae examination and kept alive in plastic bags with added oxygen. The length and weight of each fish was recorded before samples were processed.

FZT infections in fry were examined by placing 5 fry on a glass slide, compressing them with another slide, and then examining for trematodes under a stereo microscope (×4) and a compound microscope (×100). Juveniles were digested in 1% pepsin to release metacercariae, following the procedure described by the World Health Organization (*1*) and modified by Chi et al. (*12*). Identification of the metacercariae was made according to morphologic features detailed by Pearson and Ow-Yang (*21*), Scholtz et al. (*22*), and Kaewkes (*23*).

Data Analysis

SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis with fish as the study unit. Descriptive analyses of FZT infection in fry and juveniles were conducted by frequency distribution. Density of metacercariae in 1-week-old juveniles was calculated as number of metacercariae per whole fish, and density of metacercariae in 4-week-old juveniles and overwintered juveniles was calculated as number of metacercariae per gram of fish. Because of a skewed distribution of the density of metacercariae in fish, descriptive analysis was performed by means of median, Q_1 (25% percentile), Q_3 (75% percentile), minimum values, and maximum values.

Logistic regression analysis was used to evaluate prevalence differences of FZT between provinces, nurseries systems and fish species. A p value <0.05 was considered significant.

Results

FZT Species

No FZT metacercariae were found in fish fry from hatcheries in Nam Dinh, Ninh Binh, and Bac Ninh provinces. Table 1 shows the FZT species recovered from the various fish species and nursery systems examined in Nam Dinh and Ninh Binh provinces. The liver fluke *Clonorchis sinensis* was found in only 1.5% of fish. Intestinal flukes, including *Haplorchis pumilio* and *Centrocestus formosanus*, were found in 55.6% and 41.0%, respectively, of the total number of FZT-infected juveniles. Several FZT species were found in individual FZT-infected juveniles.

FZT Prevalence in Juvenile Fish

Table 2 shows the prevalence of FZTs in fish juveniles from Nam Dinh and Ninh Binh provinces and in different

Table 1. Descriptive analysis of fishborne zoonotic trematode and fish species in nursery systems in Nam Dinh and Ninh Binh provinces, northern Vietnam*

Fish species and culture system	Fishborne zoonotic trematode species, n/N (%)			
	Clonorchis sinensis	*Haplorchis pumilio*	*Haplorchis taichui*	*Centrocestus formosanus*
Overall	12/797 (1.5)	443/797 (55.6)	2/797 (0.3)	327/797 (41.0)
Fish				
Grass carp	6/313 (1.9)	213/313 (68.1)	1/313 (0.3)	186/313 (59.4)
Rohu	1/208 (0.5)	92/208 (44.2)	–	65/208 (31.3)
Mrigal	0/55 (0)	17/55 (30.9)	–	2/55 (3.6)
Pacu	0/8 (0)	6/8 (75.0)	–	5/8 (62.5)
Silver carp	5/213 (2.4)	115/213 (53.99)	1/313 (0.3)	69/213 (32.4)
Culture system				
1-week-old juveniles	0/197 (0)	87/197 (44.2)	–	138/197 (70.1)
4-week-old juveniles	5/277 (1.8)	224/277 (80.9)	1/277 (0.4)	133/277 (48.0)
Overwintered juveniles	7/323 (2.2)	132/323 (40.9)	1/323 (0.3)	56/323 (17.3)

*n, no. fish infected with specific fishborne zoonotic trematode species; N, no. fishborne zoonotic trematode–infected fish; –, no parasites found.

age groups of juveniles. Juveniles were already infected (14.1%) during the first week of exposure in the nursery ponds with a further significant increase (48.6%) after 4 weeks nursing when the juveniles were either sold for further stocking or kept in ponds during the winter months. Some additional but nonsignificant infection took place during the 5–6-month overwintering period.

FZT Density in Juvenile Fish

Among infected 1-week-old juveniles, 50% contained only 1 metacercaria/fish, 25% contained 2–4 metacercariae/fish, and 25% contained >5–18 metacercariae/fish. Among the 4-week-old juvenile fish, 50% had metacercariae densities <1.7/g fish tissue, 25% had densities of 1.7–<6.7/g fish tissue, and 25% had <6.7–173.3/g of fish tissue. In overwintered juveniles, 50% had a density <0.8/g, 25% had a density of 0.8–<2.3/g, and 25% had a density of 2.3–170.2/g. An explanation for this apparent decrease in density over the winter is that when overwintered juveniles have the same density as the non-overwintered juveniles at the start of the overwintering period, then as the fish grows during the overwinter period, the density will decrease if FZT transmission is reduced during the colder winter. The significant increase in FZT prevalence seen among overwintered juveniles compared with 4-week-old juveniles indicates that some transmission of FZTs occurs during the winter.

Table 3 shows the result of logistic analysis of FZT prevalence in 5 fish species and 3 nursery systems in Nam Dinh and Ninh Binh provinces. Fish juveniles from Ninh Binh Province had significantly higher risk for being infected with FZT than those from Nam Dinh Province ($p = 0.012$). The odds of FZT infection for grass carp was 6× higher than for Rohu ($p<0.0001$), and silver carp had a 1.3× higher risk for FZT infection than Rohu. Combined for all fish species, overwintered juveniles and juveniles cultured for 4 weeks had odds of FZT infection that were 12.9× and 3.3× higher, respectively, than 1-week-old nursed fry. The nursing system had a significant effect ($p<0.0001$) on FZT prevalence in 1-week-old juveniles.

Discussion

The finding in this study that fish become infected with FZT as early as in the first week of growth in the nurseries and have a 48.6% infection prevalence when 4 weeks of age is of great consequence for public health and the aquaculture industry. It indicates that infection in nursery juveniles may account for most of the FZT prevalence reported for fish from grow-out ponds (*12*,*17*). Furthermore, because juveniles produced at nurseries are sold and distributed to numerous grow-out farms, the risk for FZT infection becomes widely distributed throughout the fish farming areas, complicating efforts to control this zoonosis. For example, the acquisition of FZT infection by overwintered juveniles in Nam Dinh and Ninh Binh provinces has implications for grow-out farms in nearby provinces such as Thanh Hoa, Thai Binh, and Ha Nam, as well as in provinces further north, which are major customers for these fish.

The increase of FZT prevalence during the first month in the nursing ponds is much higher than that during the ≈12-month grow-out period in Nam Dinh Province (*19*). Among possible explanations for this finding are more risky management practices by nursery operators or biological factors such as age-related fish susceptibility, resistance factors, and immunity. Further studies are urgently

Table 2. Fishborne zoonotic trematode infections among juvenile fish in nurseries, northern Vietnam*

Province and fish age	No. FZT-infected/total no. (%)
Overall	797/2,524 (31.6)
Province	
Nam Dinh	448/1,761 (25.4)
Ninh Binh	349/763 (45.7)
Fish age	
1-week-old juveniles	197/1,395 (14.1)
4-week-old juveniles	277/570 (48.6)
Overwintered juveniles	323/559 (57.8)

*FZT, fishborne zoonotic trematode.

Table 3. Logistic regression analyses of fishborne zoonotic trematode prevalence found in different fish species and nursery systems in Nam Dinh and Ninh Binh provinces, northern Vietnam*

Variable	Estimate†	SE	OR (95% CI)	p value
Intercept	−1.672	0.2877		
Province				0.0123
Nam Dinh	1.026	0.3803	0.359 (0.164–0.785)	
Ninh Binh‡	0		1	
Fish				<0.0001
Grass carp	1.794	0.1668	6.013 (4.336–8.340)	
Silver carp	0.284	0.1558	1.329 (0.979–1.804)	
Mrigal	0.032	0.2260	1.033 (0.663–1.609)	
Rohu‡	0		1	
Culture system				<0.0001
1-week-old juveniles‡	0		1	
4-week-old juveniles	1.194	0.1485	3.299 (2.465–4.414)	
Overwintered juveniles	2.552	0.1684	12.831 (9.222–17.852)	

*OR, odds ratio; CI, confidence interval.
†Parameter estimate of variables in the resulting model.
‡Referent.

needed to understand the causes for this higher prevalence during the nursing phase and to develop efficient prevention management.

Grass carp, one of the favored sources of raw fish for human consumption, had a significantly higher FZT prevalence than other fish species, which is consistent with results of a previous study (*24*). This may be due to the carp's food preference for grass and aquatic plants. In these nursing ponds, the grass and aquatic plants used for feeding were often harvested outside the farm, which may have introduced FZT-infected snails into the ponds. According to Phan et al. (*19*), feeding with green vegetation collected outside the farm is a risk factor for FZT infection in cultured fish. Another factor may be that grass carp spend more time in the littoral zone where vegetation and therefore snails may be concentrated. Furthermore, grass carp might be more susceptible to FZT infection than other fish species.

The higher prevalence of FZT metacercariae in Ninh Binh Province may be due to the common practice of using night soil to fertilize ponds to increase growth of plankton, a major source of carp feed. Nam Dinh Province also has a high human and domestic animal FZT prevalence (*3*,*4*,*13*), making fecal waste from these hosts a highly risky fertilizer. To develop effective control interventions, more research on methods to inactivate FZT eggs in manure must be conducted.

The lack of infection in fish fry is consistent with the report of Thien et al. (*24*), who investigated fry from hatcheries in the Mekong Delta. Fry were raised in well-controlled hatcheries by using tanks or ponds containing filtered water free of zooplankton, snails, and other microbiota.

One limitation of our study is that bias could occur when sampling fish by using 2 types of net, i.e., cast net and scoop net. However, cast nets cannot be used in fry incubators in hatcheries or to catch 1-week-old juveniles. Different methods for examining and isolating FZT metacercariae in fish fry and juveniles are needed because compression of whole fish bodies is efficient for processing of small fry and detecting any metacercariae present. For larger fish such as juveniles, however, the thickness of the body and muscle tissue precludes using compression, and instead, pepsin digestion (a more laborious procedure) is the method of choice. We also have compared pepsin digestion for several cups of fry with compressing methods and found no significant difference in detection efficiency.

Liver fluke (*C. sinensis*) metacercariae had a low prevalence (1.5%); most FZT species found were intestinal flukes, particularly *Haplorchis* spp. and *Procerovum varium.* Although generally not considered to have a clinical role compared with liver flukes, several intestinal FZTs can cause serious pathologic effects, even death, when infecting the heart, brain, and spinal cord of humans (*1*,*9*,*11*). The dominance of *H. pumilio* trematodes in juveniles is similar to the findings of other studies of nurseries (*24*) and grow-out systems (*12*,*17*,*18*). *Stellantchasmus falcatus,* however, is a major FZT species found in fish from southern and central Vietnam. Although Trung Dung et al. (*4*) recently recovered *S. falcatus* trematodes from humans in Nam Dinh, this species was not found in fish from this province, possibly because humans acquired their infections from fish originating from provinces other than Nam Dinh.

The life cycle and epidemiology of FZTs in aquaculture systems is complicated, and fish are vulnerable to multiple risks for infection. However, by developing a hazard analysis of critical control points approach to identifying points for intervention and enlisting the collaboration between different sectors such as human health, animal health, snail vector control, and aquaculture management and extension, we believe a major reduction in FZTs in farmed fish can be achieved, with the benefit of being able to provide aquaculture products free of FZTs for human consumption.

In conclusion, the results from this study strongly suggest that a program for the prevention for FZTs in farmed fish must include nurseries and grow-out farms. An integrated program will, however, require more research on the infection events occurring in the nursery pond, particularly the regulation of snail populations, fish risk behavior, and on methods to inactivate FZT eggs in manure intended for fertilizer use.

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Pandemic (H1N1) 2009 Infection in Patients with Hematologic Malignancy

Catherine Liu, Brian S. Schwartz, Snigdha Vallabhaneni, Michael Nixon, Peter V. Chin-Hong, Steven A. Miller, Charles Chiu, Lloyd Damon, and W. Lawrence Drew

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Learning Objectives

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

- Distinguish the most common presenting symptom of pandemic (H1N1) 2009 infection in the current study.
- Analyze the course of lower respiratory tract infection with pandemic (H1N1) 2009 among patients with hematologic malignancy.
- Develop appropriate management strategies for pandemic (H1N1) 2009 infection for patients with hematologic malignancy.

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To assess outcomes of patients with hematologic malignancy and pandemic (H1N1) 2009 infection, we reviewed cases during June–December 2009 at the University of California San Francisco Medical Center. Seventeen (63%) and 10 (37%) patients had upper respiratory tract infection (URTI) and lower respiratory tract infection (LRTI), respectively. Cough (85%) and fever (70%) were the most common signs; 19% of patients had nausea, vomiting, or diarrhea. Sixty-five percent of URTI patients were outpatients; 35% recovered without antiviral therapy. All LRTI patients were hospitalized; half required intensive care unit admission. Complications included acute respiratory distress syndrome, pneumomediastinum, myocarditis, and development of oseltamivir-resistant virus; 3 patients died. Of the 3 patients with nosocomial pandemic (H1N1) 2009, 2 died. Pandemic (H1N1) 2009 may cause serious illness in patients with hematologic malignancy, primarily those with LRTI. Rigorous infection control, improved techniques for diagnosing respiratory disease, and early antiviral therapy can prevent nosocomial transmission and optimize patient care.

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Influenza is a major cause of illness and death in patients with hematologic malignancy and in hematopoietic cell transplant (HCT) recipients. In up to 30% of HCT recipients, illness progressed to lower respiratory tract infection (LRTI); death rates were 28% for patients in whom pneumonia developed (*1*). During spring 2009, infection caused by pandemic (H1N1) 2009 virus emerged in Mexico and spread rapidly throughout the world (*2*–*4*). Although it does not appear to be associated with higher death rates than seasonal influenza (*5*), pandemic (H1N1) 2009 virus has caused severe disease and death, particularly in persons with preexisting illnesses (*6*,*7*). Little is known about the clinical features and outcomes of pandemic (H1N1) 2009 infection in patients with hematologic malignancy. One recently published study suggested that pandemic (H1N1) 2009 causes mild disease in most patients with hematologic malignancy (*8*), but several reports have been published about patients with severe infection and respiratory failure (*9*–*11*). Risk factors for progression to LRTI are unknown. To characterize the clinical spectra and outcomes of pandemic (H1N1) 2009 disease in patients with hematologic malignancy, we reviewed the first 27 cases of pandemic (H1N1) 2009 among these patients in the University of California San Francisco Medical Center during June 1–December 31, 2009.

Methods

Patients and Setting

The University of California San Francisco (UCSF) Medical Center (San Francisco, CA, USA) is a large, academic medical center with an active HCT program for children and adults and extensive experience treating children and adults with hematologic malignancy. In 2009, HCTs were performed for 171 adults and 50 children; a total of 1,020 adults and 782 children were admitted to the hospital's hematology/HCT service.

At UCSF, all nasal swabs performed to diagnose respiratory viral infection in inpatients and outpatients are routinely submitted for testing to the UCSF Virology Laboratory. For infection control surveillance during the pandemic (H1N1) 2009 outbreak, the UCSF Virology Laboratory generated a list of all laboratory-confirmed cases of influenza A during June–December 2009. HCT recipients and other patients with hematologic malignancy were identified through a retrospective chart review of all laboratory-confirmed cases of influenza A during this period. We used a standardized form to capture demographic data, clinical signs and symptoms, underlying hematologic disease and other medical conditions, transplant history, immunosuppressive medications, selected laboratory tests, radiographic findings, treatment course, and clinical outcomes. Dosing and duration of antiviral treatment with oseltamivir or zanamivir, use of concomitant antimicrobial therapy, and intravenous immunoglobulin was determined by the treating providers. The study protocol was approved by the UCSF Committee on Human Research.

Laboratory Confirmation of Infection

All diagnostic testing, including repeat serial testing, was performed at the discretion of the treating provider. The standard clinical practice for detecting respiratory viral infection, including influenza, was to obtain a nasopharyngeal wash, aspirate, or flocked swab for viral direct fluorescent antibody (DFA) testing (D^3-DFA Respiratory Virus Screening and ID Kit, Diagnostics Hybrids, Athens, OH, USA) with same-day turnaround. Specimens from high-risk immunocompromised patients were submitted for multiplex PCR testing (xTAG RVP [Respiratory Viral Panel]; Luminex, Austin, TX, USA) if DFA results were negative. Results were considered consistent with pandemic (H1N1) 2009 infection when specimens were positive for influenza A by DFA or positive for influenza A matrix gene but negative for H1 and H3 hemagglutin gene subtypes by RVP. Pandemic (H1N1) 2009 infection was confirmed by using banked frozen specimens with at least 1 of 3 PCRs; the Xpert Flu A Panel (Cepheid Corp, Sunnyvale, CA, USA), the Centers for Disease Control and Prevention (CDC) real-time reverse transcription–PCR (rRT-PCR) swine influenza panel (performed at the San Francisco Department of Public Health), or a previously described PCR specific for pandemic (H1N1) 2009 performed in our laboratory (*12*). Patients were considered to have probable pandemic (H1N1) 2009 if laboratory-confirmed influenza A was detected during June–December 2009 and additional specimens were not available for confirmatory testing. CDC performed pyrosequencing to detect the H275Y mutation in the N1 neuraminidase gene associated with oseltamivir resistance.

Definitions

Upper respiratory tract infection (URTI) was defined as pandemic (H1N1) 2009 virus in nasopharyngeal specimen and compatible clinical symptoms without new pulmonary infiltrates on chest radiograph. Lower respiratory tract infection (LRTI) was defined as pandemic (H1N1) 2009 virus in a nasopharyngeal, endotracheal tube, or bronchoalveolar lavage specimen and compatible clinical symptoms with a new pulmonary infiltrate on chest radiograph or computed tomography (CT) imaging.

Statistical Analysis

We compared patient median age, absolute lymphocyte count, and duration of antiviral therapy by using the Wilcoxin rank-sum test. For categorical variables, we calculated the proportions of patients in each category. Clini-

cal characteristics, therapy, and outcomes were compared between subgroups of patients by using the Fisher exact test. We identified independent predictors of LRTI using logistic regression. Predictors significant at p<0.10 in both the univariate and multivariate analyses were retained in the final multivariate models. We conducted analyses using Stata software version 9.0 (StataCorp, College Station, TX, USA).

Results

During June 1–December 31, 2009, a total of 159 probable or laboratory-confirmed cases of pandemic (H1N1) 2009 infection were identified at our institution. Eighteen (11%) were HCT recipients and 9 (6%) had a hematologic malignancy and were included in the study. The number of identified cases peaked early in the pandemic in June, although ≈40% of the hematologic malignancy patients sought care for influenza in November (Figure 1).

Median age of patients was 43 years, and patients with URTI were younger than those with LRTI (33 vs. 53 years; p = 0.04) (Table 1). Two patients were children; both had URTI. Two thirds of cases occurred in male patients. Among HCT recipients, the median time of symptom onset after transplant was 12 months (range 0.3–45 months). A total of 10 (37%) patients had LRTI; compared with patients with URTI, these patients were older, significantly more likely to have diabetes mellitus or underlying lung disease, and more likely to be receiving corticosteroids. After controlling for sex, transplant status, presence of chronic lung disease, and corticosteroid use, we found that older age was independently associated with development of LRTI (odds ratio [OR] 1.15; 95% confidence interval [CI] 1.02–1.30; p = 0.0001).

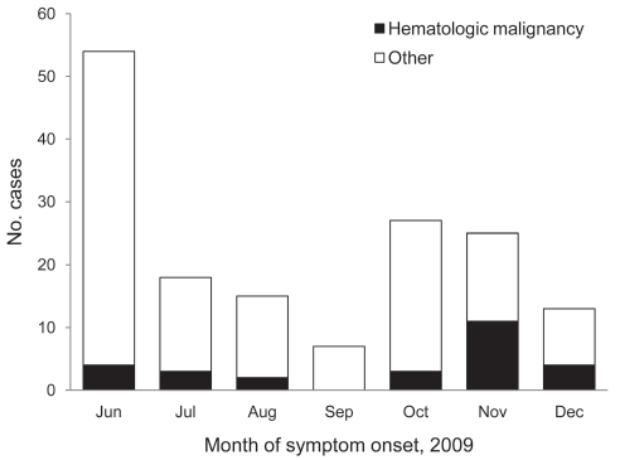

Figure 1. Pandemic (H1N1) 2009 cases among hematologic malignancy patients compared with all other patients, University of California San Francisco Medical Center, San Francisco, California, USA, June–December 2009.

Cough (85%) and fever (70%) were the most common signs; 5 (19%) patients had nausea, vomiting, or diarrhea (Table 2). Compared with patients with URTI, those with LRTI were more likely to have dyspnea (12% vs. 90%;

Table 1. Characteristics of hematologic malignancy patients with URTI or LRTI and pandemic (H1N1) 2009 virus, University of California, San Francisco, Medical Center, San Francisco, California, USA, June–December 2009*

Characteristic	Total, n = 27	URTI, n = 17	LRTI, n = 10	p value†
Median age, y (range)	43 (5–83)	33 (5–83)	53 (29–80)	0.04
Male sex	18 (67)	10 (59)	8 (80)	0.24
Underlying malignancy				
Acute lymphocytic leukemia	6 (22)	5 (29)	1 (10)	0.25
Acute myelocytic leukemia	5 (19)	4 (24)	1 (10)	0.37
Chronic lymphocytic leukemia	1 (4)	1 (6)	0	0.63
Lymphoma	6 (22)	2 (12)	4 (40)	0.11
Multiple myeloma	7 (26)	4 (24)	3 (30)	0.52
Other	2 (7)	1 (6)	1 (10)	0.61
Hematopoietic cell transplant	18 (67)	12 (71)	6 (60)	0.44
Allogeneic	7 (39)	4 (33)	3 (50)	0.43
Graft-vs.-host disease	6 (86)	3 (75)	3 (100)	0.57
Autologous	11 (61)	8 (67)	3 (50)	0.43
Median time posttransplant, mo (range)	12 (0.3–45)	12 (3–45)	11 (0.3–34)	0.81
Immunosuppressive medications				
Corticosteroid use	7 (26)	2 (12)	5 (50)	0.04
T-/B-cell depleting agent	4 (15)	1 (6)	3 (30)	0.13
Underlying concurrent conditions				
Obesity, body mass index >30 kg/m^2	2 (7)	0	2 (20)	0.13
Chronic lung disease	5 (19)	1 (6)	4 (40)	0.047
Diabetes mellitus	6 (22)	1 (6)	5 (50)	0.015
HIV infection	2 (7)	1 (6)	1(10)	0.61

*Values are given as no. (%) patients except as indicated. URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.
†p value represents statistical difference between comparison of values of patients with LRTI and URTI.

p<0.01) and less likely to have rhinorrhea (65% vs. 10%; p = 0.005) and had a lower median absolute lymphocyte count (ALC) (815 cells/μL vs. 130 cells/μL; p = 0.02). All 10 patients with LRTI were hospitalized, compared with 35% of patients with URTI (p = 0.001). Five (50%) LRTI patients required monitoring in the intensive care unit (ICU), of whom 4 required mechanical ventilation because of respiratory failure. Pulmonary infiltrates observed on CT scan of the LRTI patients ranged from focal consolidations (7/10) to ground glass opacities (6/10), centrilobular nodules (2/10), and tree-in-bud opacities (2/10).

Influenza A was diagnosed for 21 (78%) patients by DFA. DFA results were negative for 6 (22%) patients, but influenza A was subsequently diagnosed among them by PCR. Isolates for all patients were confirmed as pandemic (H1N1) 2009 virus by PCR, except for 2 for whom additional specimens were not available for confirmatory testing. These were considered probable cases of pandemic (H1N1) 2009 because no cases of seasonal H1 or H3 influenza were laboratory confirmed during this time.

Twenty-one (78%) patients received antiviral therapy, and 20 (95%) received standard-dose or high-dose oseltamivir. Two patients, both with LRTI, received inhaled zanamivir; intravenous zanamivir was later used for 1 of these patients who required mechanical ventilation and had oseltamivir-resistant virus (patient 3 in Figure 2; Table 3). Four (19%) patients received antiviral therapy within 48 hours after symptom onset; treatment started >96 hours after symptom onset for more than half the patients. Median duration of antiviral therapy was 5 days

Table 2. Clinical features, treatments, and outcomes for hematologic malignancy patients who had URTI and LRTI from pandemic (H1N1) 2009 virus, University of California San Francisco Medical Center, San Francisco, California, USA, June–December 2009*

Characteristic	Total, n = 27	URTI, n = 17	LRTI, n = 10	p value†
Signs and symptoms				
Fever	19 (70)	11 (65)	8 (80)	0.44
Cough	23 (85)	14 (82)	9 (90)	0.68
Shortness of breath	11 (41)	2 (12)	9 (90)	<0.01
Myalgias	4 (15)	3 (18)	1 (10)	0.50
Rhinorrhea	12 (44)	11 (65)	1 (10)	0.005
Sore throat	8 (30)	6 (35)	2 (20)	0.31
Gastrointestinal symptoms‡	5 (19)	3 (18)	2 (20)	0.66
Household influenza exposure§	8 (30)	1 (6)	7 (70)	0.001
Laboratory values				
ALC, cells/μL, median (range)	570 (0–16,370)	815 (150–16,370)	130 (0–1,860)¶	0.02
Absolute neutrophil count <500 cells/μL	5 (19)	3 (19)	2 (20)	0.66
Treatment#				
Antiviral drug therapy	21 (78)	11 (65)	10 (100)	0.042
Symptom onset to start of antiviral drug therapy, h**				
<48	4 (19)	3 (27)	1 (10)	0.29
48–96	5 (24)	2 (18)	3 (30)	0.50
>96	11 (52)	5 (45)	6 (60)	0.50
Type of antiviral drug therapy				
Oseltamivir, standard dose	8 (38)	5 (45)	3 (30)	0.65
Oseltamivir, high dose††	11 (52)	4 (36)	7 (70)	0.02
Zanamivir, inhaled	2 (10)	0	2 (20)	0.13
Zanamivir, intravenous	1 (5)	0	1 (10)	0.37
Median duration of antiviral drug therapy, d (range)	7 (5–49)	5 (5–14)	15 (5–49)	0.058
Intravenous immunoglobulin	6 (22)	1 (9)	5 (50)	0.015
Outcome				
Hospitalization	16 (59)	6 (35)	10 (100)	0.001
Intensive care unit admission	5 (19)	0	5 (50)	0.003
Mechanical ventilation	4 (15)	0	4 (40)	0.012
Death	3 (11)	0	3 (30)	0.041

*Values are given as no. (%) patients except as indicated. URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; ALC, absolute leukocyte count.
†p value represents statistical difference between comparison of values of patients with LRTI and URTI.
‡Nausea, vomiting, and/or diarrhea.
§Exposure to an ill household contact documented in patient's chart.
¶Two LRTI patients had ALC below the level of detection, and the value was reported by the laboratory as <100 lymphocytes/μL. For this calculation, these patients were assigned an ALC value of 0.
#The calculation for LRTI excludes 2 patients who received only 2–3 d of antiviral therapy just before they died because their anticipated duration of antiviral therapy would have been longer.
**Time of symptom was available for 20 of 21 patients who received antiviral therapy.
††Two patients received standard-dose and high-dose oseltamivir.

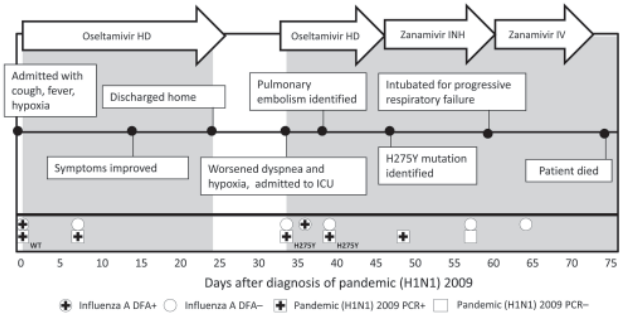

Figure 2. Clinical course for a 45-year-old woman (Table 3) hospitalized (periods indicated by gray shading) with influenza-associated pneumonia and concurrent pulmonary aspergillosis. The patient had received an autologous stem cell transplant 1 year earlier and underwent treatment with high-dose (HD) steroids for carmustine (BCNU) pneumonitis. On admission, she received HD oseltamivir (150 mg orally 2×/d) for 14 days, and antifungal therapy was initiated. Test results were positive for wild-type (WT) pandemic (H1N1) 2009 by PCR and influenza A by direct fluorescent antibody (DFA). A repeat pandemic (H1N1) 2009 PCR result was positive 1 week into treatment, but her condition later improved, and she was discharged to home. Ten days later, she was readmitted to the intensive care unit (ICU) with worsening dyspnea and again had positive test results for pandemic (H1N1) 2009; a pulmonary embolus was found. HD oseltamivir was restarted, but when pandemic (H1N1) 2009 PCR results remained persistently positive, she was switched to inhaled (INH) zanamivir and then intravenous (IV) zanamivir after intubation. PCR results indicated the H275Y mutation, confirming oseltamivir resistance. She eventually showed evidence of viral clearance but died of respiratory failure secondary to adult respiratory distress syndrome, pulmonary embolus, progressive pulmonary aspergillosis, and BCNU pneumonitis. PCR results were returned 5–11 days after specimen submission.

(range 5–14 days) and 15 days (range 5–49 days) for patients with URTI and LRTI, respectively. No concomitant bacterial infections were identified; however, 20 (74%) patients were empirically prescribed broad-spectrum antibacterial therapy. Invasive pulmonary *Aspergillus fumigatus* was concurrently diagnosed in 1 patient. Six (35%) patients with URTI were not treated with antiviral therapy and recovered without sequelae.

Five (19%) of 27 patients experienced severe complications related to pandemic (H1N1) 2009 infection that required admission to ICU, and 3 (11%) died (Table 3). Of these patients with severe infection, 3 (patients 1, 2, and 4) acquired the infection during hospitalization. None had clinical evidence of a respiratory viral infection at admission. Retrospective chart review suggested that symptoms began at 13, 16, and 11 days after admission, respectively. For all 3 patients, new onset of fever (2 with neutropenia), mild cough, progressive dyspnea, and hypoxia developed; however, the latter did not become prominent in 2 patients until 4–6 days after initial onset of symptoms. Two patients had diarrhea. Two of the 3 had ground glass opacities with areas of focal consolidation on chest CT scan; the third had frank multilobar consolidation. For each patient, initial evaluation focused on workup for typical hospital-acquired bacterial and opportunistic infections; a respiratory viral infection was not considered until 4–7 days after symptom onset, which resulted in a delay in diagnostic testing and initiation of antiviral therapy of ≈1 week. For 2 patients (2 and 4), DFA results were negative, and diagnosis was ultimately made by PCR; the result was returned postmortem for patient 4. Pandemic (H1N1) 2009 infection resulted in the deaths of 2 of these 3 patients; the third (patient 1) had substantially delayed engraftment requiring an infusion of back-up autologous stem cells; myocarditis with cardiogenic shock developed, but the patient eventually recovered. None of these cases were clustered, and the source of infection was not clearly identified but was presumed to be an ill healthcare worker (HCW) or visitor.

The third fatal case occurred in a patient who had received an autologous stem cell transplant 1 year before illness that was complicated by carmustine pneumonitis requiring steroid therapy (patient 3; Figure 2). She was initially admitted with wild-type pandemic (H1N1) 2009 infection and concurrent pulmonary aspergillosis; she improved after completing 14 days of oseltamivir therapy and initiation of antifungal therapy. Ten days after hospital discharge, she was readmitted with recurrent dyspnea and had persistent viral shedding with what was later confirmed as oseltamivir-resistant pandemic (H1N1) 2009 virus and a new pulmonary embolus. She received inhaled, and then intravenous, zanamivir and demonstrated evidence of viral clearance. However, in the context of her adult respiratory distress syndrome (ARDS), pulmonary embolus, progressive pulmonary aspergillosis, and carmustine pneumonitis, she ultimately died of respiratory failure.

Discussion

We describe a consecutive series of 27 hematologic malignancy patients with pandemic (H1N1) 2009 infection, including 3 nosocomial cases, during June–December 2009. The spectrum of illness severity ranged from mild to severe, and most patients in this and other series had signs and symptoms suggestive of an influenza-like illness (*8*). Similar to reported signs of pandemic (H1N1) 2009 in immunocompetent hosts (*13*,*14*), cough and fever were the most common signs, and nausea, vomiting, and diarrhea occurred in a greater proportion than typically observed for seasonal influenza (*13*–*15*). Although 17 (63%) patients had URTI, with 11 (65%) managed as outpatients, all 10 (37%) patients with LRTI required hospitalization. Risk factors for LRTI included chronic lung disease, diabetes mellitus, and more marked immunosuppression as measured by corticosteroid use and lymphopenia at diagnosis. Although previous observations suggest that older age protects against pandemic (H1N1) 2009 infection (*7*,*16*,*17*),

Table 3. Selected cases of severe pandemic (H1N1) 2009 infection in patients with hematologic malignancy, University of California San Francisco Medical Center, San Francisco, California, USA, June–December 2009*

Patient no./ age, y/sex	Underlying disease, time frame	ALC†	Neutro	Onset of symptoms, d before antiviral therapy	Antiviral therapy; duration, d	Duration of viral shedding, d‡	Hospital acquired	ICU/ mechanical ventilation, d	Complications; outcome
1/43/M	APML, 9 d post auto-SCT	<100†	Yes	6	SD/HD oseltamivir;§ 40 d	26	Yes	25/8	Myocarditis, cardiogenic shock; survived
2/48/M	HIV, Burkitt lymphoma	<100†	Yes	7	HD oseltamivir; 2 d	Unknown	Yes	5/4	ARDS; died
3/45/F	DLBCL, 1 year post auto-SCT, BCNU pneumonitis	80	No	5	HD oseltamivir, inhaled/IV zanamivir; 49 d	48	No	40/13	Oseltamivir resistance; died
4/75/M	Multiple myeloma	40	No	7	HD oseltamivir; 2 d	Unknown	Yes	4/3	ARDS, died
5/29/M	ALL, 1-m post allo-SCT	160	No	3	HD oseltamivir; 20 d	7	No	8/0	Pneumomediastinum; pneumopericardium, bronchiolitis obliterans; survived

*ALC, absolute lymphocyte count/μL; Neutro, neutropenia; ICU, intensive care unit; APML, acute promyelocytic leukemia; SCT, stem cell transplant; SD, standard dose; HD, high dose; DLBCL, diffuse large B-cell lymphoma; IV, intravenous; BCNU, carmustine. ARDS, adult respiratory distress syndrome; ALL, acute lymphocytic leukemia.
†Two patients had an ALC below the level of detection, and the laboratory reported the value as <100 cells/μL.
‡This time is the minimum estimated duration of viral shedding calculated on the basis of the time between the first positive to the last positive specimen collected. Because there was no standard collection interval between specimens and specimens were not collected >1 time weekly, viral shedding may have been longer than indicated. For example, patient 5 had 2 positive specimens collected 7 d apart; his next specimen, collected 15 d later, was negative.
§Oseltamivir dosing varied from HD at 150 mg 2×/d to SD at 75 mg 2×/d.

after infection is established in persons with hematologic malignancy, older age predicted an increased risk for disease involving the lower respiratory tract. These findings may be due to the lack of preexisting antibodies, which could have conferred partial immunity, in patients with hematologic malignancy.

Half of the patients with LRTI required ICU admission, and all except 1 needed mechanical ventilation. In addition to ARDS, several pandemic (H1N1) 2009–related complications were observed. One patient developed spontaneous pneumomediastinum, thought to be secondary to viral bronchiolitis from pandemic (H1N1) 2009. Spontaneous pneumomediastinum has been reported as a complication of influenza, including pandemic (H1N1) 2009, in adults and children (*18*,*19*). In another patient, severe myocarditis associated with cardiogenic shock developed; the patient ultimately had partial recovery of his left ventricular ejection fraction with treatment and supportive care. Three of the 10 patients with LRTI died, similar to published death rates on seasonal influenza pneumonia in HCT recipients (*1*).

Because decisions about antiviral therapy were made by different providers, the dose, duration, and timing of antiviral therapy relative to symptom onset varied substantially. Most patients with URTI appeared to respond to 5 days of therapy, but about one third recovered without antiviral therapy. Most patients with LRTI received high-dose oseltamivir during their treatment course, but whether this dosage is more effective than standard-dose oseltamivir is not possible to conclude. Because only 19% of patients began antiviral therapy within 48 hours after symptom onset, assessing the effect of early antiviral therapy was difficult. However, none of the 5 patients requiring ICU admission received antiviral therapy within 48 hours after symptom onset. Although the vaccination status of all patients is unknown, pandemic (H1N1) 2009 vaccine was not available during most of the study period.

Shedding of seasonal influenza virus by otherwise healthy adults is ≈5–7 days but can be >1 week for hospitalized patients (*20*) and an average of 11–12 days for HCT recipients (*1*,*21*). Initiation of therapy within the first 4 days of illness appears to enhance viral clearance (*20*). Two patients had laboratory evidence of viral shedding beyond 2 weeks; for both, antiviral therapy was started ≥5 days after symptom onset. Testing for the H275Y mutation showed that oseltamivir-resistant virus developed in 1 person after 14 days of therapy. This patient had persistent viral shedding after a week of therapy, and testing was not repeated before cessation of therapy; whether ongoing viral shedding may have selected for development of oseltamivir-resistant virus is unclear. Consistent with recommendations made by Casper et al. (*22*), we believe that hematologic malignancy patients with LRTI should be treated with high-dose oseltamivir for a minimum of 10 days. We recommend weekly

monitoring of such patients with serial viral PCR testing and extended antiviral therapy until PCR is negative, particularly for patients who have ongoing clinical symptoms or are severely immunocompromised.

Three cases of oseltamivir-resistant pandemic (H1N1) 2009 in patients with HCT have been published. These patients shed virus for 6–8 weeks, and oseltamivir-resistant virus developed after 5, 11, and 21 days of oseltamivir therapy (*23*,*24*). It was difficult to determine the relative contribution of oseltamivir-resistant pandemic (H1N1) 2009 to the overall clinical course and ultimate death of the case-patient in our report, given her ARDS and multiple concurrent pulmonary disease processes. Asymptomatic viral shedding has been described for infections with other respiratory viruses, such as parainfluenza and respiratory syncytial virus (*25*,*26*), and the recovery of 1 patient with oseltamivir-resistant virus without zanamivir therapy is notable (*23*). Further research is needed to determine the clinical significance of persistent viral shedding, role of antiviral therapy in this situation, and risk factors for oseltamivir resistance in this patient population.

Nosocomial transmission of pandemic (H1N1) 2009 to 3 patients resulted in serious complications and prolonged hospitalization of 1 patient and deaths of 2 patients. Although these cases occurred independently, ascertaining the source of transmission (HCW vs. visitor) was difficult. For each patient, influenza was not initially suspected, resulting in delayed diagnostic testing and initiation of antiviral therapy. For 2 patients, diagnosis was further delayed by initial negative DFA results and positive PCR returning 5–10 days later. Performance characteristics of the DFA test for detecting pandemic (H1N1) 2009 have been shown to vary from a sensitivity of 47% and negative predictive value of 59% (*27*) to a sensitivity of 93% and negative predictive value of 96% (*28*). In our case series, 22% of patients had negative DFA results and subsequent positive results for pandemic (H1N1) 2009 by PCR. Rapid, highly sensitive, and specific tests clearly are needed for detecting influenza, including pandemic (H1N1) 2009, in combination with rigorous infection control strategies. In response to these in-hospital transmissions, multiple measures were implemented on the hematology and blood and marrow transplant unit to prevent further transmission, including 1) visitor screening, with required symptom review, before allowing entry into patient rooms; 2) restriction to 2 visitors at any given time in patient room; 3) reinforcement of "stay at home when sick" policy among HCWs (we relied on an honor system and asked ill HCWs to call in sick rather than come in to be screened); 4) closure of common pantry area on the unit to patients and visitors; and 5) maintenance of droplet precautions for patients with pandemic (H1N1) 2009 infection throughout their hospitalization. Several hospitalwide measures were implemented: 1) exclusion of all visitors ≤16 years of age; 2) a policy of mandatory seasonal and pandemic (H1N1) 2009 vaccination for all HCWs or a requirement for those who declined to wear masks in patient-care areas; 3) reinforcement of "stay at home when sick" policy among HCWs; 4) intensive education about hand hygiene, use of appropriate precautions emphasizing early isolation of patients with influenza-like illness; and 5) proposed acquisition of in-house rapid PCR testing system for influenza.

Our findings indicate that investing in the development of enhanced diagnostic methods for respiratory disease is critical to ensure timely and accurate diagnoses. In addition, further research is needed to define the optimal dose, duration, and choice of antiviral therapy for managing influenza infections in immunocompromised patients. Finally, aggressive infection control measures are crucial for preventing transmission of pandemic (H1N1) 2009 and other respiratory viral diseases in patients with hematologic malignancy.

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Yellow Fever Virus in *Haemagogus leucocelaenus* and *Aedes serratus* Mosquitoes, Southern Brazil, 2008

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Yellow fever virus (YFV) was isolated from *Haemagogus leucocelaenus* mosquitoes during an epizootic in 2001 in the Rio Grande do Sul State in southern Brazil. In October 2008, a yellow fever outbreak was reported there, with nonhuman primate deaths and human cases. This latter outbreak led to intensification of surveillance measures for early detection of YFV and support for vaccination programs. We report entomologic surveillance in 2 municipalities that recorded nonhuman primate deaths. Mosquitoes were collected at ground level, identified, and processed for virus isolation and molecular analyses. Eight YFV strains were isolated (7 from pools of *Hg. leucocelaenus* mosquitoes and another from *Aedes serratus* mosquitoes); 6 were sequenced, and they grouped in the YFV South American genotype I. The results confirmed the role of *Hg. leucocelaenus* mosquitoes as the main YFV vector in southern Brazil and suggest that *Ae. serratus* mosquitoes may have a potential role as a secondary vector.

Yellow fever is an acute, often fulminant, disease caused by *Yellow fever virus* (YFV), the prototype member of the family *Flaviviridae*, genus *Flavivirus*. YFV is endemic to tropical regions of Africa and South America (*1*,*2*). The virus is transmitted through the bite of mosquitoes belonging to the family *Culicidae* to vertebrate hosts, especially nonhuman primates and humans.

In South America, the urban cycle involves the mosquito *Aedes aegypti* and humans, whereas in the jungle cycle, the virus is transmitted to nonhuman primates by mosquitoes in the genera *Haemagogus* and *Sabethes*, especially *Hg. janthinomys*, *Hg. albomaculatus*, *Hg. leucocelaenus*, *Sa. chloropterus*, *Sa. glaucodaemon*, *Sa. soperi*, and *Sa. cyaneus* (*2*,*3*).

Currently in Brazil, 2 yellow fever–endemic areas have been described. The area where vaccination is recommended or risk for yellow fever is recognized includes the northern and central regions, as well as Maranhão State and the eastern part of Bahia, Minas Gerais, São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul states. The area where vaccination has not been recommended includes the coastal region between Piauí and Rio Grande do Sul states (*4*). During 1989–2008, a total of 546 human cases of yellow fever and 241 deaths were recorded in Brazil (*5*).

In Rio Grande do Sul, the southernmost state in Brazil, the last cases of sylvatic yellow fever were recorded in the 1960s (*6*). After 40 years without detectable activity, the virus was isolated from mosquitoes (*Hg. leucocelaenus)* collected in 2001, during an epizootic involving free-living nonhuman primates of the species *Alouatta caraya* (black howler monkey) in the northwestern region of the state (*7*). These YFV hosts spend most of their time in the trees and only go down to the ground to feed during the day; they are extremly sensitive to YFV and die of the disease after they are infected naturally or experimentally even in lower doses.

The confirmation of YFV in nonhuman primates and in mosquitoes led to vaccination campaigns in 44 municipali-

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ties to prevent human cases and the initiation of a program of environmental surveillance for yellow fever and other arboviruses authorized by Brazil's state Ministry of Health. The goal of this program was to detect the early presence of YFV in mosquitoes and nonhuman primates (through the detection of specific antibodies). The monitoring program was improved and, in 2002, a new epizootic was recorded with virus circulation in the central region of the state, including 9 additional municipalities in the vaccination area. Subsequently, no YFV activity was recorded for 6 years.

In October 2008, the state health secretary reported an increase in the number of deaths of black howler monkeys in the northwestern region, and intensified surveillance began immediately, before the deaths from yellow fever were even confirmed (*8*). This study describes the results obtained in 2008 during entomologic surveillance in areas with records of yellow fever epizootics in 2 municipalities of the northwest region of Rio Grande do Sul State.

Figure 1. Municipalities in Rio Grande do Sul State, southern Brazil, where mosquito specimens were collected during November 2008.

Materials and Methods

Study Area

Entomologic activities were carried out in rural areas in Caibaté (54°38′W, 28°17′S) and Coronel Barros (54°03′W, 28°22′S) municipalities in the Ijuí River basin. These 2 municipalities are located 487 km and 417 km, respectively, from Porto Alegre, the Rio Grande do Sul State capital, and are ≈70 km from each other (Figure 1). Caibaté has 5,080 inhabitants, and Coronel Barros has 2,241 inhabitants, according to a 2007 census (*9*). The landscape is dominated by plantations and extensive ranching, which demonstrates the extensive human influence on the environment (*10*).

Remnant tree formations are distributed in gallery forests or in isolated forest fragments in the cultivated areas and in pasture grasslands generally associated with small rivers and creeks that offer shaded areas and water for cattle. Forests in the region are classified as deciduous stationary forest. This category is defined by the low temperatures observed during the winter, which cause the trees in the upper strata to lose their leaves because of physiologic drought caused by the cold weather (*11*).

The climate is humid subtropical, with 2 distinct seasons during the year. During the summer, the mean temperature is >20°C; during the winter, the mean temperature drops <15°C. In relation to rainfall, there are no periods with water deficit (*12*).

Field Procedures

Samples were obtained during November 19–27, 2008, in residual forests where nonhuman primate deaths had been recorded in Caibaté and Coronel Barros municipalities. Mosquitoes were captured at ground level by using entomologic net and bottle-type manual vacuums, at different times, from 8:30 AM to 4:00 PM. After sampling, the insects were frozen, transferred to cryogenic tubes, and placed in liquid nitrogen containers for transportation to the laboratory where they were stored in a freezer at –70°C until processing. A total of 358 mosquitoes were captured in Coronel Barros (n = 179; 16 pools) and Caibaté (n = 179; 20 pools) for virus isolation attempts (Table 1).

Taxonomic Identification and Viral Isolation

Cell Culture

Batches of mosquitoes were identified to species or genus level, separated into pools of 30 individuals (maximum), and macerated in 1.0 mL of phosphate-buffered saline solution with bovine albumin, containing penicillin (100 IU/mL) and streptomycin (100 μg/mL). The pools were then centrifuged at 3,000 rpm at 4°C for 10 min, filtered, and added to cultures of Vero cells. The cells were observed for 14 days; after this period, cultures were harvested and supernatants used in indirect immunofluorescent assays with polyclonal antibodies against flaviviruses (Bussuquara, Cacipacore, dengue, yellow fever, Ilhéus, Naranjal-like, Rocio, and Saint Louis encephalitis viruses) and alphaviruses (Aura, eastern equine encephalitis, Mayaro, Mucambo, Pixuna, Una, western equine encephalitis, and Trocara viruses). Mosquito batches positive for flaviviruses were retested by using indirect immunofluorescent assays with monoclonal antibodies from the identification strain isolated (*7*,*13*).

Newborn Mice

Newborn Swiss albino mice were inoculated by the intracerebral route with 0.02 mL of the same suspension used to infect cell cultures. Mice that demonstrated signs of disease were removed and tested by a complement-fixation

Table 1. Mosquitoes used for yellow fever virus isolation in municipalities of Coronel Barros and Caibaté, Rio Grande do Sul State, Brazil, November 2008

Taxonomic category	No. mosquitoes (no. batches)					
	Coronel Barros			Caibaté		
	Rincão dos Pampas	Rincão Canta Galo	Linha da Pedreira	Linha Caaró	Capão do Herval	Total
Aedes scapularis				1		1
Ae.serratus	8	3	4	32	6	53 (5)
Anopheles mediopunctatus				1		1
Chagasia sp.	2				1	3 (2)
Coquillettidia venezuelensis				1		1
Haemagogus leucocelaenus	56 (2)	83 (3)		36 (2)	14	189 (8)
Johnbelkinia sp.				1		1
Psorophora albigenu					1	1
Ps. albipes				3	38 (2)	41 (3)
Ps. ferox	1	1	3	11	12	28 (5)
Psorophora sp.	6	7				13 (2)
Sabethes albiprivus				5		5
Sa. intermedius	2			1		3 (2)
Wyeomyia sp.		3		13	2	18 (3)
Total	75 (7)	97 (7)	7 (2)	105 (12)	74 (8)	358 (36)

technique to confirm virus isolation as described (*14*,*15*).

Minimum Infection Rate

The minimum infection rate in the mosquito species from which YFV has been isolated was calculated as follows. The total number of groups of positive species of mosquito was divided by the total number of processed mosquitoes of that species × 100 (*16*).

RNA Extraction and Reverse Transcription–PCR

To extract viral RNA, the reagent Trizol LS protocol (Invitrogen, San Diego, CA, USA) was used, following the manufacturer's instructions. The cDNA was obtained directly from viral RNA by reverse transcription in vitro for the envelope (E) region. Five microliters of RNA was used and added to 1 μL (0.2 μmol/L) of the reverse oligonucleotide primer FA2554 (5′-GTATGAGTACTTGTTCAGCCAGTC-3′). This mixture was incubated for denaturation of the RNA molecule at 94°C for 2 min and at room temperature for 5 min. Next, , 14 μL of the RT solution, containing 1× buffer, 1 mmol/L of each, 10 mmol/L dithiothreitol, 40 U RNase inhibitor (RNaseOUT; Invitrogen), and 1.5 U reverse transcriptase (Superscript-II, Reverse Transcriptase; Invitrogen) were added. cDNA synthesis was carried out at 45°C for 1 h. The solution was then heated at 94°C for 10 min and incubated at 4°C until the addition of the PCR mixture for cDNA amplification.

To the final volume of 20 μL of reverse transcription were added 30 μL of the PCR mixture, which had 1× buffer, 2 mmol/L $MgCl_2$, 2.5 U DNA polymerase (Platinum Taq DNA Polymerase), 0.2 μmol/L of oligonucleotides forward (FA1223) (5′-GAAGAGAACGAAGGGACAATGC-3′) and reverse (FA2554) primers to sequencing of the YFV E region. The program used for amplification consisted of a previous denaturation at 94°C for 2 min, followed by 35 cycles, each one composed of denaturation steps at 94°C for 30 s, hybridization at 55°C for 30 s, and extension at 72°C for 2.5 min, followed by a final extension cycle at 72°C for 5 min.

Nucleotide Sequencing

For nucleotide sequencing, the amplicons were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), which uses silicon retention columns, following the protocol described by the manufacturer. The purified cDNA was sequenced by using the ABI PRISM Dye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA, USA), which uses the chain termination methods with dideoxyribonucleotides labeled with different fluorophores for each nucleotide on the 3′ end (*17*).

In the mixture for each sequencing reaction, we used 8 μL of Terminator ready reaction mixture, 2 μL (100 ng) of the PCR product, 3.5 pmol/L of the oligonucleotides FA1223 and FA2554, and water to give a final volume of 10 μL. The sequencing reaction product was precipitated by using an ethanol and isopropanol protocol and subjected to electrophoresis in an automated sequencer ABI PRISM 3130 (Applied Biosystems).

Phylogenetic Analyses and Sequences Obtained

To align and analyze the homology of the sequences obtained from the E gene, we used the computer programs SEQMAN II, EDITSEQ, and MEGALIGN included in the Lasergene package version 4.05 (DNASTAR Inc., Madison, WI, USA) were used. To construct the dendrograms and phylogenetic trees of envelope region, the Molecular Evolutionary Genetics Analysis program, version 4.1

(*18*) was used, applying the neighbor-joining method. The nucleotide distance was calculated by using the Kimura 2-parameter method and bootstrap analyses with 1,000 pseudoreplicates (*19*). To enable better rooting of the phylogenetic tree, we added the strain ASIBI (YFV prototype) and a strain of YFV from Uganda as outgroups.

Results

A total of 358 mosquitoes belonging to 14 taxonomic categories were collected. The most abundant species were *Hg. leucocelaenus* (52.8%) and *Aedes serratus* (14.8%).

From the 189 specimens of *Hg. leucocelaenus* captured, 6 (91.5%) of 7 pools were positive for YFV. YFV was not detected in 1 pool which contained 16 mosquitoes from Linha Caaró County. The calculated minimum infection rate was 1.88% for *Ae. serratus* and 3.70% for *Hg. leucocelaenus*.

YFV was isolated from 7 pools of *Hg. leucocelaenus* mosquitoes collected in Coronel Barros and Caibaté. Moreover, an additional YFV isolate was obtained from a pool of 3 *Ae. serratus* mosquitoes sampled in Coronel Barros (Table 2). All YFV-positive pools showed positive results with the 3 techniques used, except strain BeAr 754962, which was only positive by cell culture and by reverse transcription–PCR.

A phylogenetic analysis with 6 YFV isolates recovered from hematophagous arthopods collected in Rio Grande do Sul (5 from *Hg. leucocelaenus* and 1 from *Ae. serratus*) was carried out after 1,205 nt of E gene were sequenced and compared with sequences of other YFV strains from South America, including a nucleotide sequence of an isolate of *Hg. leucocelaenus* collected in 2001 in Rio Grande do Sul State. The 2008 YFV strain that circulated in the state during the yellow fever outbreak belongs to the South American I genetic lineage. Although it was the same genotype as YFV from *Hg. leucocelaenus*/2001, it was genetically distinct (Figure 2).

Discussion

Yellow fever still represents an infectious disease that causes a high proportion of illness and death. Forty years since the last human yellow fever case in Rio Grande do Sul state, an epizootic was reported in nonhuman primates; the virus was isolated from mosquitoes in southern Brazil in 2001 with no record of infection in humans (*7*). Surprisingly, specimens of *Hg. janthinomys*, considered the main sylvan vector of the YFV in the Americas, were not collected in this region, and *Hg. leucocelaenus* was the most abundant species captured.

Hg. leucocelaenus is a broadly distributed species in South America, although its presence has not yet been documented on the west side of the Andes or in southern Chile and Argentina. This species is found in forests and has diurnal and acrodendrophilic habits, although the mosquito can take blood from hosts at ground level (*20*). In Brazil, this species is most abundant and common in forests in the southern region (*21*).

In Rio Grande do Sul State, *Hg. leucocelaenus* was first documented in the municipality of Taquara in 1932 (*22*). In 1939, it was detected in Santa Rosa, Santo Augusto, and São Luiz Gonzaga municipalities in the northwestern region of the state (*21*).

YFV was isolated from *Hg. leucocelaenus* mosquitoes in 1938 from 16 specimens collected in Rio de Janeiro State (*23*) and in 1944 from a pool with 6 specimens from a forest close to Villavicencio, Colombia (*21*). Sixty years later, during an epizootic in rural areas in the northwestern region of Rio Grande do Sul State, 2 strains of YFV were isolated from 21 specimens of these mosquitoes in a pool with 6 insects captured in the canopy and another pool of 15 *Hg. leucocelaenus* mosquitoes captured at ground level in Santo Antonio das Missões municipality (*7*). At that time, researchers suggested that *Hg. leucocelaenus* mosquitoes could be a secondary vector of YFV, in addition to having a major role in the epidemiology of this arbovirus in the Southern Cone region of South America.

A biologic vector can be classified as a main or primary vector or as an auxiliary or secondary vector, according to the transmission area it is associated with. According to Forattini (*20*), auxiliary vectors contribute to the action of the main vectors when they coexist with, or can take the role of, the main vector, but at a local or regional level. In contrast, the main vectors fulfill this role in broad biogeographic regions.

Table 2. Mosquitoes positive for yellow fever virus, by lot number, collected in Coronel Barros and Caibaté municipalities, Rio Grande do Sul State, Brazil, November 2008

Date	Time	Municipality	Locality	Lot	Species	No.
25	10:00 AM–2:00 PM	Coronel Barros	Rincão Canta Galo	754954	*Haemagogus leucocelaenus*	30
25	10:00 AM–2:00 PM	Coronel Barros	Rincão Canta Galo	754955	*Hg. leucocelaenus*	30
26	10:05 AM–2:15 PM	Coronel Barros	Rincão Canta Galo	754956	*Hg. leucocelaenus*	23
25	10:00 AM–2:00 PM	Coronel Barros	Rincão Canta Galo	754957	*Aedes serratus*	03
19	8:30 AM–11:30 PM	Coronel Barros	Rincão dos Pampas	754962	*Hg. leucocelaenus*	29
27	9:40 AM–1:40 PM	Coronel Barros	Rincão dos Pampas	754963	*Hg. leucocelaenus*	27
26	9:30 AM–12:00 PM	Caibaté	Capão Herval	754984	*Hg. leucocelaenus*	14
25	9:00 AM–4:00 PM	Caibaté	Linha Caaró	754993	*Hg. leucocelaenus*	20

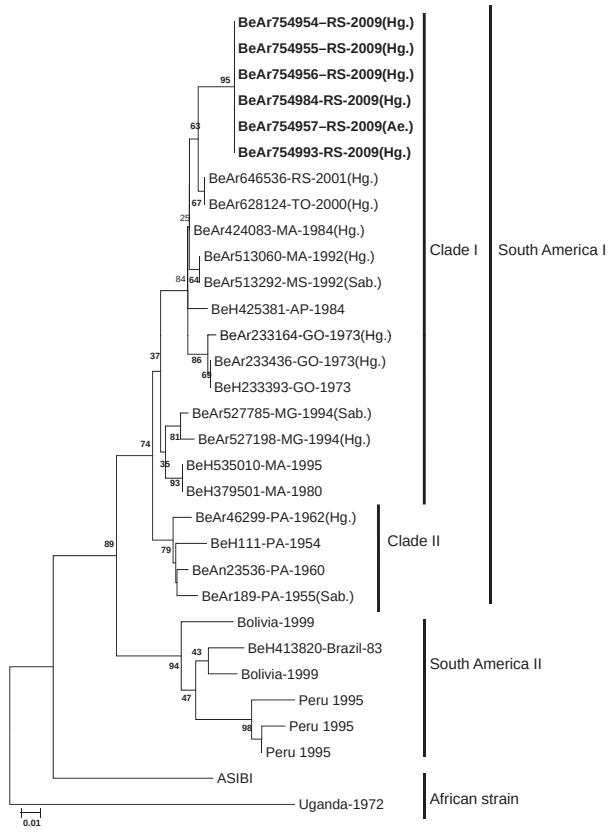

Figure 2. Phylogenetic analysis of partial (1,205 nt) structural region of the envelope gene of 6 yellow fever virus (YVF) isolates (**boldface**) sequenced from samples recovered from hematophagous arthopods collected in Rio Grande do Sul State, southern Brazil, November 2008. Comparison is shown with sequences of 17 genotype I YFV strains from Brazil and with sequences of 6 reference strains of genotype II from South America (Peru, Bolivia, and Brazil) obtained from GenBank. The analysis was performed by the neighbor-joining method; the nucleotide distance was calculated by the Kimura 2-parameter method. Bootstrap values were calculated after 1,000 replicates and are listed only in the main branches. The sequences of YFV strain Asibi and Uganda were used as outgroups. Scale bar indicates a divergence of 10%.

Until now, *Hg. leucocelaenus* has been the only species in the genus with a confirmed presence in Rio Grande do Sul (*24*). Thus, our data support the role of *Hg. leucocelaenus* mosquitoes as the primary vector of the sylvan yellow fever in the state. However, this finding could be broadened to include other states in the southern Brazil.

In São Paulo State, entomologic investigations (*25*) in several municipalities verified that *Hg. leucocelaenus* was one of the most abundant and most frequently captured species in all studied regions during an outbreak of sylvan yellow fever in 2001, but YFV was only isolated from *Hg. janthinomys* mosquitoes. The authors of that study demonstrated the capacity of *Hg. leucocelaenus* to adapt to secondary and degraded environments and, although they collected mosquitoes of other species (*Hg. janthynomis*/*capricornii* and *Hg. spegazzinii*), they highlighted the possibility that *Hg. leucocelaenus* mosquitoes might also be involved in the maintenance cycle of YFV in the area (*25*).

All mosquitoes obtained in Caibaté and Coronel Barros municipalities were collected close to the ground in remnants of deciduous forests, surrounded by soy plantations. Because the continuous canopy of these forests does not exceed 20 m (*12*), and because of the low flight range of *Hg. leucocelaenus* mosquitoes associated with the active search for blood demonstrated by females (*20*), sampling mosquitoes at ground level likely enables collection of an a sizeable number of individuals for taxonomic identification and viral isolation. Thus, canopy platforms are not needed, as they are in other regions of the country, to collect *Hg. janthinomys* and other species. In 2001, when YFV was isolated in 2 pools with 23 mosquitoes, 22 specimens of *Hg. leucocelaenus* were collected at ground level (*7*).

At the Evandro Chagas Institute in northern Brazil (eastern Amazon region), ≈98% of all YFV isolates have been obtained from mosquitoes in the genera *Haemagogus* and *Sabethes*. Only occasionally were species from other genera such as *Ae. fulvus*, *Ae. scapularis*, and *Psorophora albipes* found to be infected, but with only 1 isolate (*2*); thus, these genera lack importance in the maintenance of YFV in nature.

At the Rincão Canta Galo locality, in Coronel Barros municipality, 3 specimens of *Ae. serratus* were collected from which YFV was isolated (strain BEAR 754957). These mosquitoes inhabit temporary puddles on the ground, are abundant in forest environments, and their feeding activity is diurnal with peaks at dusk. Although the females' choice of blood meals is eclectic, they prefer to take blood from large mammals (*20*,*26*).

Records indicating that mosquito species have been naturally infected with other arboviruses suggest that this species can successfully maintain and transmit other pathogens. *Ae. serratus* mosquitoes have been found to be naturally infected with Oropouche virus in the Amazon Region (*27*); with Aura virus in Pará, Brazil, and in Misiones Province, Argentina (*28*,*29*); and with Trocara virus in the Amazon regions of Brazil (Pará State) and Peru (*30*,*31*). Moreover, *Ae. serratus* mosquitoes are considered a secondary vector of Ilheus virus (*27*).

In relation to the molecular study, the distribution of YFV isolates in the present study was monophyletic; they formed a subgroup inside clade I of the South American genotype I. Notably, however, 2 clades (I and II) grouped randomly with strains from different states in Brazil. From these results, we can hypothesize that clades have been formed on the basis of the date of virus circulation and not on the basis of their geographic distribution. Isolates from

southern Brazil show an elevated genetic divergence when compared with strains in clade II, designated "Old Pará" by Vasconcelos et al. (*33*). These results were previously found in the analyses of the junction region N terminal of the nonstructural protein 5/nontranslated 3′ region of 79 samples of YFV from Brazil (*33*).

Thus, the YFV isolates from our study are genetically distinct from other YFV isolates from humans, mosquitoes, and nonhuman primates obtained in other states in Brazil during the 1960s, 1970s, 1980s, and 1990s. An interesting result was that the isolates of the present study showed a homology of 83% with an isolate of YFV from a *Haemagogus* mosquito collected in Tocantins in 2000 (*32*) and with a YFV strain recovered in Rio Grande do Sul in 2001 (*7*).

In summary, this study confirmed the key role of *Hg. leucocelaenus* mosquitoes as a vector of yellow fever in Rio Grande do Sul State. It also demonstrated the natural infection of *Ae. serratus*, which suggests that the latter species might serve as a potential secondary vector of YFV in southern Brazil, and identified that the genotype I South American, clade I was responsible for the yellow fever outbreak and epizootic in Rio Grande do Sul State in 2008. Finally, comparative studies with strains of YFV from primates and humans from the same geographic region should be conducted, using molecular clock analysis to understand the transmission dynamics of the virus.

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Reemergence of Rabies in Chhukha District, Bhutan, 2008

Tenzin, Basant Sharma, Navneet K. Dhand, Nilkanta Timsina, and Michael P. Ward[1]

From January through July 2008, rabies reemerged in the Chhukha district of southwestern Bhutan. To clarify the distribution and direction of spread of this outbreak, we mapped reported cases and conducted directional tests (mean center and standard deviational ellipse). The outbreak resulted in the death of 97 animals (42 cattle, 52 dogs, and 3 horses). Antirabies vaccine was given free of charge to ≈674 persons suspected to have been exposed. The outbreak spread south to north and appeared to follow road networks, towns, and areas of high human density associated with a large, free-roaming, dog population. The outbreak was controlled by culling free-roaming dogs. To prevent spread into the interior of Bhutan, a well-coordinated national rabies control program should be implemented in disease-endemic areas.

Rabies is a fatal zoonosis caused by rabies virus or rabies-related viruses (genus *Lyssavirus*) and transmitted by the bite of a rabid animal (*1*). Domestic dogs are the main (>95%) source of human rabies infection. An estimated 55,000 persons die of rabies in Asia and Africa each year (*2*), >20,000 in India alone (*3*).

In Bhutan, rabies is endemic to the southern districts that border India (*4*,*5*). Domestic dogs are the main reservoir and are responsible for spillover infection of other domestic animals, especially cattle. Sporadic human deaths have also been reported in south-central and southwestern rabies-endemic areas of Bhutan (*6*,*7*).

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On January 23, 2008, a clinical case of rabies in a cow in Dala, a subdistrict of the Chhukha district, was reported and later confirmed by fluorescent antibody test (*8*,*9*). The cow had reportedly been bitten ≈3 weeks earlier by a stray dog with suspected rabies. On the same day, another case was reported (and later confirmed by fluorescent antibody test) in a stray dog in the town of Tshimalakha, Bjachho subdistrict. A retrospective epidemiologic field investigation found that an unreported rabies outbreak in dogs had occurred in the southern villages of Dala subdistrict during November and December 2007.

We report a rabies outbreak in the 3 subdistricts of Chhukha district, Bhutan: Dala, Bongo, and Bjachho (Figure 1). To help develop future control programs, our objectives were to 1) describe the spatio–temporal patterns of the outbreak, 2) generate hypotheses about rabies introduction and spread, 3) assess the relationship between animal rabies and public health, and 4) estimate the cost of the outbreak.

Materials and Methods

Data Sources

Outbreak data were obtained from the Veterinary Information System database and included case date, number and species of animals affected, location (village X and Y coordinates), subdistrict, and date and type of intervention activities implemented during the outbreak. Data on number of human exposures, reasons, and type and number of postexposure prophylaxis doses administered were acquired from local hospitals. The study was conducted January 23–July 31, 2008.

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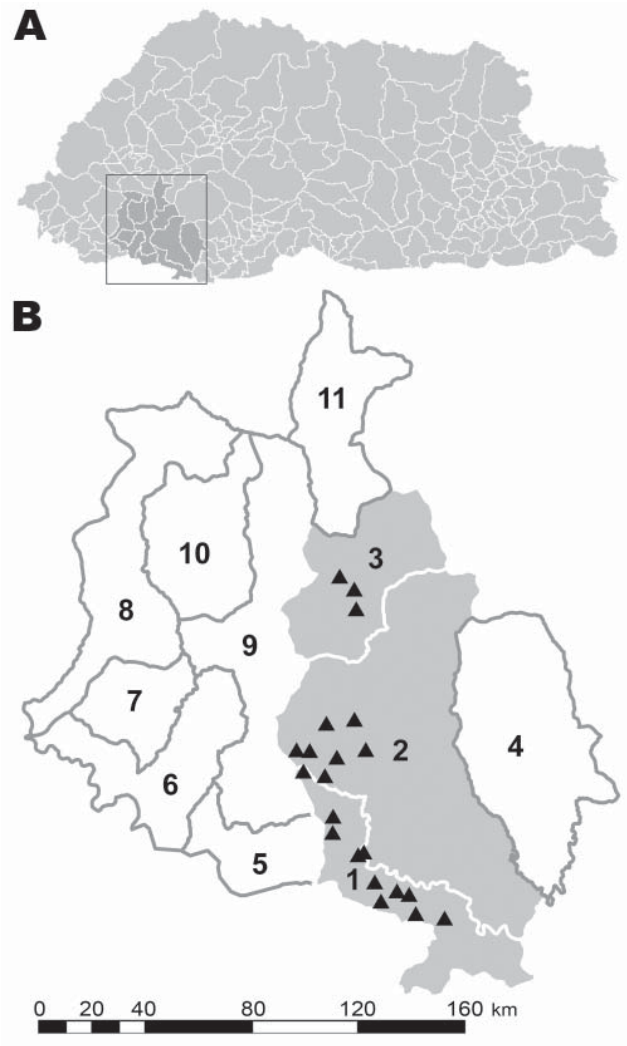

Figure 1. A) Bhutan, with the Chhukha district enclosed. B) The 11 subdistricts of Chhukha district. 1, Dala; 2, Bongo; 3, Bjachho; 4, Genata; 5, Sampheling; 6, Phuentsholing; 7, Logchina; 8, Dungna; 9, Geling; 10, Metap; 11, Chapcha. Gray shading indicates the study areas (1–3); triangles (▲) indicate locations of rabies outbreaks.

Data Analysis

Animal Patterns

The attack rate (no. rabies cases/1,000 animals at risk) was calculated for the outbreak period (*10*). Animal census data for 2008 or dog population data recorded during a vaccination campaign in 2006 in the main towns of Chhukha district were used to calculate attack rates (Table 1) (*11*). The χ^2 test was used to compare differences in dog and cattle attack rates among subdistricts. We tabulated the number of persons exposed in each subdistrict, number of persons who received postexposure prophylaxis, and reasons for doing so.

Temporal Patterns

The distribution of cases over time was investigated by counting the biweekly number of cases. The relationship between intervention measures (e.g., culling and impounding of free-roaming dogs) and the time series of cases was assessed by counting the number of cases before and after implementation of these control measures.

Spatio-Temporal Patterns

The reported cases were mapped (ArcGIS 9.3; ESRI, Redlands, CA, USA) by using a Bhutan shape file (datum: GRS [Geodectic Reference System] 1980, Spheroid; projection: GCS [Geographic Coordinate System] Bhutan Drukref03, Transverse Mercator). The mean center of cases (average X and Y coordinates, useful for tracking changes in distribution) and a standard deviational ellipse (a measure of directional spread), weighted by date of report of cases (*12–16*), was calculated (Spatial Statistics Tools; ArcGIS 9.3).

Economics

The cost of the outbreak was analyzed by using 3 simple, direct-cost calculation methods (*17*). The first calculation was direct cost associated with cattle deaths = number of cattle deaths (n = 42) × average cost of cattle (existing market price of local cattle in Bhutan was Bhutanese ngultrum [Nu.] 10,000). The second calculation was cost of postexposure prophylaxis for humans = total number of human exposures (n = 674) × 5 vaccine doses/person × cost of vaccine (Nu. 450/dose in Bhutan), which was provided free of charge and paid for by the Ministry of Health, Bhutan. The third method was cost of the surveillance and control program, which was calculated on the basis of actual expenditure incurred (removal and impounding of dogs, awareness program), vaccination of ≈200 dogs (at Nu. 25/vaccine dose), and travel and logistics costs for the outbreak response team (paid by the Department of Livestock, Bhutan).

Table 1. Attack rates for reported rabies cases, Chhukha district, Bhutan, January 23–July 31, 2008

Subdistrict and species	No. cases/total population	Attack rate (95% confidence interval)
Dala		
Cattle	18/4,194	4 (3–7)
Dogs	12/601	20 (11–34)
Bongo		
Cattle	21/2,898	7 (5–11)
Dogs	32/1,343	24 (17–33)
Bjachho		
Cattle	3/772	4 (1–11)
Dogs	8/707	11 (6–22)

Results

Animal Patterns

During the study period, 97 cases of rabies (42 in cattle, 52 in dogs, 3 in horses) were reported in the subdistricts of Dala (18 cattle, 12 dogs), Bongo (21 cattle, 32 dogs and 3 horses), and Bjachho (3 cattle, 8 dogs) (Table 1). Incidence was 5 (95% confidence interval 4–7) and 20 (95% confidence interval 15–26) cases per 1,000 population at risk for cattle and dogs, respectively. Incidence did not differ significantly between the 3 subdistricts for dogs (χ^2 3.65, p = 0.16) or cattle (χ^2 3.12, p = 0.21) (Table 1).

Temporal Patterns

The epidemic peak occurred during weeks 11 and 12 (April 3–16), and 65% of cases were reported between weeks 9 and 18 (April and May). The epidemic lasted for 27 weeks and ended in July (Table 2).

Spatio-Temporal Patterns

The outbreak (mean center X = 2,700,680 meters; Y = 1,014,350 meters) had an ellipsoid (south-to-north) distribution (Figure 2, panel A). The mean center during consecutive 2-month intervals moved northward (Figure 2, panel B). These distributions overlapped and had a south-to-north direction; however, during the final phase (June–July), the outbreak was distributed west to east and spread in the main town areas of Gedu in Bongo subdistrict and its surrounding villages (Figure 2, panel B). The distribution of cases followed the road network and towns with high human density and high numbers of free-roaming dogs (Figure 2, panel A; some road network data not shown).

Human Exposure Patterns

A total of 674 persons were reported to have been exposed to animals with suspected rabies. Most (77%) exposures were related to contact with rabid animals while either conducting zoosanitary measures or feeding sick animals and by consuming meat and dairy products derived from suspected rabid animals (Table 3). All persons were given antirabies vaccine (5 doses/person) in the hospital. No human deaths were reported during this outbreak.

Outbreak Control

In the outbreak areas, free-roaming dogs were culled during weeks 6–9, 15–20, and 24; a total of 500 dogs were impounded during weeks 16 and 17 and remained in shelters until the outbreak subsided. In the adjacent unaffected areas, ≈200 dogs were vaccinated against rabies. The general public and school students in the outbreak areas were made aware, through public meetings and media announcements, of the dangers of rabies. Culling and impounding of free-roaming dogs is believed to have controlled the outbreak; no cases were detected after July (Table 2).

Outbreak Cost

The direct outbreak cost was estimated to be Nu. 2.75 million (≈US $59,923; 1 US $ = Nu. 46). This cost included cattle deaths (≈Nu. 42,000; 15%); postexposure prophylaxis for humans (≈Nu. 1,516,500; 55%); and implementation of the rabies control program (Nu. 820,000; 30%). The control program cost included ≈Nu. 500,000 for culling, impounding, and awareness programs; ≈Nu. 5,000 for vaccination of domestic dogs; and ≈Nu. 315,000 for the rapid response team (field surveillance and control activities).

Table 2. No. rabies cases in animals reported biweekly by subdistrict, Chhukha district, Bhutan, January 23–July 31, 2008*

	DalaV*		Bongo†			Bjaccho‡		
Weeks	Cattle	Dogs	Cattle	Dogs	Horses	Cattle	Dogs	Total
1–2	2	0	0	0	0	3	3	8
3–4	1	0	0	0	0	0	0	1
5–6	0	0	0	0	0	0	2	2
7–8	5	0	0	0	0	0	0	5
9–10	1	1	4	3	0	0	1	10
11–12	4	3	6	4	0	0	1	18
13–14	1	2	2	5	0	0	1	11
15–16	0	2	0	9	0	0	0	11
17–18	3	4	0	6	0	0	0	13
19–20	1	0	0	3	0	0	0	4
21–22	0	0	2	0	0	0	0	2
23–24	0	0	3	1	0	0	0	4
25–26	0	0	4	1	2	0	0	7
27	0	0	0	0	1	0	0	1
Total	18	12	21	32	3	3	8	97

*Free-roaming dogs were culled during weeks 6–9 (Mar 27–Feb 27) and week 24 (Jul 3–9).
†Free-roaming dogs in Gedu town were culled during weeks 15–20; dogs were impounded during week 17.
‡Dogs in Tshimalakha and Tshimasham towns were impounded during weeks 16 and 17.

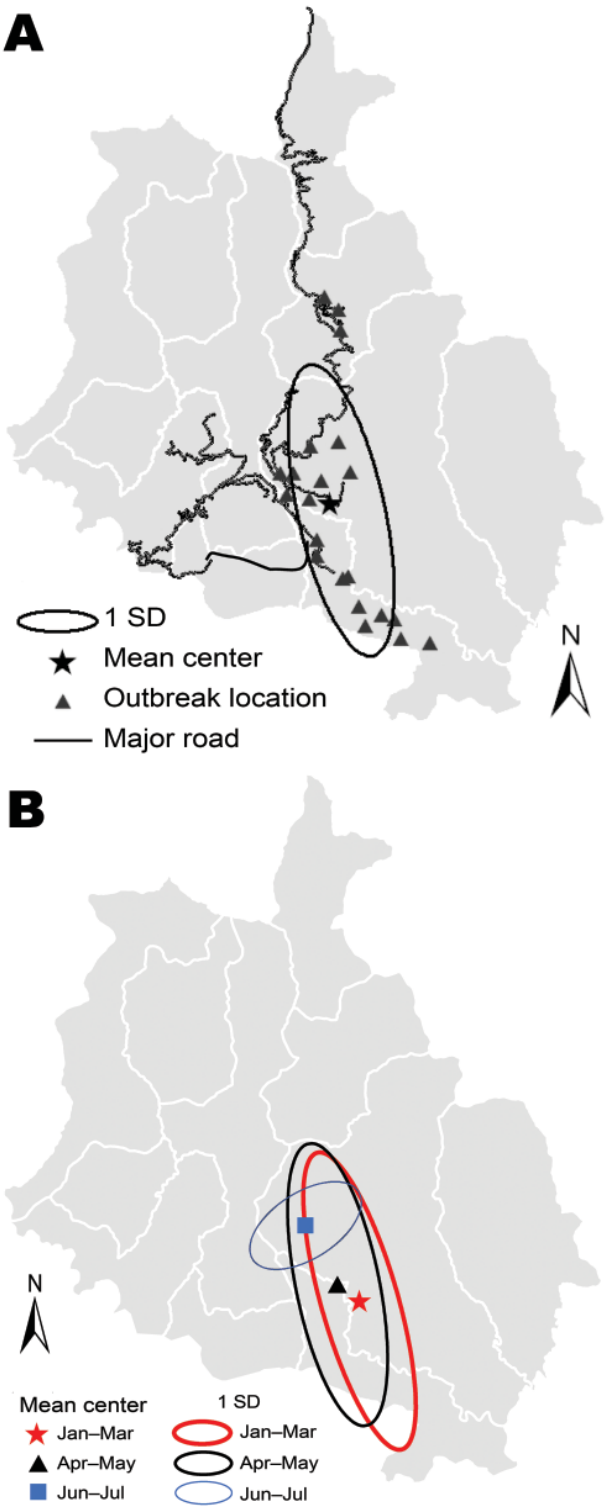

Figure 2. Spatio-temporal patterns of rabies outbreak in the Chhukha district, Bhutan, January 23–July 31, 2008. A) Pattern for the complete outbreak period. B) Patterns during consecutive 2-month intervals during the outbreak period. Jan–Mar period includes January 23–31 (total 69 days; total for other periods 61 days).

Because other indirect costs were not taken into account, these costs are likely underestimates.

Discussion

The rabies outbreak in the Chhukha district initially occurred in dogs in the villages in the southern parts of Dala. The index case dog probably bit several other dogs, resulting in sustained animal-to-animal spread among the free-roaming dog population. After this initial focus of infection, infected free-roaming dogs might have spread the disease by biting cattle and other dogs.

The outbreak spread from south to north and seemed to follow the road network and town areas (Figure 2) that had many free-roaming dogs. High numbers of free-roaming dogs would have provided opportunities for infected dogs to transmit the virus to susceptible dogs and then to cattle in the region. Later, the movement of infected free-roaming dogs from some of these towns might have been responsible for the spread of the disease and spillover infection to cattle in surrounding villages (*5*). However, the culling of free-roaming dogs possibly removed this rabies reservoir from the outbreak areas, resulting in a drastic reduction in the number of cases by June 2008 (*18*). This corroborates anecdotal field evidence that immediate removal of reservoirs can facilitate the control of a rabies outbreak. In contrast, in a similar large rabies outbreak in eastern Bhutan from May 5, 2005, through the end of 2007, mass culling was not implemented because of the religious sentiments of the local people (*5*); in this outbreak, widespread dissemination of rabies persisted for much longer.

Postexposure prophylaxis is crucial for preventing rabies in humans after exposure to any rabid animals. Globally, >10 million persons (mostly in Asia) receive postexposure vaccination against rabies (*18*,*19*). In the Chhukha district outbreak, ≈674 persons were given full courses (at 0, 3, 7, 14, and 28 days; Essen regimen) of antirabies vaccine, provided free by hospitals. However, most exposures likely carried low risk, e.g., feeding sick (rabid) cattle, touching carcasses while conducting zoosanitary procedures, and consuming cooked meat and dairy products derived from cattle that had died of rabies (because of lack of knowledge about rabies). Except for the few who handled meat or carcasses or were bitten by dogs, others fell under the World Health Organization exposure category I (touching or feeding animals, licks on intact skin); rabies vaccination is usually not recommended for such exposures (*19*–*22*). Probably the fear of rabies sensitized the public and ultimately led to mass vaccination of people. Similar mass vaccination after consumption of dairy products from cattle with suspected rabies or handling of rabid animals and contact with confirmed rabies patients has been reported in Bhutan and elsewhere in the world (*5*,*21*–*23*). There are no specific guidelines to assess such nonbite exposure

Table 3. No. human exposures to suspected rabies virus, Chhukha district, Bhutan, January 23–July 31, 2008

Subdistrict and exposure	No. persons exposed
Dala	
Contact with rabid animals	30
Bongo	
Dog bite	130
Contact with rabid animals	132
Consumption of meat or dairy products from rabid animals	120
Other animal bite	22
Bjachho	
Consumption of meat from rabid animals	116
Consumption of dairy products from or contact with rabid animals	124
Total	674

groups. Should a large-scale exposure occur in the future, use of specific criteria and risk assessment for antirabies vaccination may prevent unnecessary use of scarce vaccine resources, whereas public awareness education might prevent future episodes and potential foodborne transmission (*18*,*21*,*22*). Furthermore, in addition to the existing 5-dose intramuscular Essen regimen followed in Bhutan, other postexposure prophylaxis regimens, such as the intradermal method approved by the World Health Organization Expert Committee, should be reviewed because this method is immunogenic, effective, requires fewer doses of vaccine, and costs 70% less than the conventional intramuscular regimen (*19*,*20*,*24*,*25*).

The estimated cost of this outbreak was large by Bhutan standards and reflects the extent of rabies in a resource-limited country (*2*,*4*,*17*). Globally, it has been estimated that > US$ 1 billion per year is spent on rabies prevention programs, mostly on postexposure prophylaxis (*18*,*19*). Similarly, in the Chhukha district outbreak, 55% of the estimated total costs were associated with postexposure prophylaxis for humans. Although vaccinations were free for the recipients, the cost to the Ministry of Health was high. Because the program to eliminate rabies in dogs contributes to the elimination of rabies in humans (or reduces the cost of postexposure prophylaxis), public health and animal health efforts should emphasize the need for control and elimination of rabies in animal reservoirs. In Bhutan, the resources allocated to rabies control in the animal health sector are inadequate and often lead to low vaccination coverage of dogs. Therefore, financial resources should be shared by the public health sector for effective implementation of rabies control and dog management programs.

In conclusion, the Chhukha district rabies outbreak spread consistently from south to north, following the distribution of roads and towns that had large free-roaming dog populations. Rapid culling of in-contact and unvaccinated free-roaming dogs controlled this outbreak. A similar strategy should be considered for any future rabies outbreaks in Bhutan. Because of the risk for spread of rabies from the southern rabies-endemic zone to the rabies-free interior of Bhutan, a well-coordinated national rabies control program should be implemented to prevent and control rabies in Bhutan.

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Mortality Risk Factors for Pandemic Influenza on New Zealand Troop Ship, 1918

Jennifer A. Summers, Nick Wilson, Michael G. Baker, and G. Dennis Shanks

We describe the epidemiology and risk factors for death in an outbreak of pandemic influenza on a troop ship. Mortality and descriptive data for military personnel on His Majesty's New Zealand Transport troop ship Tahiti in July 1918 were analyzed, along with archival information. Mortality risk was increased among persons 25–34 years of age. Accommodations in cabins rather than sleeping in hammocks in other areas were also associated with increased mortality risk (rate ratio 4.28, 95% confidence interval 2.69–6.81). Assignment to a particular military unit, the field artillery (probably housed in cabins), also made a significant difference (adjusted odds ratio in logistic regression 3.04, 95% confidence interval 1.59–5.82). There were no significant differences by assigned rurality (rural residence) or socioeconomic status. Results suggest that the virulent nature of the 1918 influenza strain, a crowded environment, and inadequate isolation measures contributed to the high influenza mortality rate onboard this ship.

To plan and prepare appropriately for future influenza pandemics, public health authorities need to better understand the epidemiology of previous pandemics. Much remains obscure about the epidemiology of the influenza pandemic of 1918–19, the spread of which depended on the transportation of large numbers of troops during World War I.

Pandemic influenza outbreaks among closed military populations are problematic and sometimes show high mortality rates. Reports on this topic have been published. These include descriptions of 1918 pandemic outbreaks in U.S. and Australian troop and civilian ships in 1918–19 (*1–5*), descriptions of 1918 pandemic outbreaks in military camps in the United States, the United Kingdom, and New Zealand (*2*,*3*,*6–8*), and more recent influenza outbreaks onboard naval and civilian ships (*9–12*).

Some studies have investigated specific risk factors for death from the 1918 pandemic. Evidence has shown that lower socioeconomic status increased mortality risk (*13*,*14*) and that young adults, for as-yet-unexplained reasons, had disproportionately higher mortality rates (*13–16*). Rural living versus urban living is another risk factor that has been investigated and has showed conflicting results (*17–20*). Lower mortality rates were observed among seasoned troops (>6 months experience) compared with newly recruited troops, possibly because of previous exposure to respiratory pathogens in seasoned troops (*8*,*21*,*22*).

The purpose of this study was to examine the 1918 outbreak on His Majesty's New Zealand Transport (HMNZT) Tahiti (Figure 1) and to identify mortality risk factors among persons onboard. During and after World War I, HMNZT Tahiti made numerous trips, transporting reinforcements and supplies from New Zealand to Europe, and bringing home New Zealand troops (Figure 2). On July 10, 1918, HMNZT Tahiti departed New Zealand with the 40th Reinforcements, a unit that consisted largely of infantry replacements. The voyage across the Indian Ocean and around the Cape of Good Hope was uneventful. HMNZT Tahiti was to join a convoy in Freetown, Sierra Leone, before heading to England. Upon reaching Freetown, reports of disease ashore resulted in all ships in the convoy being quarantined at port (*7*,*25*). However, a conference was attended by captains and wireless operators from every ship in the convoy onboard the His Majesty's Ship Mantua. The Mantua had experienced an influenza outbreak onboard 2 days after leaving the United Kingdom on August 1, 1918, and is thought to have been responsible for bringing the

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Figure 1. His Majesty's New Zealand Transport Tahiti in Wellington Harbor (c. 1914–1919). Photograph was taken by an unidentified photographer (*23*).

second wave of the 1918 pandemic to western Africa from England (*5*,*26*).

HMNZT Tahiti left Freetown on August 26, 1918, as part of the convoy after being resupplied by local workers (who were another possible source of infection with the new pandemic influenza strain). On the day of sailing, influenza case-patients began to be admitted to the onboard hospital. Over the next few weeks of the voyage, influenza developed in >1,000 of the 1,217 persons onboard (*25*). By the time HMNZT Tahiti reached Plymouth, England, on September 10, 1918, a total of 68 men had died onboard the ship (*23*,*27*). Eight other men and 1 nurse who had been on the ship died of influenza in England. HMNZT Tahiti, the worst affected ship in the convoy, was referred to as the death ship, and a Court of Inquiry was held to investigate this outbreak.

Historical Context and Mortality Data

Historical information was obtained from the official report of the outbreak held in Wellington from Archives New Zealand (*27*), the Inquiry Report from the Transport Epidemic Committee to the House of Representatives of New Zealand, dated December 9, 1918, and the written account of Colonel E.J. O'Neill as officer commanding the 40th Reinforcements (*25*). Individualized data on all military personnel on the July 1918 sailing of HMNZT Tahiti recorded in the Cenotaph database were obtained from the Auckland War Memorial Museum (*28*). An electronic dataset (Roll-of-Honor) covering all deaths among New Zealand military personnel during World War I was obtained from Peter Dennis (Australian Defence Force Academy, University of New South Wales, Canberra, Australian Capital Territory). The Roll-of-Honor and Cenotaph databases were matched to identify persons onboard HMNZT Tahiti whose death from the disease had been listed. The precise cause of death was only reported in the Cenotaph database for 3 of 77 case-patients and was recorded as influenza or pneumonia. One death recorded as a drowning was included because a recently published study showed that the drowning occurred when a febrile soldier aboard HMNZT Tahiti threw himself into the sea (*29*).

Demographic Data

Few records in the Cenotaph database included age data (n = 16). Therefore, the age of those persons aboard HMNZT Tahiti during the voyage was determined for 864 persons (77.4%) on the basis of the soldier's date of birth from the Roll of Casualties held at Archives New Zealand (*30*) and an online database for births, deaths, and marriages in New Zealand (*31*).

Preenlistment occupations were coded for occupational class as per a New Zealand–specific system for historical classification of occupational class (*32*) by using 1919 codes and a website (http://caversham.otago.ac.nz/electors/erform.php). This classification provided results such as laborer (code 9) and company manager (code 1). If an occupation was not listed, the classification for a different census year (e.g., 1924) or the closest match (e.g., orchardist to gardener) was used. Only 13 (1.16%) records had no occupation or could not be coded.

All records with an enlistment address (n = 15) or next-of-kin address (n = 1,088) were given a rurality score on the basis of the rural/urban classification in a previous study (*17*). Because some (n = 167) of these addresses could not be readily classified, further work to assign a rurality score was conducted by using an estimate of likely population levels in 1918 and Google Maps (*33*).

Figure 2. His Majesty's New Zealand Transport Tahiti with World War I troops alongside a wharf (c. 1915). This photograph was presumably taken in a Wellington, New Zealand, wharf, given the gauge of the railway tracks and the crane type. Photograph was taken by David J. Aldersley (*24*).

A scoring system for grading rurality was developed on the basis of occupation and address. All occupations were ranked for likelihood of being a rural-based job: definitely rural = 4 (e.g., farmer); probably rural = 2 (e.g., a fence builder or other occupations); and 0 (e.g., accountant). The final rurality index ranged from 0 (urban) to 8 (rural), which is the combined score of the address rurality score and the occupation rurality score.

Military Data

Military rank was divided into categories on the basis of a key military text (*34*) and other available information regarding the New Zealand Expeditionary Force. These categories were officers, noncommissioned officers, healthcare workers, and others. The Cenotaph database information was used to classify persons by their military units. Most persons onboard HMNZT Tahiti belonged to specific companies within the 40th Reinforcements. All military personnel (n = 30) with embarkation dates before HMNZT Tahiti sailed on July 10, 1918, were identified as persons with previous military experience >1 month of service. The first embarkation date was used to estimate months in military service.

Statistical Analyses

The association of demographic, socioeconomic, and other variables with mortality risk was analyzed by using univariate and multivariate analyses. In multivariate logistic regression analyses, 1 model considered the demographic and sociodemographic factors, and the more fully adjusted model also included military unit. All analyses used Stata version 10 (StataCorp LP, College Station, TX, USA).

Total Number of Cases

The total number of persons onboard HMNZT Tahiti at the time of the outbreak was 1,117 military personnel plus 100 crew (total 1,217 persons). This total included 6 deserters (who embarked in New Zealand but left the ship before the outbreak) but it was not possible to identify these persons and remove them from the dataset. The outbreak onboard reached its illness peak on August 29, 1918, and the peak number of deaths (*20*) occurred on September 4 (Figure 3). The Inquiry Report showed that the military commander estimated that 800 were sick on the peak day (on the basis of those who did not have breakfast and those who had duties caring for the sick) of the outbreak, and the overall mortality rate was 68.9 persons/1,000 population (*25*).

Age Patterns and Mortality Rates

The average age of those onboard HMNZT Tahiti was 26.7 years. Those ≥40 years of age (the smallest age group) had the highest mortality rate (140 persons/1,000 population (Figure 4). Ages were grouped into larger groups than shown in Figure 4 for further analysis. The mortality rate for persons 25–34 years of age was 108.1 persons/1,000 population, which was higher than that for persons 20–24 years of age (70 persons/1,000 population) and was higher than that for all other age groups combined (crude rate ratio [RR] 1.80, 95% confidence interval [CI] 1.08–2.92).

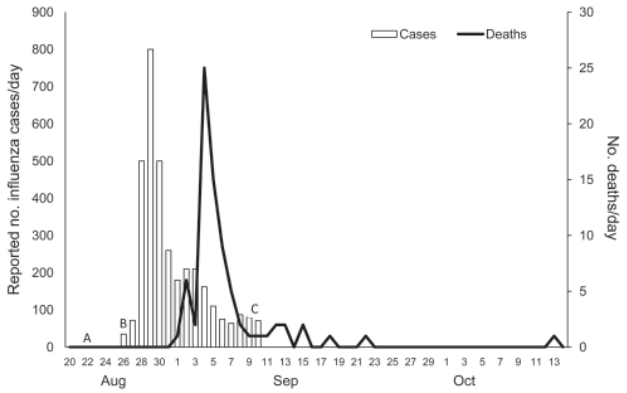

Figure 3. Cases of influenza and mortality rates for persons aboard His Majesty's New Zealand Transport (HNZMT) Tahiti during an outbreak of pandemic influenza, 1918. Reported cases of influenza are approximate and the definition of a case was not precisely described. A, August 22, 1918, HMNZT Tahiti arrives in Sierra Leone; B, August 26, 1918, HMNZT Tahiti leaves Sierra Leone; C, September 10, 1918, HMNZT Tahiti arrives in England (subsequent deaths occurred in hospitals in England).

Military Rank

Officers had the highest mortality rate among military personnel (83.3 persons/1,000 population). However, because only 1 officer died, this result was not significant when compared with the rates for noncomissioned officers and also the rate for all other ranks combined.

Occupation and Rurality

No variations in the mortality rates were found for different occupational classes, rural occupations, and rurality of address. Additionally, no differences in mortality rates could be attributed to rurality scores (Table 1).

Crowding and Military Unit

On the basis of postoutbreak data in archival sources (*25*,*27*), the mortality rate by types of accommodation could be analyzed. This comparison showed a higher mortality rate for persons in cabins with bunks (39/267, 146.1 persons/1,000 population) than for persons in other areas in which hammocks were used (28/820, 34.1 persons/1,000 population) (*25*). This difference was significant (crude RR 4.28, 95% CI 2.69–6.81).

The 8 military units onboard HMNZT Tahiti (40th A, B, C, and E companies, 40th Field Artillery, 40th all groups,

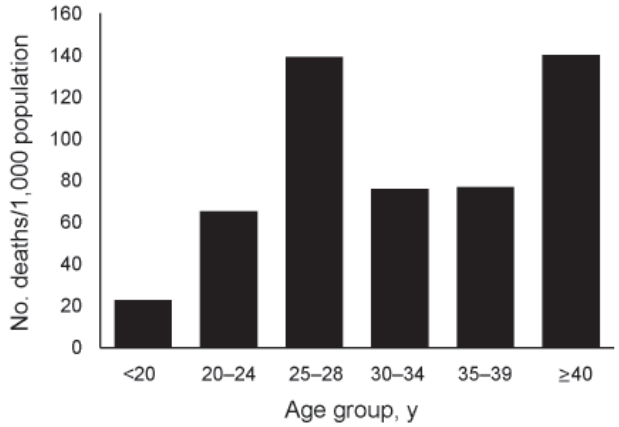

Figure 4. Mortality rates for persons aboard His Majesty's New Zealand Transport Tahiti, by age group, during an outbreak of pandemic influenza, 1918.

Medical Corps and Nursing, and all other groups) were housed separately. Only the 40th Field Artillery, which had a mortality rate of 152.4 persons/1,000 population, had a significantly increased mortality rate (crude RR 2.72, 95% CI 1.16–6.36). Anecdotal evidence in the Inquiry Report suggests that this unit was housed in cabins.

Military Experience

No significant difference in mortality rates was found between persons with military experience and those without experience. Numbers were too small to assess whether the number of months in military service was associated with mortality risk in this outbreak.

Multivariate Analyses

Two logistic regression models were used to analyze risk for death among those onboard HMNZT Tahiti (Table 2). In the more fully adjusted model (model 2), age was independently associated with increased mortality risk. Being in the Field Artillery (versus all other military units) was also independently associated with increased mortality risk (adjusted odds ratio 3.04, 95% CI 1.59–5.82). Military rank, occupational class, and rurality were not associated with mortality risk in either model.

Table 1. Mortality rates during pandemic influenza outbreak, by rurality score, aboard His Majesty's New Zealand Transport Tahiti, 1918*

Rurality score†	No. deaths	Mortality rate/ 1,000 persons	Crude mortality rate ratio (95% CI)
0 (urban)	32	81.0	1.0 (reference)
1–2	18	60.6	0.75 (0.43–1.31)
3–4	12	60.3	0.74 (0.39–1.41)
5–6	7	59.8	0.73 (0.33–1.63)
7–8	8	88.9	1.10 (0.52–2.30)

*CI, confidence interval.
†Calculated by using occupation and address. Highest score was rural occupation plus rural address.

Conclusions

A shipboard epidemic of influenza resulted when persons onboard HMNZT Tahiti were infected in Sierra Leone. The Inquiry Report states that "The disease appeared in severe and epidemic form on August 26." (*25*). The date coincides with the outbreak of the more severe second wave of the pandemic in western Africa (*26*). During the earlier stages of the voyage, the report states that "the number of sick has been remarkably low" (*25*).

The estimated cumulative incidence of pandemic influenza (90%) on HMNZT Tahiti was similar to the highest levels on other ships from Australia, such as the Ooma (88%) (*1*,*4*), and much higher than the estimated cumulative incidence of one of the worst affected US troop ships, USS Leviathan, which had a cumulative incidence of 20% (*2*,*3*). One of the highest reported mortality rates on any ship during the pandemic was that of the Atua, which sailed November 2, 1918 (98.2 persons/1,000 population) (*1*), which was similar to that observed for HMNZT Tahiti, although the Atua was a much smaller ship that was carrying 163 persons.

The nature of the sleeping area (cabins with bunks rather than hammocks) was associated with increased mortality risk in this outbreak. The Court of Inquiry stated that one of the main reasons for the high mortality rate in this outbreak was poor ventilation systems onboard HMNZT Tahiti (*25*). The system of closing port holes at night and during danger periods (bad weather and U-boats in the water) and ineffective wind sails resulted in insufficient ventilation to sleeping areas. It was recommended that some form of artificial ventilation be introduced in the future. Anecdotal evidence from troops interviewed after the outbreak reported that the cabins had poorer ventilation than other accommodations (*25*). This situation may have been caused by makeshift conversions of cabins on HMNZT Tahiti, which potentially blocked ventilation, even though the space allotted to each person was approximately equivalent in both types of accommodation (≈110 ft^3 of airspace/person). Good ventilation may play a role in preventing or limiting spread of viral influenza by airborne transmission. One study of an isolated influenza outbreak onboard a commercial airliner suggested that an inoperative ventilation system was the cause of the high attack rate (*35*). Additionally, 1 study reported that open-air treatment was associated with reduced morality rates during the 1918 pandemic (*36*). However, more recent analysis of viral influenza transmission suggests that the infection is transmitted primarily by contact, followed by droplets, and to a lesser extent by airborne transmission (*37*,*38*).

Military personnel assigned to the 40th Reinforcements Field Artillery had a higher risk of dying from pandemic influenza than any other military unit on HMNZT Tahiti. Evidence from the inquiry suggests that all Field Artillery personnel were lodged in cabins (*25*). However, the Field Artillery personnel were unlikely to be the only unit placed in the cabins, given the numbers in the inquiry.

Although the inquiry found that HMNZT Tahiti was no more crowded than other similar troop ships, it was originally fitted for ≈650 passengers and crew (*39*), noticeably fewer than the 1,217 persons onboard during the July 1918 sailing. This crowding was caused by shipping shortages during World War I, which led to placing as many troops onboard a ship as possible. Isolation measures onboard HMNZT Tahiti, such as clearing deck space for temporary hospitals, were insufficient because the number of patients exceeded the capacity of the onboard hospital (*25*). A crowded environment and inadequate isolation appear to have exacerbated the influenza outbreak, enabling transmission of influenza virus through contact and droplets. These findings serve as a reminder to healthcare planners that the effects of an influenza outbreak within an institutionalized population, such as in hospitals, prisons, and ships, can be devastating without proper preparation beforehand to deal with the variety of potential transmission routes.

Older age was independently associated with increased mortality risk (by logistic regression). This finding was largely reflected in increased risk among persons 25–34 years of age than in persons <25 years of age. This finding is consistent with those of previous research (*13–15*) and with the total New Zealand population, in which the worst affected group was 30–34 years of age, which had a mortality rate of 15.5 persons/1,000 population (*7*). This rate is less than one fourth of the rate on HMNZT Tahiti. This difference may have been caused by crowding, with those onboard HMNZT Tahiti being exposed to higher infective doses of influenza virus or bacterial infections (e.g., *Streptococcus pneumoniae*). Persons onboard HMNZT Tahiti may also have not been exposed to the first wave of the pandemic, and therefore had no immunity to the new pandemic strain (*8*) because there is no evidence of a first pandemic wave in New Zealand before the July 1918 sailing of HMNZT Tahiti (*7*).

The medical and nursing personnel were overwhelmed by the mass casualty event caused by the influenza outbreak; many of them were incapacitated by illness when they were most needed. The use of strychnine, digitalis, and alcohol as stimulants for treating sick personnel onboard may have adversely affected mortality rates, but it is unlikely that any of the medications available in 1918 would have changed the outcome for most soldiers. Injections of an unspecific mixed catarrhal vaccine were given in the weeks before the outbreak (*27*), but what affect, if any, this vaccine may have had is unknown. Nevertheless, another study during this period found that a possibly similar vaccine, also described as a mixed catarrhal vaccine, could have had a favorable affect on influenza-related mortality rates (*40*).

Socioeconomic status and military rank did not appear to effect mortality rates. Additionally, lower occupational status was not related to higher mortality rates, which suggested that any potential differences in nutritional or health status before embarkation or during the voyage did not play any major role in mortality risk. Classifications of rurality by using preenlistment occupation, address, and rurality score did not show any differences in mortality rates. New recruits (first embarkation) were just as likely to die during the outbreak as seasoned troops, which is not consistent with results of previous research (*8,21,22*). However, the numbers of experienced soldiers were small in this particular outbreak.

There are many limitations in studying past events because of transcription and other recording errors. Mili-

Table 2. Multivariate analyses of risk for death during pandemic influenza outbreak onboard His Majesty's New Zealand Transport Tahiti, 1918*

	Model 1: demographics and sociodemographics†		Model 2: model 1 plus military unit‡	
Variable	aOR (95% CI)	p value	aOR (95% CI)	p value
Demographic and sociodemographic				
Age, continuous	1.03 (1.00–1.06)	0.071	1.04 (1.00–1.07)	0.025
Military rank, officer plus NCO vs. all other ranks§	0.51 (0.24–1.12)	0.093	0.49 (0.23–1.08)	0.077
Occupational class based on prewar occupation, groups 7–9 vs. groups 1–6¶	0.72 (0.44–1.18)	0.196	0.83 (0.50–1.38)	0.468
Rurality score, continuous	0.97 (0.87–1.08)	0.558	1.00 (0.90–1.12)	0.943
Other				
Military unit, 40th Reinforcements New Zealand Field Artillery vs all other units combined			3.04 (1.59–5.82)	0.001

*CI, confidence interval; aOR, adjusted odds ratio for death during the outbreak; NCO, noncommissioned officer.
†Hosmer-Lemeshow χ^2 8.47, degrees of freedom 8, p = 0.389.
‡Hosmer-Lemeshow χ^2 13.06, degrees of freedom 8, p = 0.110.
§Healthcare workers were included in other ranks.
¶Groups 1–6 indicate higher status occupations.

tary data were, understandably, never designed to capture detailed epidemiologic information. The lack of a proper case definition and only approximate estimates of case numbers in this outbreak limit their value for estimating epidemiologic parameters such as reproduction number. Use of preenlistment address and next-of-kin address as proxies for rurality may not give an accurate estimate of the geographic exposure of a person. The use of preenlistment occupation as a measure of socioeconomic status is also problematic because many persons may have been assigned to particular reserved occupations for the war effort, which did not reflect their prewar occupation. Conscripted men aboard HMNZT Tahiti may not have been representative of those remaining in New Zealand but they would have had to pass minimum medical standards to be in the military.

The outbreak on HMNZT Tahiti likely represents a worst-case scenario in which nonimmune soldiers were intensively exposed to a highly pathogenic virus while experiencing crowding and ineffective isolation measures. Perhaps the best use of the tragic story of HMNZT Tahiti is as a reminder that although the influenza pandemic that began in 2009 was relatively mild, influenza is capable of causing devastating mass casualties, especially in closed and crowded populations.

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Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, South Korea

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To identify oseltamivir resistance, we analyzed neuraminidase H275Y mutations in samples from 10 patients infected with pandemic (H1N1) 2009 virus in South Korea who had influenza that was refractory to antiviral treatment with this drug. A neuraminidase I117M mutation that might influence oseltamivir susceptibility was detected in sequential specimens from 1 patient.

Since April 2009, pandemic (H1N1) 2009 has spread worldwide and caused the first influenza pandemic of the 21st century. Pandemic (H1N1) 2009 virus initially showed resistance to amantadine but susceptibility to oseltamivir (*1*). Thereafter, 285 cases of oseltamivir-resistant pandemic viral infection were reported worldwide on April 14, 2010 (*2*). However, information is limited about oseltamivir-resistant pandemic influenza in South Korea. Monitoring of community circulation of oseltamivir-resistant viruses has not yet detected any evidence of oseltamivir resistance in South Korea. To identify these viruses, we conducted specific surveillance of antiviral drug–resistant infection in patients whose illness did not resolve after antiviral treatment.

The Study

The study was reviewed and approved by ethics committees of relevant institutions and hospitals. After patients provided informed consent, we obtained >150 clinical specimens from patients in various hospitals in South Korea. Respiratory specimens (>60% nasopharyngeal swab specimens) were obtained during October 2009–January 2010 from patients whose illness had been clinically refractory to antiviral treatment since October 2009.

Viral RNAs were isolated from specimens of 10 patients (Table 1) by using the QIAamp viral RNA Mini Kit (QIAGEN, Crawley, UK). PCR products of the neuraminidase (NA) and matrix 2 (M2) genes were generated by reverse transcription–PCR with primers for NA (forward: 5′-AAATTAACGGGCAATTCCTCTCT-3′; reverse: 5′-CCGAAAATCCCACTGCATATGTAT-3′) and M2 (forward: 5′-CTAGCTCCAGTGCTGGTCTGA-3′; reverse: 5′-CTCAGGCACTCCTTCCGTAGA-3′). DNA sequences of NA and M2 reverse transcription–PCR products were analyzed by using the Big-Dye Terminator Sequencing Reaction Kit and an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). A total of 58 NA sequences and 52 M2 sequences were obtained and analyzed by

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Learning Objectives

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

- Describe the pattern and characteristics of resistance seen with antiviral agents in pandemic (H1N1) 2009 in South Korea

Editor

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Table 1. Characteristics of 10 patients infected with oseltamivir-resistant pandemic (H1N1) 2009 virus, South Korea, 2009

Patient	Age, y/ sex	Antiviral treatment and date	Date of specimen collection	Type of clinical specimen	Underlying condition
A	5/M	Oseltamivir, 30 mg 2×/d (Oct 29–Nov 4); 60 mg 2×/d (Nov 5–9)	Nov 6	Nasopharyngeal swab	Mitochondrial cytopathic changes (bedridden)
B	46/M	Oseltamivir, 150 mg 2×/d (Nov 6–15); zanamivir, 10 mg 2×/d (Nov 16–26)	Nov 18	Nasopharyngeal swab	Leukemia
C	1/F	Oseltamivir, 30 mg 2×/d (Nov 16–21); 60 mg 2×/d (Nov 22–Dec 8)	Nov 21 and 22	Nasopharyngeal swab	Brain damage (fatal)
D	2/M	Oseltamivir, 90 mg 2×/d (Nov 26–30); 180 mg and amantadine, 65 mg 2×/d (Dec 2–7)	Nov 22, Dec 2	Nasal suction	None
E	3/F	Oseltamivir, 30 mg 2×/d (Dec 1–5 and 7–11)	Dec 1, 7, and 9	Oropharyngeal swab (Dec 1, 7); nasopharyngeal swab (Dec 9)	Asthma
F	3/F	Oseltamivir, 45 mg 2×/d (Dec 10–15); 75 mg 2×/d (Dec 16–20)	Dec 18	Oropharyngeal swab	Delayed development
G	1/F	Oseltamivir, 30 mg 2×/d (Dec 1–5); peramivir, 75 mg 1×/day (Dec 12–18)	Dec 1, 5, and 15	Nasal/oropharyngeal swab	Myelodysplasia (fatal)
H	63/M	Oseltamivir, 150 mg 2×/d (Dec 7–15); zanamivir, 10 mg 2×/d (Dec 16–20)	Dec 16	Oropharyngeal swab	Diabetes
I	58/M	Oseltamivir, 150 mg 2×/d (Dec 16–18); peramivir, 600 mg 1×/d, amantadine, 100 mg 2×/d a day, ribavirin, 300 mg 1×/d (Dec 19–25); zanamivir, 10 mg 2×/d (Dec 26–2010 Jan 1)	Dec 26	Nasopharyngeal swab	Cancer
J	60/M	Oseltamivir, 75 mg 2×/d (Nov 30–Dec 2)	Dec 1	Viral RNA	Diabetes, cardiac disorders (fatal)

using the sequence analysis tool in the Influenza Sequences and Epitopes Database for detecting oseltamivir-resistance mutations (*3*).

Ten patients were detected who had oseltamivir-resistant pandemic (H1N1) 2009 virus with the H275Y substitution in viral NA (Table 2). Oseltamivir resistance was associated with oseltamivir treatment on the basis of H275Y changes from the oseltamivir-sensitive genotypes to oseltamivir-resistant genotypes of viral NA in consecutive samples from the same patient. Furthermore, a novel NA (I117M) substitution that may be associated with oseltamivir resistance (*4*,*5*) was detected in specimens from 1 patient (patient G) who had myelodysplasia and received oseltamivir and peramivir (Tables 1, 2).

In addition, we cultured viral isolates from clinical specimens (patients A and C) and evaluated antiviral susceptibility by measuring the dose of oseltamivir and zanamivir required for 50% inhibition (IC_{50}) of NA activity. One isolate of pandemic (H1N1) 2009 virus with an oseltamivir-sensitive genotype (H275 in its NA) was susceptible to oseltamivir (IC_{50} 1.18 nmol/L) and zanamivir (IC_{50} 0.42 nmol/L). Viral isolates from patients A and C with an oseltamivir-resistant genotype (Y275 in NA) were resistant only to oseltamivir (IC_{50} 713.2 nmol/L and 359.4 nmol/L, respectively). Susceptibility to zanamivir was not altered whether NA contained Y275 or H275 (IC_{50} 0.13 nmol/L and 0.78 nmol/L, respectively).

Patients with oseltamivir-resistant pandemic (H1N1) 2009 were treated during hospitalization with oseltamivir alone or with a combination of other antiviral drugs (Table 2). Active surveillance that evaluated spread of oseltamivir-resistant viral infections among hospital staff, family members, and other patients who had contacted with or cared for the patients showed no evidence of virus transmission in the hospitals.

Conclusions

During pandemic (H1N1) 2009, oseltamivir-resistant viral infections were found mainly in immunocompromised patients who were treated with antiviral drugs and chemoprophylaxis (*6*). In our study, genetic characteristics of oseltamivir resistance in virus isolates from all 10 patients showed the H275Y substitution in NA, resulting in phenotypic resistance to oseltamivir but not to zanamivir. In addition, a novel NA mutation (I117M) was detected in virus from patient G (Tables 1, 2). The NA 117 residue is near R118 and may have an effect on this residue, 1 of 3 arginine residues that bind the carboxylate region of the sialic acid substrate (*5*,*7*). This finding implies that changes at NA residue 117 may influence oseltamivir susceptibility of the virus. For example, the NA I117V mutation in avian influenza virus (H5N1) was reported to increase resistance to oseltamivir (*5*,*6*). The 117 residue is also highly conserved in all N1 typed viruses (*5*). These results indicate that the I117M substitution in NA of pandemic (H1N1) 2009 virus may influence oseltamivir resistance of this virus.

Multiple specimens were available from patients D, E, and G for analyzing antiviral resistance mutations in

Table 2. Amino acid differences in HA and NA of viruses from 10 patients infected with oseltamivir-resistant pandemic (H1N1) 2009 virus, South Korea, 2009*

Strain or patient	Sample†	Amino acid sequences																	
		NA		M2	HA														
		275	117	31	22	56	83	116	119	120	128	134	155	197	203	249	283	321
Kor/01‡§		**H**	**I**	N	K	N	S	S	I	T	S	A	G	A	**S**	V	K	V
A‡	1	Y	I	N	K	N	S	S	I	T	S	A	G	**T**	T	V	K	V
B	1	Y	I	N	K	N	S	**N**	**I**	**I**	S	A	G	A	T	V	K	V
C‡	1	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	K	V
	2	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	K	V
D	1	**H**	I	N	K	N	S	S	I	T	**P**	A	G	A	T	**M**	K	V
	2	Y	I	N	K	N	S	S	I	T	**P**	A	G	A	T	**M**	K	V
E	1	**H**	I	N	K	N	S	S	I	T	S	A	G	A	T	**L**	K	V
	2	**H**	I	N	K	N	S	S	I	T	S	A	G	A	T	**L**	K	V
	3	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	**L**	K	V
F	1	Y	I	N	K	N	S	S	I	T	S	**T**	G	A	T	V	K	V
G	1	**H**	**M**	N	**R**	N	S	S	I	T	**P**	A	G	A	T	V	K	V
	2	**H**	**M**	N	**R**	N	S	S	I	T	**P**	A	G	A	T	V	K	V
	3	Y	**M**	N	**R**	**S**	S	S	I	T	**P**	A	**E**	A	T	V	K	V
H	1	Y	I	N	K	N	S	S	**M**	T	S	A	G	A	T	V	K	V
I	1	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	**E**	V
J¶	1	Y	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cal/07#		**H**	I	N	K	N	**P**	S	I	T	S	A	G	**T**	**S**	V	K	**I**
Cal/04**		**H**	I	N	K	N	**P**	S	I	T	S	A	G	A	**S**	V	K	**I**

*HA, hemagglutinin; NA, neuraminidase; M, matrix. Gene sequences for 16 clinical specimens from 10 patients were submitted to GenBank (accession nos. CY060453–CY060498). Amino acids that differ are shown in **boldface**. NA sequence numbering uses N1 nomenclature.
†Matches the temporal order shown in the Figure.
‡Viral isolation and phenotyping of oseltamivir resistance were performed.
§Pandemic A (H1N1) virus from South Korea (GenBank accession nos. GQ132185, GQ131025, and GQ131023).
¶Specimen from patient J was viral RNA. –, not available.
#A/California/07/2009 virus (GenBank accession nos. GQ377078, FJ969537, and FJ969540).
**A/California/04/2009 virus (GenBank accession nos. FJ969517, FJ969513, and GQ280797).

NA. Analysis of viruses from these patients showed that the H275Y mutation emerged in the later stages of viral infection, during oseltamivir treatment; infective viruses initially had an oseltamivir-sensitive genotype (H275) (Table 2; Figure).

A mixture of oseltamivir-sensitive (H275 in NA) and oseltamivir-resistant (Y275 in NA) viruses was observed in the second specimen from patient E and the third specimen from patient G (Figure). These specimens showed double signals (nucleotides T and C) for TAC and CAC encoding tyrosine (Y) and histidine (H), respectively. There are 2 possible explanations for mixtures of oseltamivir-resistant and oseltamivir-sensitive viral genotypes. The first explanation is reinfection with Y275 virus after a previous infection with H275 virus. The second explanation is an H275Y mutation caused by selective pressure from oseltamivir treatment. Our molecular epidemiologic data (Table 2) support the second explanation that selective pressure from oseltamivir treatment caused the H275Y substitution in NA because sequential viruses from the same patient had identical HA sequences in the absence of additional infection (*8*).

Multiple specimens from the same patient showed identical hemagglutinin sequences despite amino acid changes (H to Y) at position 275 of NA (hemagglutinin sequences of patients D, E, and G) (Table 2). This finding indicates that temporally sequential viruses from a patient were likely generated from the same viral progenitor without further viral infection. Conversely, our findings also support absence of epidemiologic links among current cases of oseltamivir resistance, given that viruses from 9 patients in this study had their own signature hemagglutinin amino acid sequences (Table 2).

Spreading and clustering of oseltamivir-resistant strains in the general population has not been observed in South Korea. However, in the present study, a comparatively high rate of emergence (19%) of oseltamivir-resistant viral infections was observed in unhealthy patients treated with antiviral drugs. We also detected a novel substitution (I117M) in NA, which might influence susceptibility to NA inhibitors. Our findings demonstrate that oseltamivir-resistant quasispecies of pandemic (H1N1) 2009 viruses can be generated. This suggestion indicates that continuous surveillance is required to evaluate emergence and circulation of drug-resistant pandemic (H1N1) 2009 viruses and possible reassortment with other viruses that have oseltamivir resistance.

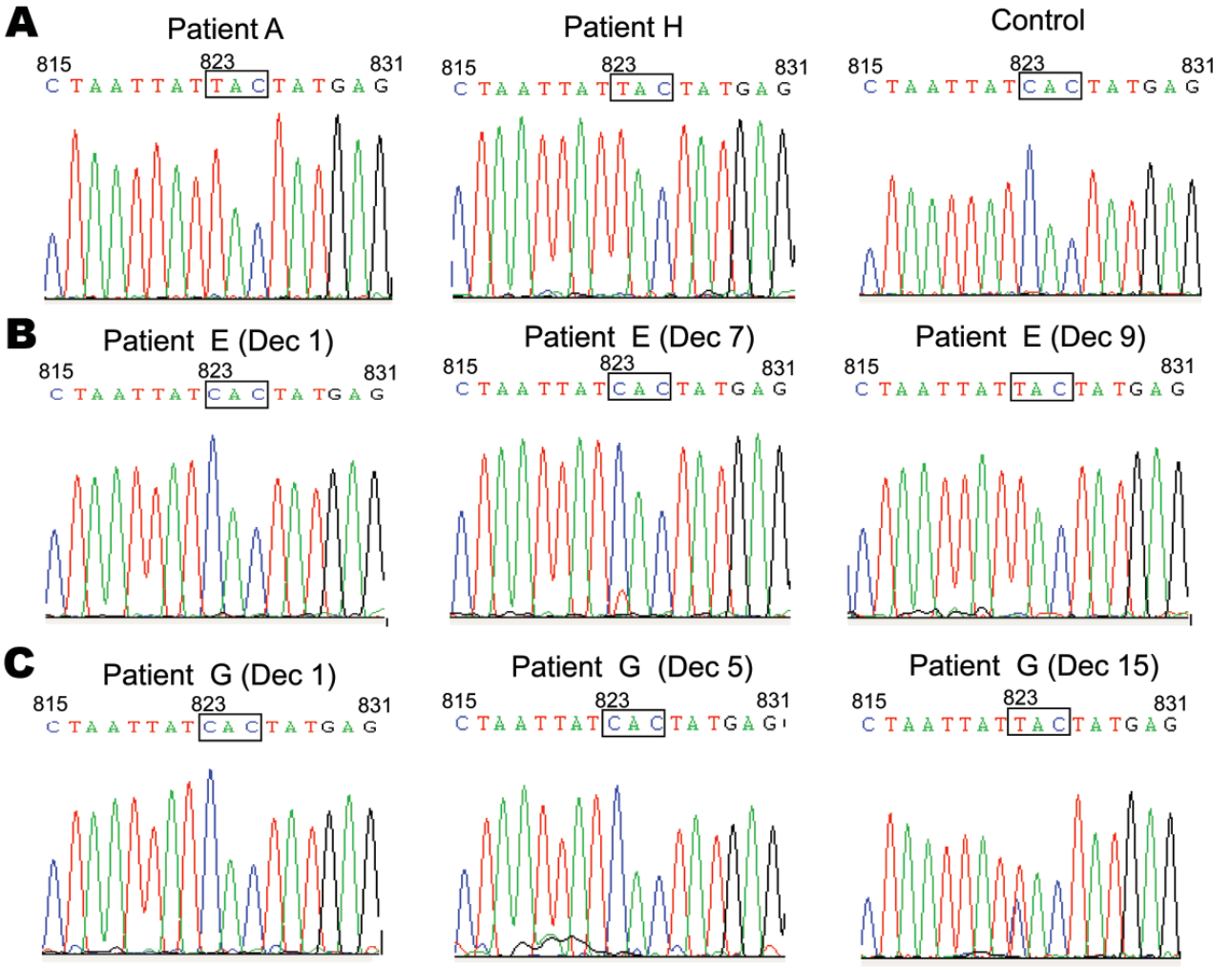

Figure. Evolution of oseltamivir-sensitive H275 in pandemic (H1N1) 2009 virus to oseltamivir-resistant virus (mutation Y275) in the neuraminidase (NA) gene, South Korea. A) NA sequences of viruses from patient A, patient H, and strain A/Korea/01/2009 (H1N1) (Control) (Table 2). Viruses from these 2 patients had triplet TAC (encoding tyrosine in NA 275), indicating that these patients were infected with oseltamivir-resistant virus, in contrast to A/Korea/01/2009 (H1N1) of the oseltamivir-sensitive control showing CAC (encoding histidine) in NA 275. B) NA sequences from specimens obtained on different dates from patient E. This patient was initially infected with oseltamivir-sensitive virus with CAC in NA 275 (2009 Dec 1). Virus then showed mutation 823C (blue dominant peak) in the boxed CAC and a minor signal 823T (small red peak) in NA 275 (2009 Dec 7). In the third specimen (2009 Dec 9), only TAC in NA 275 was observed. C) Oseltamivir-sensitive virus infection (CAC in NA 275) was observed in the first 2 specimens from patient G (2009 Dec 1 and Dec 5). Mixed signals of dominant 823T (red peak) and minor 823C (blue peak) forms were seen in most recent specimen (2009 Dec 15), indicating a mixed infection with oseltamivir-resistant virus and oseltamivir-sensitive virus. Nucleotide coordinates (815 and 831) refer to NA gene sequence of pandemic (H1N1) 2009 virus. Amino acid residue 275 in the NA gene is encoded by 3 nt indicated in boxes.

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Ocular Thelaziosis in Dogs, France

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During 2005–2008, veterinary practitioners reported ocular infection by *Thelazia* spp. nematodes in 115 dogs and 2 cats in southwestern France. Most cases were detected in Dordogne, particularly in 3 counties with numerous strawberry farms, which may favor development of the fruit fly vector. Animal thelaziosis may lead to emergence of human cases.

Thelazia spp. (Spirurida, Thelaziidae) nematodes live in the conjunctival sac of warm-blooded vertebrates. These nematodes are responsible for epiphora, conjunctivitis, keratitis, and corneal ulcers (*1*–*3*). *Thelazia* spp. nematodes are transmitted by different species of flies feeding from the lacrimal secretions of the definitive hosts. Among the 10 species, *T. californiensis* and *T. callipaeda* parasitize carnivores and sometimes humans. *T. californiensis* is confined to the western United States and has never been reported in Europe (*1*). *T. callipaeda*, the "oriental eye worm," is common in the former Soviet republics and in India, Thailand, People's Republic of China, and Japan (*2*), where it causes infections in humans, dogs, and cats (*3*). Wild mammals, such as foxes and lagomorphs, are reservoir hosts for the nematodes. During the past decade *T. callipaeda* infection was proven to be widespread among dogs and cats from northern (Aosta valley) to southern (Basilicata region) Italy (*4*). In Ticino, a region of southern Switzerland, a retrospective study identified 106 *T. callipaeda*–positive dogs and 5 positive cats during 2005–2007 (*5*). Recently, the first autochthonous case of thelaziosis in a dog was described in southern Germany (*6*). Locally transmitted cases of thelaziosis were first reported in 4 dogs and 1 cat that lived or spent time in the department of Dordogne in southwestern France (*7*).

The Study

At the end of 2008, we contacted veterinary practitioners from 938 veterinary practices in 16 departments in France by regular mail. The survey covered a large part of southwestern France (Figure 1), where the first thelaziosis cases in dogs and cats were reported in 2007 (*7*). Veterinary practitioners were asked whether they had diagnosed ocular thelaziosis in a dog or a cat during the previous 3 years. For each clinical case, a short questionnaire asked for a description of the animal (i.e., sex, age, breed, use), description of the place where the animal lived, and treatment protocol.

A total of 117 clinical cases of thelaziosis (115 dogs and 2 cats) was reported in 22 veterinary practices from 9 departments (Ariège, Dordogne, Gironde, Haute-Garonne, Lot-et-Garonne, Puy-de-Dôme, Pyrénées-Atlantiques, Tarn, and Tarn-et-Garonne). Most (104 [89%]) cases were diagnosed from 10 practices in Dordogne (Figure 1). In each of the other departments, only a few (1–6) cases were diagnosed. Furthermore, most of the infected animals in other departments had spent time in Dordogne a few months before clinical signs developed. In Dordogne, most cases were from the center of the department, with 3 counties overrepresented (60 cases in Vergt, 16 cases in Saint-Pierre-de-Chignac, and 9 cases in Villamblard) (Figure 2). In these counties, strawberry production is predominant and may favor development of the fruit fly vector, *Phortica variegata*; in other areas of Dordogne, other types of fruit production (plum or apple) are reported. All infected dogs and the cats were 6 months–14 years of age and privately owned. Ninety-one (78%) of the 117 animals lived in a

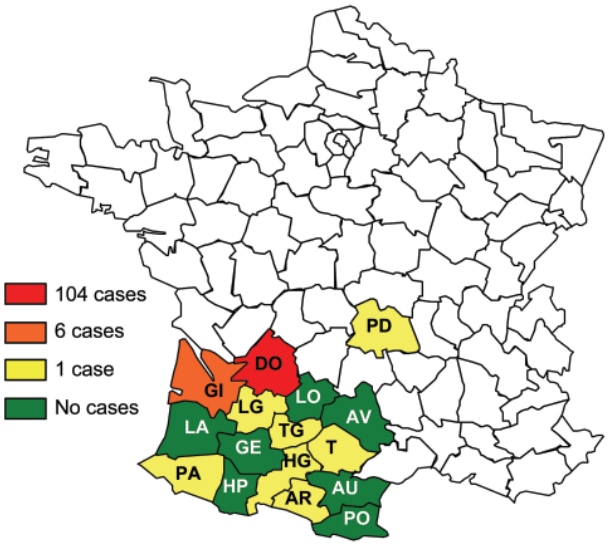

Figure 1. Departments in which the epidemiologic survey for thelaziosis was conducted and number of cases of canine and feline thelaziosis, France, 2005–2008. Clinical cases of thelaziosis were reported in 9 departments. PO, Pyrénées-orientales; AR, Ariège; AU, Aude; AV, Aveyron; DO, Dordogne; GE, Gers; GI, Gironde; HG, Haute-Garonne; HP, Hautes-Pyrénées; LA, Landes; LG, Lot-et-Garonne; LO, Lot; PA, Pyrénées-Atlantiques; PD, Puy-de-Dôme; T, Tarn; TG, Tarn-et-Garonne.

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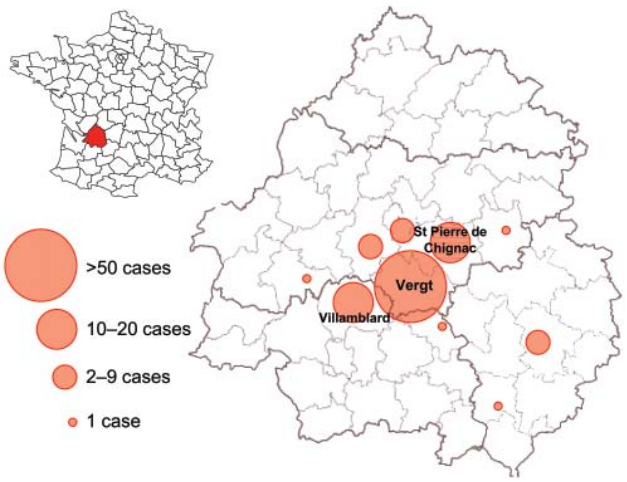

Figure 2. Department of Dordogne (with its 4 arrondissements and 50 counties) and distribution of clinical cases of thelaziosis in dogs and cats, France, 2005–2008. Most cases were reported in the counties of Vergt, Saint Pierre de Chignac, and Villamblard.

small village; 22 were farm dogs. Twenty-six animals lived in a city, but all had free access to the outdoors.

The animals were referred to veterinary practitioners for unilateral or bilateral conjunctivitis. For all animals, nematodes were observed on the eye surface. The first cases of thelaziosis were detected in 2 dogs and 1 cat in the county of Vergt at the end of 2005. During 2006, a total of 27 cases were detected in late summer and autumn; animals may have been contaminated by infected vectors during the peak of the male *Phortica* spp. fly population in summer 2006. During 2007 and 2008, clinical cases were detected throughout the year. The apparent absence of seasonality for detecting adult *Thelazia* spp. nematodes in definitive hosts is in accordance with previous observations in areas in Italy to which *Thelazia* spp. nematodes are endemic (*8*).

Nematodes were collected from the eyes of 19 dogs and 1 cat and morphologically identified according to Skrjabin et al. (*9*). To determine the haplotype sequence, we processed specimens using the specific amplification of a partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*, 605 bp), as previously described (*10*). The sequences obtained were identical to the sequence representing haplotype 1 of *T. callipaeda* (GenBank accession no. AM042549) previously reported in Italy and Switzerland but they displayed a 1.3%-nt difference from the haplotype recently detected in Germany (*6*).

Discussion

Before 2005, thelaziosis had been reported only sporadically in France (*11*,*12*), occurring in dogs that had spent time during summer in northern Italy. In 2007, Dorchies et al. described 5 locally transmitted thelaziosis cases from southwestern France (*7*). The present investigation indicates that Dordogne and, more precisely, the county of Vergt should now be considered as an area to which ocular thelaziosis is endemic. This area is near the Atlantic Ocean and is part of the Aquitaine Region (44°–45°N, ≈0°). Its altitude ranges from 112 m to 246 m, and it has an oceanic climate with an average of 800 mm annual rainfall. This area is at the same latitude as Aosta valley in northern Italy, where thelaziosis in dogs is regularly reported. It belongs to the putative areas in which the drosophilid species *P. variegata*, the *T. callipaeda* vector, could be present according to a predictive geoclimatic model in Europe (*13*).

The *T. callipaeda* nematode may have been introduced in France by importation or dispersal of vectors and/or reservoir hosts. The dispersal of infected vectors is unlikely because fruit flies are not as robust as other vectors, such as mosquitoes, and are not known to disperse by wind. Introduction by an infected animal seems to be more likely. Adult parasites may have been introduced by a dog (or a small number of dogs) that spent time in a thelaziosis-endemic area in Italy or southern Switzerland during 2005. Another explanation for the introduction of thelaziosis in Dordogne would be migration of infected wild animals (such as foxes) from Switzerland or Italy. However, Dordogne is far from these areas (500 km–600 km) and separated by the Alps. In such circumstances, the possibility of population exchanges is limited. Our final explanation could be the importation of wild hares for hunting in Dordogne. Introduction of infected hares from Italy already has been implicated in outbreaks of animal and human cases of tularemia in Dordogne (*14*). A recent investigation in southern Italy demonstrated the existence of an active sylvatic life cycle of *T. callipaeda* nematodes (*15*). Further studies in the county of Vergt should include investigation of eye worms in wild mammals.

Conclusions

Once introduced in Dordogne, this parasite might have found appropriate conditions for the perpetuation of its life cycle. Our investigation showed that cases in companion animals were located where strawberry production was predominant. *T. callipaeda* nematodes may be transmitted to humans, and animal thelaziosis in Dordogne may lead to emergence of human cases.

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Dr Ruytoor graduated from the Veterinary College of Alfort, France. Her research interests include the emergence of canine thelaziosis in southwestern France.

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Emergence of African Swine Fever Virus, Northwestern Iran

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In 2008, African swine fever was introduced into Georgia, after which it spread to neighboring Armenia, Azerbaijan, and the Russian Federation. That same year, PCR and sequence analysis identified African swine fever virus in samples from 3 dead female wild boars in northwestern Iran. Wild boars may serve as a reservoir.

African swine fever (ASF) is a notifiable, highly contagious, lethal, hemorrhagic disease in domestic pigs (*1*,*2*). ASF virus (ASFV) (International Committee on Taxonomy of Viruses database no. 00.002.0.01.001), an enveloped double-stranded DNA virus, is the only known DNA arbovirus (*3*). Maintenance and transmission of ASFV involves cycling of virus between soft ticks of the genus *Ornithodoros* and wild pigs (warthogs, bush pigs, and giant forest boars) (*1*,*2*). The virus can also be acquired through ingestion of contaminated feed.

The syndrome caused by ASFV in pigs was initially described in Kenya and later in most other African countries (*1*,*4*). In Africa, it causes a long-term, persistent infection in warthogs and bush pigs (*2*,*3*,*5*). Clinical diagnosis of ASF is difficult because signs of ASF and other hemorrhagic diseases are similar and because virulence varies among ASFV isolates (*1*,*2*,*5*,*6*).

In June 2007, ASFV was identified in the Caucasus region, including Georgia, Russian Federation, and Armenia (*2*). Diagnosis near the port of Poti, Georgia, was based on clinical findings, and virus identification was later confirmed by laboratory investigations. ASFV might have been introduced into Georgia by ships carrying contaminated pork or pork products from other countries. After entering Georgia, the virus extended into Armenia in August 2007. The probable route of virus entry into Armenia was movement of infected pigs and wild boars across the border (*7*). By the end of 2007, an outbreak had occurred in Yerevan and Ararat, after which 1 additional case occurred in February 2008.

In December 2007, the Russian Federation reported its first ASF outbreak since the 1970s. The virus may have entered through neighboring Georgia (*7*,*8*). In January 2008, presence of ASF was officially confirmed in northwest Azerbaijan, ≈180 km east of the Georgia border (village of Nic). Because most residents of Nic keep pigs in backyard smallholdings, ASFV may have entered Nic in contaminated pork (or pork products) or in infected wild boars (*7*,*8*).

In December 2008 and January 2009, ASFV spread to wild boars in northwestern Iran. As in Georgia, initial diagnosis was based on clinical signs and postmortem examination. Virus identification was subsequently confirmed by laboratory investigations.

The Study

The wild boar population in northwestern Iran is 12,000–13,000. Boars affected by ASFV show weakness, difficulty walking, dragging of the hind legs, dysentery, and sudden death. The first report of dead boars came from 2 villages (Oshdibin, and Namngah) in the Jolfa area and then from other cities such as Ahar, Sarab, Maragheh, and Marand. The disease spread to the city of Khoie, located in another province (West Azarbaijan). Postmortem histopathologic investigations of tissue samples of 3 dead, female, wild boars found characteristic signs of ASF, such as disseminated intravascular coagulation with multiple hemorrhages (Figure).

Viral DNA from 6 tissue samples (kidney, liver, lung, large intestine, heart, and spleen) from the 3 dead boars was extracted by using the JETQUICK Tissue DNA Spin Kit (GENOMED GmbH, Lohne, Germany) according to the manufacturer's instructions. ASFV was detected by PCR and SYBR Green real-time PCR on a Rotor-Gene 65H0 (Corbett Life Sciences, Sydney, New South Wales, Australia) in all samples from each boar; primers used were P72 D, U (major capsid protein), and $PPA_{1,2}$ (in the viral protein 73 coding region of the genome) (*6*,*9*). Melting curve analysis showed that an elevated temperature of 86.7°C could generate a specific peak in this curve. The sequences of the PCR products derived by using PPA primer pairs were analyzed by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and showed 100% similarity to submitted sequences of Georgia isolates. A partial sequence of the isolates from Iran has been submitted to GenBank under accession no. FJ897727.

Conclusions

To confirm a specific pathogen and trace the possible sources of infection, genetic characterization of the virus strain associated with disease is crucial (*1*,*2*). Real-time

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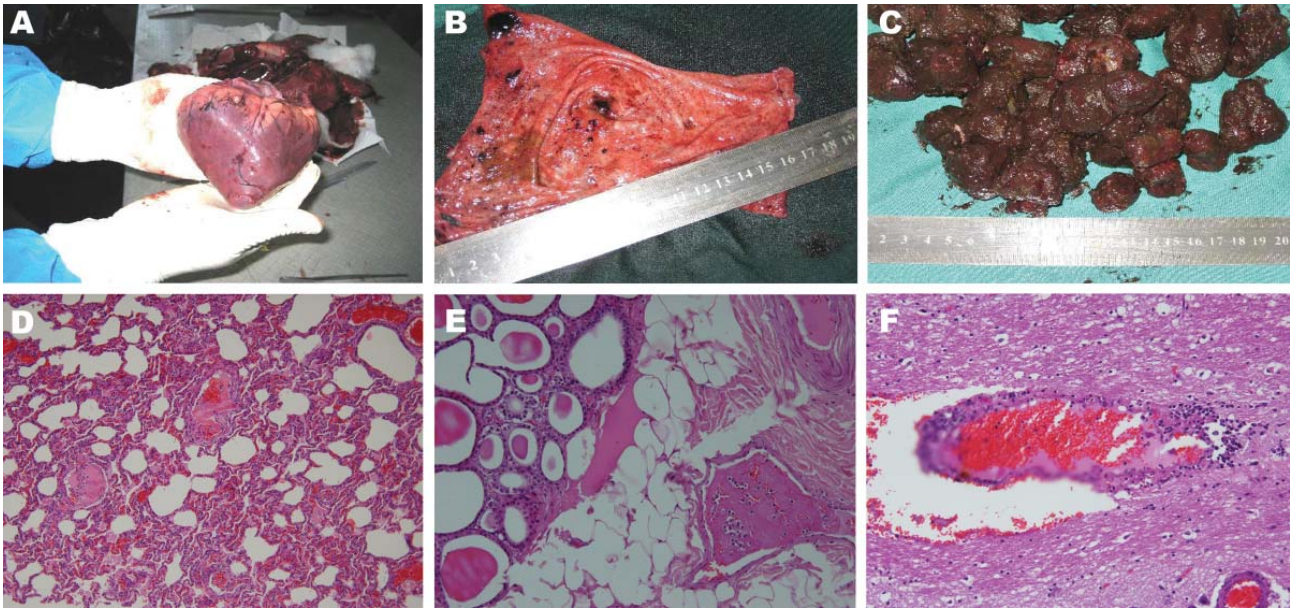

Figure. Acute form of African swine fever in wild boars. A) Petechial and larger ecchymotic hemorrhages beneath the epicardium. B) Severe hyperemia and petechial and larger ecchymotic hemorrhages in mucosa of urinary bladder. These hemorrhages are common in acute infectious fever and hemorrhagic diathesis. C) Blood-tinged colon contents with fecal balls covered by thick, blood-stained mucus. D) Congestion and fibrinous thromboses in pulmonary vessels and thickening of alveoli (hematoxylin and eosin stain; original magnification ×100). E) Fibrinous thrombus in a venule within interlobular adipose tissue of the thyroid gland (hematoxylin and eosin stain; original magnification ×200). F) Blood vessel congestion, perivascular hemorrhage, lymphocytic perivascular cuffing, and infiltration with degenerating lymphocytes (hematoxylin and eosin stain; original magnification ×200).

PCR and PCR are the most practical ways to differentiate infectious agents that cause similar clinical signs (*5*,*6*,*9*). The 100% similarity between our isolates and those from Georgia suggests that they might have originated from Georgia, probably brought into Iran by infected wild boars crossing the Aras River during the disease incubation period.

The main obstacles to ASF eradication are wildlife reservoirs, limited ability to control movement of infected pigs and wild boars, inadequate laboratory support for rapid and accurate diagnosis, and lack of an effective vaccine (*4*). *Ornithodoros* spp. ticks may contribute to virus persistence in the Caucasus region, including northwestern Iran. Although in our study ticks were not detected on the boar carcasses, they should be considered as potential transmission vectors. *O. lahorensis* ticks are found in areas with a cold climate and are mainly found near sheep and cows, so they are not likely to be isolated from an animal. In the absence of *Ornithodoros* spp. ticks, transmission to pigs could occur through contact with infected pigs or through feeding of virus-contaminated products (*7*,*8*).

The pig industry differs among Caucasus countries. In Azerbaijan, Chechnya, and southern Russian Federation, the pig industry is not as large as it is in Georgia and Armenia (*7*). In these countries with limited or no pig farms, the virus could be spread by infected wild boars, and the disease could become endemic as it has in Spain and Sardinia (*3*,*7*,*8*,*10*–*12*). Because Iran has no commercial pig facilities, the ASFV reservoir would be wild boars, which could transmit disease to neighboring countries that have pig industries, resulting in considerable economic losses.

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Mycobacterium tuberculosis Infection of Domesticated Asian Elephants, Thailand

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Four Asian elephants were confirmed to be infected with *Mycobacterium tuberculosis* by bacterial culture, other diagnostic procedures, and sequencing of 16S–23S rDNA internal transcribed spacer region, 16S rRNA, and gyrase B gene sequences. Genotyping showed that the infectious agents originated from 4 sources in Thailand. To identify infections, a combination of diagnostic assays is essential.

During the past 2 decades, infections of captive African and Asian elephants with *Mycobacterium bovis* and *M. tuberculosis* have been diagnosed worldwide (*1*–*4*). Transmission of these infections to other mammals and veterinary personnel has also been observed (*5*). To date, *M. tuberculosis* infection has not been reported in elephants in Thailand. Four elephants referred to the National Elephant Institute (NEI) Hospital during 2005–2008, three of which showed signs of weakness and chronic weight loss, and 1 showed serous nasal discharge. Tuberculosis was confirmed by using conventional and molecular diagnostic assays.

The Study

The ElephantTB *Stat-Pak* (Chembio Diagnostic Systems, Inc, Medford, NY, USA), which detects antibodies specific to M. tuberculosis in elephants, was performed. Trunk wash sampling of elephants 1, 2, and 4, according to the Guidelines for the Control of Tuberculosis in Elephants, 2008 (6), was followed by culture for bacteria (Technical Appendix Table 1, http://www.cdc.gov/eid/content/16/12/1949-Techapp.pdf). Necropsy of elephants 1, 3, and 4 was performed at 21 months, 7 days, and 33 months after admission, respectively, and lesion tissues were collected for bacterial culture, Ziehl-Neelsen (ZN) staining, and histopathologic examination (Technical Appendix Table 2, http://www.cdc.gov/eid/content/16/12/1949-Techapp.pdf)..

A serum sample from elephant 1 was negative for *M. tuberculosis* at admission, but a sample obtained 10 months later was positive. Bacteria could not be grown from trunk wash samples. Necropsy showed that elephant 1 had tuberculous lesions in the respiratory tract, mediastinal lymph nodes, liver, kidney, and spleen. Histopathologic examination showed caseous necrosis; infiltration of lymphocytes; and accumulation of macrophages and giant cells in lung tissue, lymph nodes, and liver. ZN staining identified acid-fast bacilli. Mycobacteria were cultured from lesion tissue.

A serum specimen from elephant 2 was negative for mycobacteria at admission, but a second sample was positive 23 months later. Bacteria that were positive by ZN staining were cultured from a trunk wash sample. This elephant is still alive and being kept in a restricted area.

Serum samples from elephant 3 were negative at days 1 and 7 after admission, and the elephant died a few hours after the second sample was tested. A stored serum sample from elephant 3, obtained 4 months earlier was also negative. The animal was severely ill and in lateral recumbency. Necropsy showed tuberculous lesions in the lungs, upper trachea, and mediastinal lymph nodes. Histopathologic examination showed caseous necrosis and accumulation of macrophages and giant cells in the lung and lymph nodes. ZN staining showed acid-fast bacilli. Mycobacteria were cultured from lesion tissues.

A serum specimen from elephant 4 was positive at admission. Initially, *M. avium* bacteria were grown from cultures of trunk wash samples. At necropsy, tuberculous lesions were found in the respiratory organs and mediastinal lymph nodes. Histopathologic examination showed accumulation of macrophages and edema in the lung tissues. ZN staining did not show acid-fast bacilli. However, mycobacteria were cultured from lesion tissues.

Bacteria cultured from trunk wash and tissue samples were further identified by PCR reactions by using 16S rRNA, 16S–23S-rDNA internal transcribed spacers (ITS) (*7*,*8*), and gyrase B (*gyrB*) primers (Table 1). The subsequent sequencing was conducted by using an ABI 3070 system (Applied Biosystems, Foster City, CA, USA). Unambiguous sequences were compared with data available in GenBank (www.ncbi.nih.gov/BLAST) and analyzed by using ClustalW version 1.4 (www.ebi.ac.uk/Tools/clustalw/). The 16S rRNA and ITS sequencing confirmed that bacteria from lesion tissues of elephants 1, 3, and 4

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Table 1. Primers used to identify bacteria cultured from trunk wash and tissue samples from domesticated Asian elephants, Thailand, 2003–2008*

Primer	Forward	Reverse
16s rRNA	5′-AgA gTT TgA TCC Tgg CTC Ag-3′	5′-ACg gCT ACC TTg TTA CgA CTT-3′
ITS	5′-TTg TAC ACA CCg CCg gTC a-3′	5′-TCT CgA TgC CAA ggC ATC CAC C-3′
gyrB	5′-TCG GAC GCG TAT GCG ATA TC-3′	5′-ACA TAC AGT TCG GAC TTG CG-3′

*ITS modified from (*7*,*8*); *gyrB* modified from (*9*). ITS, internal transcribed spacer; *gyrB*, gyrase B.

and from a trunk wash sample of elephant 2 belong to the *M. tuberculosis* complex. The *gyrB* sequences of isolates from elephants 2, 3, and 4 were identical to those of *M. tuberculosis* strain American Type Culture Collection (ATCC) 27294 and others (Table 2); the *gyrB* sequence of the isolate from elephant 1 differed at position 482, which is similar to the *M. tuberculosis* strain KPM KY679, the ancient TbD_1-positive strain (*9*–*11*). The mycobacterial interspersed, repetitive-unit variable number tandem repeat typing of the exact tandem repeat-A (ETR-A) locus was performed according to protocols of Fleche et al. (*12*). The sequence of the ETR-A locus showed that different types of *M. tuberculosis* were present in elephants 2, 3, and 4 because the sequence had 3, 2, and 4 repeats of the typical 75-bp sequence, respectively.

Conclusions

We report *M. tuberculosis* infection in elephants in Thailand. Clinical signs shown by these 4 elephants varied considerably. Elephant 2 showed nasal discharge only; in contrast, elephant 3, showed severe clinical signs and lateral recumbency. Elephant 3 had no antibodies, which may indicate an anergic status of the mycobacteria-specific immune response (*13*). Histopathologic examination showed that this elephant was severely affected by the infection. Elephant 2 is still alive; cultures of trunk wash samples contain mycobacteria. The elephant was seropositive for *M. tuberculosis* antigens as defined by the *StatPak* assay. The other 2 elephants (1 and 4) showed anorexia, chronic weight loss, and comparable lesions at necropsy, but diagnostic assays showed variable results. Trunk wash culture, considered to be the standard for confirmation of *M. tuberculosis* complex infection in elephants, has its limitations, as described elsewhere (*14*). This study, which included 3 elephants positive for mycobacteria in tissue culture at necropsy, showed that bacterial cultures of only 2 of 60 trunk wash samples were positive for mycobacteria. The study indicates that serologic tests or other diagnostic procedures could not unequivocally identify infected animals, perhaps because of differences in specific immune responsiveness among species and length of time after infection (*13*). However, the combination of the different diagnostic observations after infection holds promise for improving the likelihood of confirmed *M. tuberculosis* infection.

Sequence analysis of 16S and ITS indicated *M. tuberculosis* complex bacteria in each elephant. Nucleotide sequence polymorphism in the *gyrB* gene of the mycobacteria isolates (*9*–*11*) confirmed the identity of *M. tuberculosis* for all 4 elephants. *M. tuberculosis* may be classified into ancestral and modern strains based on *M. tuberculosis*–specific deletion (TbD1) (*15*). *M. tuberculosis* isolated from elephant 1 had a *gyrB* gene sequence identical to strains of the ancient TbD_1-positive strain (Table 2). The other 3 elephants were infected with strains identical to *M. tuberculosis* ATCC 27294, the modern type, potentially related

Table 2. *gyrB* gene sequence comparisons of 4 *Mycobacterium tuberculosis* isolates from domesticated Asian elephants, Thailand, 2003–2008*

	Gene position						
Organism	41	122	482	677	776	816	824
M. tuberculosis ATCC	C	G	G	T	C	G	C
M. tuberculosis TbD1	C	G	C	T	C	G	C
M. africanum	C	G	G	T	C	T	C
M. canetti	C	G	G	T	C	T	T
M. microti	T	G	G	T	C	T	T
M. bovis	C	A	G	T	T	T	T
M. caprae	C	A	G	G	T	T	T
Elephant 1 isolate	C	G	C	T	C	G	C
Elephant 2 isolate	C	G	G	T	C	G	C
Elephant 3 isolate	C	G	G	T	C	G	C
Elephant 4 isolate	C	G	G	T	C	G	C

*Nucleotide variability at relevant positions of the *gyr B* gene in the genome of mycobacteria isolated from the 4 infected elephants as compared with those in established *M. tuberculosis* ATCC 27294 (GenBank accession no. GQ247736.1), *M. tuberculosis* KPM KY679 (accession no. AB014215.1), *M. africanum* (accession no. AB014192.1), *M. canetti* (accession no. AJ749915.1), *M. microti* (accession no. AB014205.1), *M. bovis* (accession no. AB018554.1), and *M. caprae (*accession no. AJ276122.1). Modified from Gutierrez et al. (*11*). Shading corresponds to sequence stretch in the strains that are identical to the sequences in *M. tuberculosis*: yellow, performed ATCC strain; blue, position 482 performed TbD1 strain; tan, performed other *M. tuberculosis* complex strain. *gyrB*, gyrase B; ATCC, American Type Culture Collection.

to major epidemics like the Beijing, Haarlem, and African *M. tuberculosis* clusters (*15*).

On the basis of these molecular studies, we believe that *M. tuberculosis* was probably transmitted to these 4 elephants from humans. In addition, mycobacterial interspersed, repetitive-unit variable-number tandem-repeat typing of the ETR-A gene *M. tuberculosis* strains in elephants 2, 3, and 4 showed different numbers of the typical 75-bp repeat. Therefore, we conclude that the sources of infection were of different origins. Annual health checks of mahouts and veterinarians who were in contact with the infected animals for >4 years at the NEI did not identify any persons with positive results by chest radiograph when tested as part of the tuberculosis control program in Thailand. To control *M. tuberculosis* complex transmission from humans and other species to wild animals, including elephants, or from wild animals to humans, assays that enable early diagnosis of infection are necessary. Because no assay unequivocally defines the infectious status, a combination of diagnostic approaches is essential.

Further investigation of tuberculosis transmission and surveillance and monitoring of this disease in Thailand will enhance the understanding of its epidemiology. Increased epidemiologic knowledge is essential to control and prevent tuberculosis in elephants.

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Hantaviruses and Hantavirus Pulmonary Syndrome, Maranhão, Brazil

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To confirm circulation of Anajatuba virus in Maranhão, Brazil, we conducted a serologic survey (immunoglobulin G ELISA) and phylogenetic studies (nucleocapsid gene sequences) of hantaviruses from wild rodents and persons with hantavirus pulmonary syndrome. This virus is transmitted by *Oligoryzomys fornesi* rodents and is responsible for hantavirus pulmonary syndrome in this region.

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) cause a viral zoonosis transmitted by rodents belonging to the families Muridae and Cricetidae. Each hantavirus is predominantly associated with a specific rodent species in a specific geographic region. However, infection of other rodent species can occur as a spillover phenomenon (*1*).

Hantavirus disease has 2 recognized clinical forms, hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS) (*2*). The respiratory form of the disease was described in June 1993 during an epidemic of severe respiratory disease caused by Sin Nombre virus in the United States (*3*). A few months later, 3 HPS cases were identified in 3 siblings in Juquitiba, São Paulo State, Brazil (*4*). During 1993–2009, a total of 1,246 HPS cases (264 in the Amazon region) were reported in Brazil, and new hantaviruses were identified (Juquitiba virus, Castelo dos Sonhos virus, Araraquara virus, Anajatuba virus, and Rio Mearim (*5*).

During 2003–2005, an ecoepidemiologic study was conducted in the municipality of Anajatuba, Maranhão, Brazil, to identify reservoirs of hantaviruses after identification of 3 HPS cases (*6*). Two new hantaviruses, Anajatuba virus and Rio Mearim virus, were isolated from *Oligoryzomys fornesi* (rice rat) rodents and *Holochilus sciureus* (marsh rat) rodents, respectively, and genetically characterized (*5*). To confirm circulation of Anajatuba virus in Maranhão, Brazil, we conducted a serologic survey (immunoglobulin [Ig] G ELISA) and phylogenetic studies (nucleocapsid gene sequences) of hantaviruses obtained from wild rodents and persons with HPS.

The Study

Anajatuba (3°16′S, 44°37′W; population 23,907) and Santa Rita (3°9′S, 44°20′W; population 31,033) (www.ibge.gov.br), are located in the western floodplain of the Maranhão River in Maranhão State, Brazil (Figure 1, panel A). The region has chains of lakes with extensive swamps and flooded fields, forest areas, and rice fields extending from the outskirts of the urban area. The climate is tropical and humid (average temperature range 26°C–28°C), and the rainy season is during January–July (*5*,*6*).

Data for 5 cases of HPS in men (age range 25–30 years, 3 from Anajatuba and 2 from Santa Rita) are shown in Table 1. In a cross-sectional serologic survey in residents of Anajatuba, 293 serum samples (8.1% of the population studied and 1.2% of the total population of the municipality) were obtained; 153 (52%) residents were women. Fifty-four samples were obtained from urban residents, and 239 samples were obtained from rural residents. All samples were tested by using an ELISA to detect IgM and IgG as described (*7*).

The main findings of the serologic study are shown in Table 2. A male:female ratio of 2:1 was observed in urban and rural areas. Factors investigated for increasing risk for exposure to hantaviruses included living near rice paddies; engaging in farming or fishing; having wild rodents around the household; having contact with wild rodents in the workplace, school, or domestic surroundings; and storing rice in the household.

In May 2003 and May 2005, two rodent captures approved by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Instituto Chico Mendes de Conservação da Biodiversidade were conducted in São Roque, Anajatuba (Figure 1, panel B). Trapping was conducted ≤50 m from residences of 3 deceased HPS case-patients in accordance with accepted rodent capture and

[1]Deceased.

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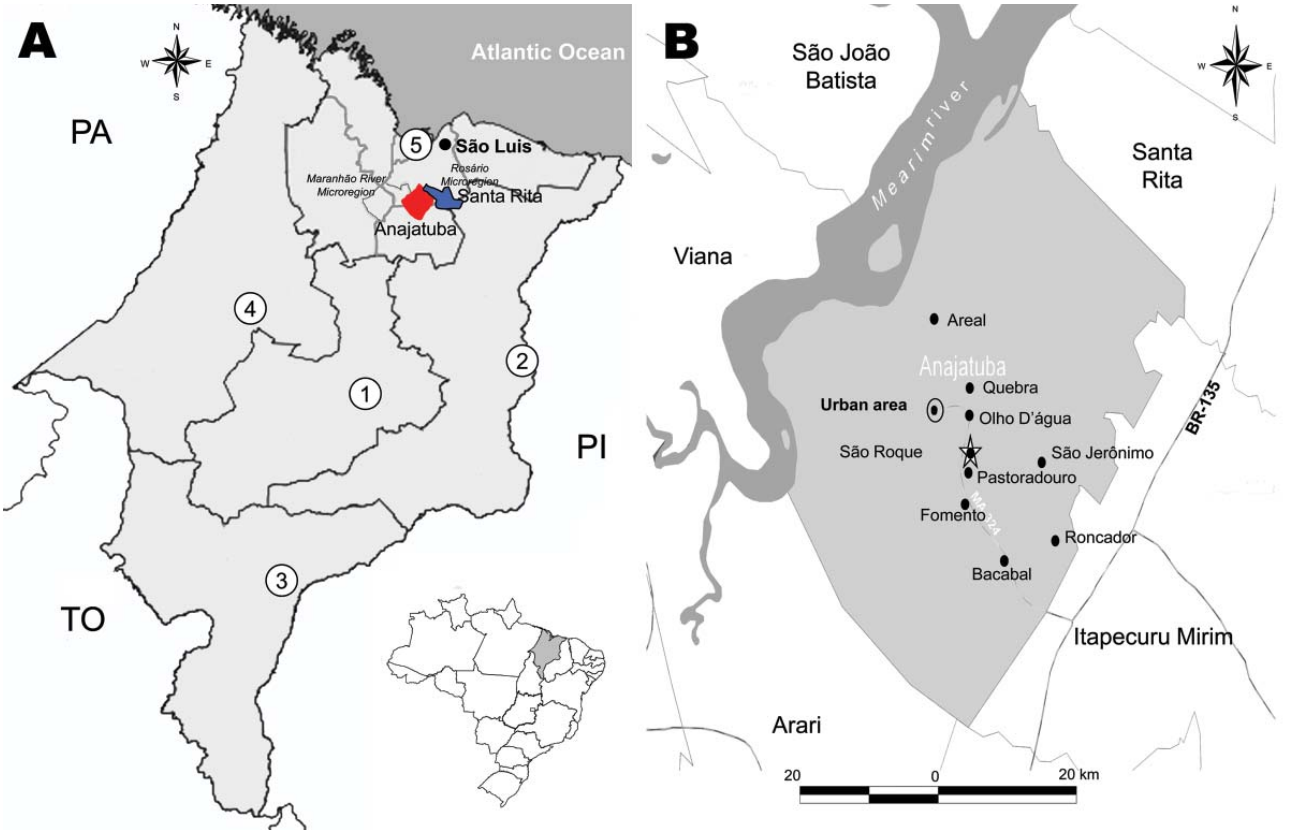

Figure 1. A) Regions of Anajatuba (red) (Maranhão River Microregion) and Santa Rita (blue) (Rosario Microregion), Maranhão, Brazil, where hantavirus pulmonary syndrome (HPS) cases were found. PA, Para; TO, Tocantins; PI, Piaui; 1, central region; 2, eastern region; 3, southern region; 4, western region; 5, northern region. B) Towns in Anajatuba where a serologic survey for HPS in humans was performed. Dotted oval, São Roque; star, rodent capture location; ovals, locations where HPS cases were found.

handling procedures and standard biosafety protocols for anesthetizing and killing rodents, and biometric analysis was conducted (*8*). Fragments of liver, lung, spleen, heart, and kidney were obtained. Taxonomic identification was performed according to procedures of Bonvincino and Moreira (*9*).

Biologic samples (blood and viscera fragments) were obtained from 216 captured rodents: 96 (44%) captured in 2003 and 120 (56%) captured in 2005. The most common species captured in 2003 were *Necromys lasiurus* rodents (n = 62, 64%) and *Akodon* sp. rodents (n = 27, 28%). The most common species captured in 2005 were *N. lasiurus*

Table 1. Characteristics of 5 human case-patients with hantavirus pulmonary syndrome and 3 rodents infected with hantavirus, Maranhão, Brazil*

Sample origin†	Municipality/town	Patient age, y	Sample collection date	Symptom duration, d	Clinical outcome	ELISA results		GenBank accession no.
						IgG	IgM	
Human								
Be H 666379‡	Anajatuba/São Roque	24	2003 Mar 25	1	Died	Neg	Pos	HM238889
Be H 668281	Santa Rita/Conceição	21	2003 May 14	6	Recovered	Pos	Pos	–
Be H 670957‡	Anajatuba/Fomento	24	2003 Jul 22	4	Recovered	Pos	Pos	HM238890
Be H 672862‡	Santa Rita/NA	39	2003 Oct 21	10	Recovered	Neg	Pos	HM238885
Be H 708080	Anajatuba/Roncador	28	2006 Jun 12	NA	Died	Neg	Pos	–
Rodent				Species				
BeAN669104‡	Anajatuba/São Roque	NA	2003 May 27	*Necromys lasiurus*		Pos	ND	HM238886
BeAN690936‡	Anajatuba/São Roque	NA	2005 May 18	*Oligoryzomys fornesi*		Pos	ND	HM238887
BeAN690985‡	Anajatuba/São Roque	NA	2005 May23	*O. fornesi*		Pos	ND	HM238888

*Ig, immunoglobulin; Neg, negative; Pos, positive; NA, not available; ND, not done.
†Serum samples were obtained from humans (males), and lung tissue samples were obtained from rodents (males).
‡Amplicons for these samples were sequenced.

Table 2. Testing for hantavirus among residents of urban and rural areas of Anajatuba, Maranhão, Brazil, May 2005*

Zone	Town	Total population	No. (%) persons sampled	No. (%) persons positive†
Urban	Limirique and Porção do Junco (neighborhood)	1,059	54 (5.09)	9 (16.7)
Rural	Areal	375	42 (11.2)	3 (7.1)
	Bacabal	790	57 (7.2)	7 (12.3)
	Olho d'água	193	51 (26.4)	5 (9.8)
	Quebra	584	38 (6.5)	5 (13.2)
	São Roque and Pastoradouro	634	51 (8.0)	3 (5.9)
Rural zone total		2,576	239 (9.3)	23 (9.6)
Total		3,635	293 (8.1)	32 (10.9)

*Data were provided by the Health Municipal Secretary of the Anajatuba Municipality, 2005.
†By immunoglobulin G testing.

rodents (n = 105, 87%) and *O. fornesi* rodents (n = 2, 2%); the remaining rodents were from other genera.

Blood samples collected from wild rodents were also tested by using an IgG ELISA (*10*). IgG against hantavirus was detected in 2 (100%) of 2 *O. fornesi* rodents captured in 2005 and 6 (4%) of 167 *N. lasiurus* rodents (3 of 62 captured in 2003 and 3 of 105 captured in 2005) (Table 1).

Virus RNA was extracted from IgM-positive human serum or blood samples and lung fragments from IgG-positive rodents by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Nested reverse transcription–PCR and hemi-nested reverse transcription–PCR were used for amplification of partial nucleocapsid gene sequences from human and rodent samples, respectively, by using primers described (*11*). Purified amplicons were obtained by using the GFX PCR DNA and Gel Band Purification Kit (Healthcare, Little Chalfont, UK) and sequenced. Amplicons (434 bp) generated from HPS cases in humans (2 from Anajatuba and 1 from Santa Rita) and from 3 of 8 lung samples from hantavirus IgG-positive rodents were sequenced (Table 1).

Phylogenetic trees were constructed by using neighbor-joining, maximum-parsimony, maximum-likelihood, and Bayesian methods implemented in PAUP 4.0b.10 (*12*), PHYML (*13*), and BEAST (*14*). Modeltest version 3.6 (*15*) was used to determine the best nucleotide substitution model based on Akaike information criteria. Analyses were conducted by using confidence values estimated from mean nucleotide divergence obtained for different Old World and New World hantavirus sequences by using MEGA version 3.0 software (www.megasoftware.net) Estimated values were ≤45%, ≤25%, 22%, and 15% and were used for grouping viruses in clusters, clades, subclades, and species, respectively.

All phylogenetic methods showed similar topologies, and the ML maximum-likelihood construction was select-

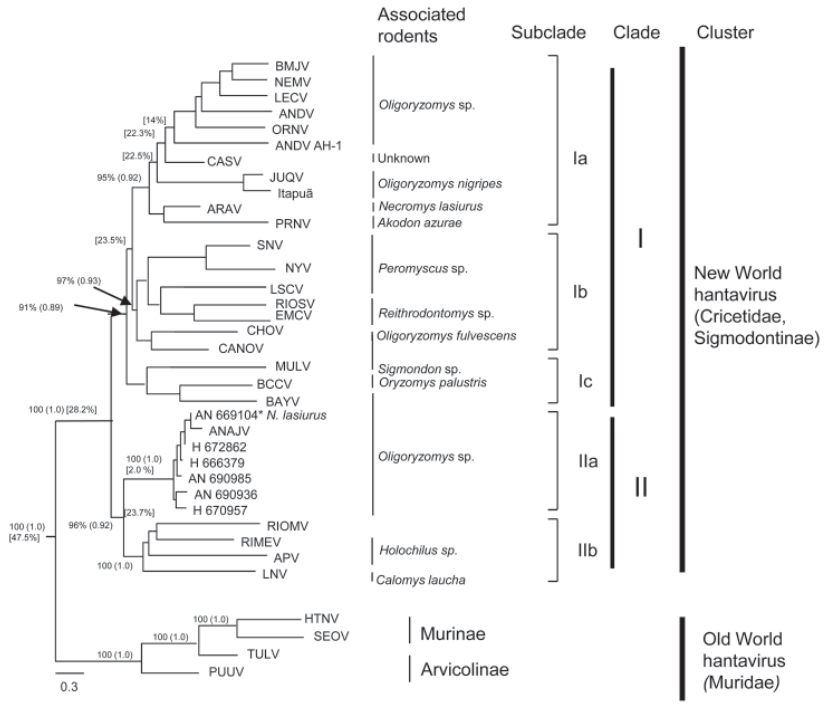

Figure 2. Phylogenetic analysis of partial small RNA segments of hantaviruses, Maranhão, Brazil, by using maximum-likelihood and Bayesian methods. Bayesian and bootstrap values (in parentheses) are shown over each main tree node. Values in brackets indicate mean divergence between groups. Arrows indicate exact position of these 2 values. Scale bar indicates nucleotide sequence divergence. BMJV, Bermejo virus; NEMV, Neembuco virus; LECV, Lechiguanas virus; ANDV, Andes virus; ORNV, Oran virus; CASV, Castelo dos Sonhos virus; JUQV, Juquitiba-Araucaria virus; ARAV, Araraquara virus; PRNV, Pergamino; SNV, Sin Nombre virus; NYV, New York virus; LSCV, Limestone Canyon virus; RIOSV, Rio Segundo virus; ECMV, El Moro Canyon virus; CHOV, Choclo virus; CANOV, Cano Delgadito virus; MULV, Muleshoe virus; BCCV, Black Creek Canal virus; BAYV, Bayou virus; ANAJV, Anajatuba virus; RIOMV, Rio Mamoré virus; RIMEV, Rio Mearim virus; APV, Alto Paraguay virus; LNV, Laguna Negra virus; HTNV, Hantaan virus; SEOV, Seoul virus; TULV, Tula virus; PPUV, Puumala virus.

ed for representing the final tree. Bootstrap and Bayesian posterior probability values are shown in Figure 2.

Two major clusters were observed (New World and Old World hantavirus groups) and had a genetic distance of 28.2% (inclusion value 25%). The New World group was divided into clades I and II. Clade I was divided into 3 subclades (genetic divergence 23.5%), Ia, Ib, and Ic. Clade II was divided into 2 subclades (genetic divergence 23.7%), IIa and IIb. The strains used in this study were closely related to Anajatuba virus and were included in the IIa subclade (genetic divergence 2%) (online Technical Appendix, www.cdc.gov/EID/content/16/12/1952-Techapp.pdf).

Nucleotide and amino acid homology between Anajatuba virus (*5*) and the strains isolated in this study in Maranhão were 98.3% and 100%, respectively. These strains were included in a group related to rodents belonging to the genus *Oligoryzomys*, although sample Be AN 669104 was obtained from an *N. lasiurus* rodent, which suggests spillover transmission between rodent species.

Conclusions

We showed that Anajatuba virus is responsible for human HPS cases and that *O. fornesi* rodents are its likely reservoir. Anajatuba virus infections of *N. lasiurus* were spillover infections. Human hantavirus infections are common among persons in the Baixada Maranhense region, but cases of HPS are rare. However, educational and health surveillance programs are needed to prevent hantavirus transmission.

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Wild Chimpanzees Infected with 5 *Plasmodium* Species

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Data are missing on the diversity of *Plasmodium* spp. infecting apes that live in their natural habitat, with limited possibility of human-mosquito-ape exchange. We surveyed *Plasmodium* spp. diversity in wild chimpanzees living in an undisturbed tropical rainforest habitat and found 5 species: *P. malariae*, *P. vivax, P. ovale*, *P. reichenowi*, and *P. gaboni.*

Despite ongoing and, in some regions, escalating morbidity and mortality rates associated with malaria-causing parasites, the evolutionary epidemiology of *Plasmodium* spp. is not well characterized. Classical studies of the blood pathogens of primates have found protozoa resembling human malaria parasites in chimpanzees and gorillas (*1*); however, these studies were limited to microscopy, negating conclusions regarding evolutionary relationships between human and ape parasites. Recent studies that used molecular approaches showed that captive and wild chimpanzees (*Pan troglodytes*) and lowland gorillas (*Gorilla gorilla*), as well as captive bonobos (*Pan paniscus*), harbor parasites broadly related to *P. falciparum* (*2*–*5*); wild and captive gorillas and captive bonobos and chimpanzees are sometimes infected with *P. falciparum* itself (*4*–*6*). Further, captive chimpanzees and bonobos have been shown to have malaria parasites related to human *P. ovale* and *P. malariae* (*6*–*8*); *P. vivax* has been identified in various monkeys and 1 semiwild chimpanzee (*5*,*9*). Recently, *P. knowlesi*, a simian malaria species, became the fifth human-infecting species (*10*), highlighting the possibility of transmission of new *Plasmodium* spp. from wild primates to humans.

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The Study

To investigate the prevalence of different *Plasmodium* spp. in wild great apes living in their natural habitat (tropical rainforests), we analyzed tissue samples from 16 wild West African chimpanzees that died primarily of anthrax or respiratory disease in Taï National Park, Côte d'Ivoire. A generic real-time PCR that detects all known *Plasmodium* spp. was used to test all samples for the parasite. Sequence analysis of the *CytB* gene and small subunit rRNA genes was conducted for real-time PCR–positive samples to determine the strain present; 1,140 bp of the *CytB* gene and 765 bp of the *18S* gene of the *Plasmodium* genome were amplified by classic PCR. Resulting products were sequenced either directly or after cloning for rRNA gene and when initial sequence information showed the possible presence of 2 different species (Table).

Phylogenetic analyses of sequences obtained confirmed the presence of 5 species: *P. reichenowi* and *P. gaboni*, which had been found previously (*2*,*3*); but also *P. vivax*, *P. ovale*, and *P. malariae*–like strains (Figures 1, 2). The most prevalent species was *P. reichenowi* (6/16), which had representatives in subclusters *P. gaboni* and *P. reichenowi*. The other species were rare, seen only 1 (*P. ovale* and *P. vivax*) or 2 (*P. malariae*) times. Two chimpanzees showed co-infections with multiple *Plasmodium* spp. (Figures 1, 2), 1 infected with *P. reichenowi* and a *P. malariae*–like strain and the other with *P. reichenowi* and *P. gaboni*.

Is the observed high prevalence of *Plasmodium* spp. typical for wild chimpanzees or related to reduced immune function associated with the severe infection that was the primary cause of death in each case? To investigate this question, we tested DNA extracted from fecal samples of apparently healthy chimpanzees collected over the past 8 years (n = 30) (*11*) of the same study population by using the generic real-time PCR followed by amplification of the *CytB* gene. Of these samples, 21 (70%) were positive for *Plasmodium* spp. by real-time PCR. Because of low copy numbers in feces, phylogentic analyses were limited to 2 samples in which *P. reichenowi* of the *P. gaboni* subcluster was confirmed.

To determine if the observed high prevalence of plasmodia was a site- or chimpanzee subspecies–specific phenomenon, we tested 30 randomly selected fecal samples of individually known apparently healthy wild Eastern chimpanzees from the Budongo Forest in Uganda. Overall prevalence of *Plasmodium* spp. was lower than in West African

Table. Tissue and fecal samples from wild chimpanzees examined for *Plasmodium* species, Tai National Park, Cote d'Ivoire, and Budongo Forest, Uganda*

Type of sample and name or species of chimpanzee	Genetic sequence copies/mg tissue	*Plasmodium* species detected	GenBank accession nos.
Necropsy			
Loukoum	530	*P. gaboni*	GU815507 (*CytB*), GU815523 (*18S*)
Noah	50	*P. gaboni*	GU815508 (*CytB*), GU815524 (*18S*)
Orest	2.2 x 10	*P. gaboni*	GU815509 (*CytB*), GU815525 (*18S*)
Candy	65	*P. reichenowi*	GU815510 (*CytB*), GU815526 (*18S*)
Atra	100	*P. reichenowi*	GU815511 (*CytB*)
Louise	160	*P. reichenowi*	GU815512 (*CytB*), GU815527 (*18S*)
EastChip 06	105	*P. reichenowi*, *P. gaboni*	GU815512–13 (*CytB*)
Olduvai	130	*P. reichenowi*, *P. malariae*	GU815514–15 (*CytB*), GU815528–29 (*18S*)
Leo	850	*P. malariae*	GU815516 (*CytB*), GU815530 (*18S*)
Kady	105	*P. ovale*	GU815517 (*CytB*), GU815531 (*18S*)
Sagu	760	*P. vivax*	GU815518 (*CytB*), GU815532 (*18S*)
Dorry	Neg		
Virunga	Neg		
Ophelia	Neg		
Akruba	Neg		
Akwaba	Neg		
Fecal samples, n = 30	Positive qPCR results		
P. t. verus	21 (2)	*P. gaboni*	GU815519 (*CytB*)
P. t. schweinfuthii	12 (3)	*P. reichenowi P. gaboni*	GU815520–22 (*CytB*)

*All chimpanzees were *Pan troglodytes verus* from Tai except *P. t. schweinfuthii* chimpanzees, which were from Budongo Forest. Parentheses indicate the number of samples for which sequences were obtained and used for phylogenetic tree analyses. Neg, negative; qPCR, quantitative PCR.

chimpanzees but still relatively high (40%); *P. reichenowi* and *P. gaboni* were identified in 3 samples.

Our results demonstrate that the prevalence of different *Plasmodium* spp. in wild chimpanzees is similar to that of untreated human populations in sub-Saharan Africa (www.who.int/malaria). Throughout sub-Saharan Africa, *P. falciparum* is more predominant in humans than are other *Plasmodium* spp. Considering the lack of clinical signs of malaria in chimpanzees from which fecal samples were collected and those that had died of respiratory disease or anthrax, *Plasmodium* spp. infections appear to be asymptomatic or at least nonlethal in wild chimpanzees. However, signs of illness are rarely observed in wild primates because infected animals often mask weakness to maintain social position and avoid attack by predators (*12*). Recently developed technologies for the noninvasive determination of temperature in wild chimpanzees may enable more effective examination of the relationship between the primary clinical feature of malaria (i.e., cyclical fevers) and *Plasmodium* spp. infection (*13*).

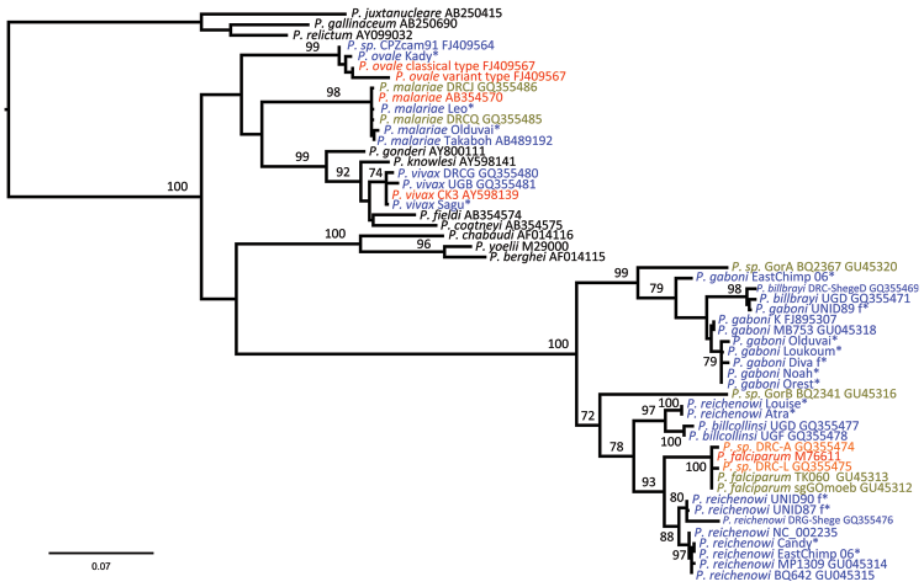

Figure 1. Maximum-likelihood trees of *Plasmodium* spp. obtained from the analysis of a 1,087-bp *CytB* alignment. Blue indicates sequences determined from chimpanzee hosts, green, bonobos, gray, gorillas, and red, humans; black indicates sequences obtained from nonprimate hosts. *Plasmodium* spp. sequences derived from chimpanzees in this study are marked with an asterisk. Bootstrap values are shown when ≥70. The tree was rooted using avian plasmodium sequences. Accession numbers of all sequences used are shown in the Table. Scale bar indicates nucleotide substitutions per site.

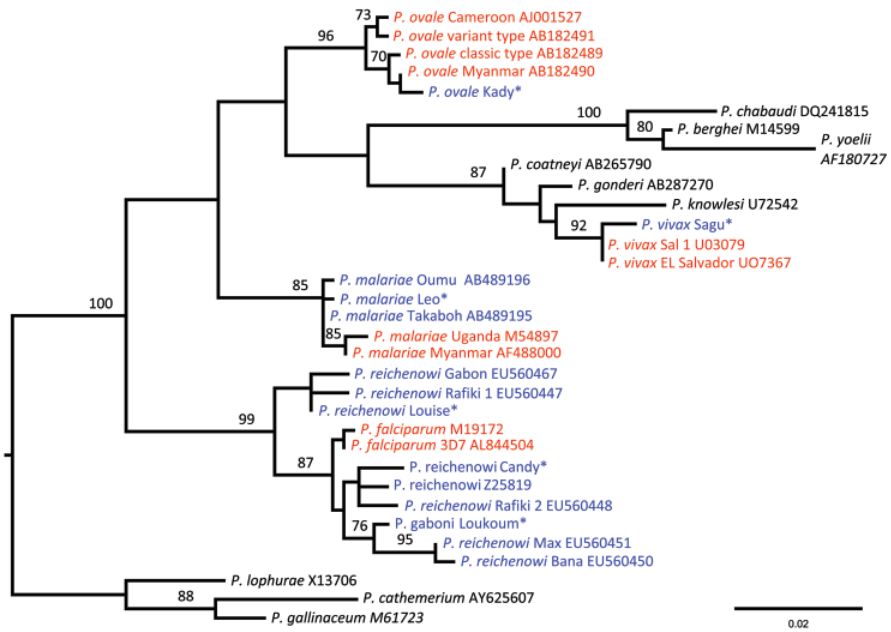

Figure 2. Maximum-likelihood tree of *Plasmodium* spp. obtained from the analysis of a 621 bp–long 18S alignment. Blue indicates sequences determined from chimpanzee hosts; green, bonobos; gray, gorillas; and red, humans. Black indicates sequences obtained from nonprimate hosts. *Plasmodium* spp. sequences derived from chimpanzees in this study are marked with an asterisk. Bootstrap values are shown when ≥70. The tree was rooted using avian plasmodium sequences. Accession numbers of all sequences used are shown in the Table. Scale bar indicates nucleotide substitutions per site.

P. ovale was previously described from captive chimpanzees and *P. malariae* from captive chimpanzees and captive bonobos have been described (*5*–*8*). Our study results demonstrate that *P. malariae* and *P. ovale* occur in wild chimpanzees that inhabit pristine contiguous forest with extremely limited exposure to humans, suggesting the natural existence of these parasites in wild great apes.

Because of a Duffy-negative condition in 95%–99% of the human population in western and central continental Africa, transmission of *P. vivax* does not seem to occur. However, *P. vivax* infections are common in travelers returning from these areas (*11*). Even though we cannot totally exclude the possibility of introduction of *P. vivax* in the chimpanzee population through humans, our discovery of *P. vivax* in wild chimpanzees living exclusively within their natural habitat suggests that wild African apes may be a natural reservoir.

Our study shows the existence of *P. reichenowi* and related strains in wild chimpanzees as described for chimpanzees and gorilla by others (*2*–*4*,*6*). Infections with strains of the *P. reichenowi* group (sometimes referred to as the species *P. gaboni*, *P. billbrayi*, and *P. billcollinsi*) appear to occur widely in wild and captive great apes in Africa with some variation between chimpanzee subspecies from biogeographically distinct sites. The wild chimpanzees examined demonstrated no infections with classic human *P. falciparum*. This lack of infection is likely caused by low human presence in their habitat and, consequently, few or no infected vectors, low sample size, or a missing receptor in chimpanzees (*14*). More investigations are needed because recently *P. falciparum* infections have been described for 2 captive chimpanzees (*6*). The situation is clearer for captive and wild lowland gorillas (*Gorilla gorilla*) for which infections and receptors have recently been described (*4*). Infections have also been documented for captive bonobos (*5*).

Conclusions

Previous examination of the role of our closest phylogenetic relatives, the great apes, in the evolution and persistence of human plasmodia has been limited by a lack of data from wild ape populations where opportunities for human-mosquito-ape malaria exchange are minimal. Interpretation of patterns of malaria infection in captive ape populations, such as sanctuaries and zoos, must consider the ample opportunities for human-to-ape transmission of such parasites, negating the opportunity to investigate the evolutionary origins and public health–related risks of these parasites. Conversely, our examination of these parasites in wild chimpanzees with no contact to the periphery of the rainforest habitat (online Technical Appendix Figure, www.cdc.gov/EID/content/16/12/1956-Techapp.pdf) demonstrates that these apes are most likely naturally infected with *P. ovale*, *P. vivax*, and *P. malariae*, 3 types of plasmodia rarely observed in humans of the region. Whether wild great apes are the origin or reservoirs of these *Plasmodium* types requires further investigation. These results may have implications for global efforts to eradicate malaria in humans, including vaccine development based on animal variants of human parasites.

Addendum

While this article was in press, Liu et al. published a study showing strong evidence that *P. falciparum* originat-

ed in gorillas (*15*). Their study also recovered other plasmodia, complementing our findings. As recommended in our conclusions section, the Liu et al. study was based on a large number of samples from wild great apes.

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Online Flutracking Survey of Influenza-like Illness during Pandemic (H1N1) 2009, Australia

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We compared the accuracy of online data obtained from the Flutracking surveillance system during pandemic (H1N1) 2009 in Australia with data from other influenza surveillance systems. Flutracking accurately identified peak influenza activity timing and community influenza-like illness activity and was significantly less biased by treatment-seeking behavior and laboratory testing protocols than other systems.

A variety of surveillance methods were used to monitor the incidence and severity of influenza A pandemic (H1N1) 2009 in Australia. Severity of illness was measured by number of hospitalizations, intensive care unit (ICU) admissions, and deaths. Influenza disease incidence was monitored through laboratory-confirmed cases, general practitioner sentinel surveillance of influenza-like illness (ILI), emergency department visits for ILI, absenteeism data from large employers, and the Flutracking surveillance system (*1*).

Flutracking is a national weekly online survey of ILI (completed by >8,000 participating community members each week in 2009); it is the only ILI surveillance system that provides comparable data across Australia's states and territories. Flutracking integrates participants' ILI symptom information with their influenza vaccination status (*2*). Flutracking surveillance has correlated well with other Australian influenza surveillance systems in describing the timing and scale of the 2007 and 2008 seasonal influenza epidemics (*3*,*4*). We compared Flutracking data with data from other routine influenza surveillance systems during the 2009 pandemic wave in New South Wales (NSW), Australia's most populous state.

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The Study

From May 4, 2009, through October 31, 2010, participants received an automatically generated weekly email link to the online questionnaire, which asked whether they had experienced fever or cough and how many days they had been absent from work or normal duties because of these signs (recruitment details in *2*,*3*; location of participants at www.flutracking.net). Each individual response usually took <15 seconds. Participants who had previously reported not receiving seasonal influenza vaccine were asked whether they had received influenza vaccination in the prior week during each weekly survey. If they answered yes, the question was automatically deleted from their subsequent weekly surveys. Participants were permitted to enroll at any time during the surveillance period. Participants could respond on behalf of household members, and children ≥12 years of age could complete their own survey. During online enrollment, participants provided the following information: their month and year of birth; whether they had received a seasonal influenza vaccine in the preceding year; whether they worked face to face with patients in hospitals, nursing homes, doctors' clinics, or as community health workers; and their residential postal code.

The weekly proportion of participants with ILI signs or symptoms was calculated as the proportion of participants for that week who reported both fever and cough within the previous 7 days. These proportions were compared with influenza activity recorded in 2009 by other established New South Wales influenza surveillance systems, i.e., number of patients who visited emergency departments with ILI symptoms (*5*), laboratory-confirmed influenza A antigen tests (PCR and direct immunofluorescence) (*5*), Google Flu Trends data (aggregated Google search data used to estimate current influenza in Australia) (*6*), workplace absenteeism data (*5*), and Australian Sentinel Practice Research Network (ASPREN) general practice ILI data (*7*).

Surveillance data were compared with data from 2007 and 2008. NSW was selected because no other states had sufficient Flutracking participants in 2007 and 2008 to allow year-to-year comparisons. The number of NSW participants who completed ≥1 survey in the 2009 Flutracking surveillance system was 3,447.

The concordance across NSW influenza surveillance systems was high for ILI peak weeks during the past 3 years. During 2009, Flutracking, laboratory influenza notifications, and Google Flu Trends peaked 1 week before emergency department ILI, workplace absenteeism, and ASPREN ILI surveillance (Table).

A comparison of the weekly scale of NSW Flutracking fever and cough symptom rates during 2007, 2008, and 2009 showed that the peak attack rate of 6.8% in 2009 was significantly lower than that of 9.4% in 2007 and

Table. Peak ILI attack week and attack rates across influenza surveillance systems in New South Wales, Australia, 2007–2009*

	Peak week of ILI (week ending)			Peak ILI/influenza-related values		
Surveillance system/weekly measure used	2007	2008	2009	2007	2008	2009
Flutracking, fever and cough rate, %	Aug 5	Aug 24	Jul 12	9.4	5.8	6.8
No. laboratory notifications	Aug 5	Aug 31	Jul 12	133	69	1,167
No. ED ILI visits	Aug 19	Aug 31	Jul19	374	170	1,024
Google Flu Trends						
Influenza-related search term counts	Jul 22	Aug 31	Jul 12	1,933	1075	1,022
Workplace absenteeism, weekly rate, %	Jul 15	ND	Jul 19	1.5	ND	1.4
ASPREN, ILI/1,000 consultations, %	Aug 12	Sep 7	Jul 19	73.7	62.8	74.3

*ILI, influenza-like illness; ED, emergency department; ND, no data collected; ASPREN, Australian Sentinel Practice Research Network.

only slightly higher than the peak rate of 5.8% in 2008 (Figure). However, peak weekly NSW laboratory notifications were almost 9- and 17-fold higher in 2009 than in 2007 and 2008, respectively. Peak emergency department ILI patient visits were almost 3- and 6-fold higher in 2009 than in 2007 and 2008, respectively (Table; Figure).

The attack rate pattern for NSW Google Flu Trends data was similar to that of Flutracking; attack rates for 2009 were slightly lower than those for 2008 and about half those of for 2007. ASPREN ILI rates were slightly higher in 2009 than in 2007 and 2008. Workplace absenteeism data demonstrated a slightly more severe influenza season in 2007 than in 2009 (Table).

When the surveillance systems were compared, laboratory notifications and emergency department surveillance appeared to be more affected by health-seeking behavior and changes in physician's testing protocols and may not have reflected true community ILI rates, in contrast to Flutracking, Google Flu Trends, workplace absenteeism, and ASPREN. Potential biases in laboratory notifications and emergency department surveillance may vary, depending on the pandemic phase. For example, during the protect phase of the pandemic, testing for influenza was recommended only for those admitted to the hospital for ILI or when test results could alter clinical care of a patient. Before the protect phase (during the contain phase), testing for pandemic (H1N1) 2009 virus was conducted to confirm diagnosis for anyone with ILI.

Flutracking's finding of a 2009 peak ILI rate similar to those of previous years was also consistent with NSW mortality data. The number of NSW deaths attributed to influenza or pneumonia suggested that the 2009 influenza season did not result in excess overall deaths but rather a redistribution of deaths with a relative increase of deaths in younger age groups (*8*). The low ILI rate found by Flutracking was initially viewed with suspicion because other near real-time surveillance (laboratory notifications and emergency department surveillance) suggested a high pandemic (H1N1) 2009 attack rate compared with rates for previous years. However, Flutracking results were consistent with other pandemic influenza attack rate estimates in NSW and other countries (*9*–*12*).

Because Flutracking does not rely on the health sector for ILI or laboratory reporting, it is not biased by changes in testing, treatment seeking, jurisdictional protocols, or resource constraints. Flutracking, Gripenet, and other similar Internet-based surveillance could potentially facilitate near real-time comparison of ILI activity between regional jurisdictions and among countries to assist with monitoring the global spread of influenza (*13*).

Conclusions

During the initial pandemic (H1N1) 2009 outbreak, Flutracking demonstrated its ability to accurately identify peak influenza activity timing and the relative magnitude of community influenza activity when compared with influ-

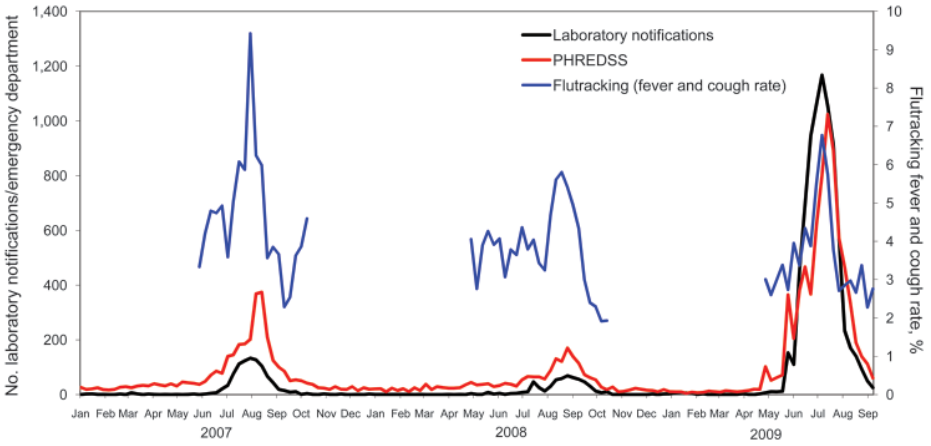

Figure. Flutracking fever and cough rates, counts of emergency department visits for influenza, and number of laboratory notifications for influenza, New South Wales, Australia, 2007–2009. PHREDSS, Public Health Real Time Emergency Department Surveillance System.

enza tracking efforts in previous years. Its results were also less affected by treatment-seeking behavior and by laboratory testing protocols during different pandemic phases than was health system–based surveillance.

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Bartonella henselae in Skin Biopsy Specimens of Patients with Cat-Scratch Disease

Emmanouil Angelakis, Sophie Edouard, Bernard La Scola, and Didier Raoult

During the past 2 years, we identified live *Bartonella henselae* in the primary inoculation sites of 3 patients after a cat scratch. Although our data are preliminary, we report that a cutaneous swab of the skin lesion from a patient in the early stage of cat-scratch disease can be useful for diagnosis of the infection.

Bartonella henselae is the main causative agent of cat-scratch disease (CSD). Little is known about the organism's pathogenesis in long-lasting lymphadenopathy, but an immunopathogenesis is assumed (*1*). *B. henselae* is infrequently grown from the lymph nodes of humans, and only in a few cases was *B. henselae* isolated from patients with CSD (*2*,*3*). In experiments with mice, *B. henselae* was eliminated within a few days to 1 week after systemic (intraperitoneal or intravenous) infection (*4*). Moreover, on the basis of molecular methods, we recently identified that the scalp eschars from 2 patients who were bitten by a tick contained *B. henselae* (*5*). In this study, our objective was to determine if *B. henselae* was present in the papule, which is developed in the scratch line. We report isolation of *B. henselae* from a swab specimen and the skin biopsy specimens sampled from the skin papule of 3 patients with CSD.

The Study

From January 2007 through February 2010, we tested 92 skin biopsy specimens from patients suspected of having CSD. DNA was extracted by using a QIAamp Tissue Kit (QIAGEN, Valencia, CA, USA) and was used as a template in a previously described real-time reverse transcription–PCR (RT-PCR) specific for a portion of the *Bartonella* 16S–23S intergenic spacer region and the *PAP31* gene for detection of *B. henselae* (*6*). *B. henselae* was identified in 4 skin biopsy specimens (4.3%). For each patient, we received a skin biopsy specimen from the skin papule, a lymph node biopsy specimen, and paired serum samples. For 1 patient, we also received a swab from a skin papule. Immunoglobulin G and M titers were determined by using an immunofluorescent antibody assay (*7*).

Skin biopsy specimens and the swab were cultured in human embryonic lung fibroblasts by using the centrifugation shell-vial technique (3.7 mL; Sterilin Ltd., Felthan, UK); 12-mm round coverslips seeded with 1 mL of medium containing 50,000 cells and incubated in a 5% CO_2 incubator at 37°C for 3 days were used to obtain a confluent monolayer (*8*). Cultures were surveyed for 4 weeks and detection of bacteria growth was assessed every 7 days on coverslips directly inside the shell vial by using Gimenez and immunofluorescence staining. We obtained a positive culture from 3 patients, and detailed histories are described below (Table).

Patient 1 was a 38-year-old man who had fever (40°C) and asthenia. The patient was a cat owner who had been scratched 8 days before onset of symptoms. Clinical signs were right axillary lymphadenitis and an inflammatory reddish skin lesion on the right hand with epitrochlear adenopathy, which appeared 2 days before he sought treatment. Abdominal ultrasound showed small hepatic abscesses. After the skin biopsy sample was obtained, doxycycline (200 mg/d) was given for 1 week. The patient fully recovered.

Patient 2 was a 17-year-old man with an inflamed red skin lesion on the right foot and epitrochlear adenopathy. The patient reported that he was scratched ≈1 week earlier by his cat and that the skin lesion appeared the day before he sought treatment. Right inguinal lymphadenitis was also identified during examination.

Patient 3 was a 20-year-old man had an inflammatory skin lesion on the left hand. He had a cat scratch 9 days before; the skin papule appeared 1 day before he sought treatment. Left axilliary lymphadenitis was identified during the examination and abdominal ultrasound showed hepatomegaly.

Skin biopsy specimens and lymph nodes from all patients were positive by real-time RT-PCR; patient 2 also had a positive swab specimen. Moreover, all patients had serum samples positive for *B. henselae* by immunofluorescent antibody assay. We detected gram-negative bacilli (Figure), which were identified as *B. henselae* by real-time RT-PCR (*6*), in the cultures of the skin biopsies and swab specimen. Patients 2 and 3 recovered without treatment.

Conclusions

We isolated *B. henselae* from skin biopsy specimens of 3 patients with CSD. Patients with CSD usually have gradual regional lymph node enlargement, accompanied by a papule, which develops in the scratch line after 3–10 days; the papule may persist for only a few days or as long as 2–3 weeks (*9*). Histopathologic of the skin lesion is similar to lymph node changes, consisting of a diffuse

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Table. Assessment and testing results for 3 patients with cat scratch disease who had skin biopsy specimens positive for *Bartonella henselae*, France, 2010*

Patient no.	Fever	Lymphadenopathy	Cat contact	Serologic results for *Bartonella* spp.	RT-PCR results for *B. henselae*			Culture results (incubation period)	
					Skin biopsy	Lymph node	Swab	Skin biopsy	Swab
1	Yes	Right auxiliary and epitrochlear lymphadenitis	Yes	Positive	Yes	Yes	ND	Positive (2 wk)	ND
2	No	Right inguinal and epitrochlear lymphadenitis	Yes	Positive	Yes	Yes	Yes	Positive (3 wk)	Positive (3 wk)
3	No	Left auxiliary lymphadenitis	Yes	Positive	Yes	Yes	ND	Positive (2 wk)	ND

*RT-PCR, reverse transcription–PCR; ND, not done.

inflammatory cell infiltrate associating numerous neutrophils and histiocytes mixed with scattered eosinophils and plasma cells (*9*). Other more unusual skin manifestations include morbilliform eruptions, urticaria, erythema nodosum, erythema multiforme, and erythema marginatum (*9*). *B. henselae* in the skin papule was first proposed by Wear et al., who reported that the primary inoculation site and the lymph nodes of patients with CSD contained the same small Gram-negative bacilli (*10*). Using immunohistochemical stain, Lin et al. found *B. henselae* in the cytoplasm of histiocytes within the granulomatous lesions in 9 lymph nodes and 1 skin biopsy specimen from patients with CSD (*11*). Avidor et al. identified *B. henselae* in inflammatory papules and pustules of 2 patients with CSD (*12*). Our group recently identified *B. henselae* in patients with scalp eschars and neck lymphadenopathy after tick bites (5). Moreover, Fournier et al. detected *B. henselae* in 2 skin biopsy specimens of a primary papule from patients in Australia clinically suspected of having CSD (*2*).

Swabs of lesions for the diagnosis and culture of *B. henselae* are not widely used. Fournier et al. found that swabs from 6 primary skin papules from patients clinically suspected of having CSD were positive for *B. henselae*; a positive culture was also obtained from 1 cutaneous swab (*2*). For rickettsial diseases, in 2006, the diagnosis of 1 case of scrub typhus was based on PCR results of the patient's eschar (*13*). Wang et al. identified 3 cases of Queensland tick typhus caused by *Rickettsia australis* and 1 case of African tick bite fever caused by *R. africae* by the use of PCR in dry and sterile saline moistened swabs collected from the eschar margin (*14*). *B. henselae* is often isolated from cutaneous tumors in AIDS and immunocompromised patients with bacillary angiomatosis (*15*); however, all our patients were immunocompetent.

In conclusion, we found live *B. henselae* in the primary inoculation site after a cat scratch. An incubation period of 2–3 weeks was necessary to obtain *B. henselae* isolates from the skin lesion, therefore, cultures are not proposed for point-of-care diagnosis. To reduce the delay in diagnosis, real-time RT-PCR enables rapid detection and identification of CSD in skin biopsy specimens and swabs. Probably crucial for the isolation of *B. henselae* was the fact that the skin biopsy specimens and the swab were sampled early after appearance of the skin papule and that patients did not receive treatment. Two of 3 patients recovered without antimicrobial drug treatment, which leads us to believe that treatment with antimicrobial drugs is not necessary for immunocompetent patients. A cutaneous swab of the skin lesion in the early stage of CSD infection may replace the more painful skin or lymph node biopsies.

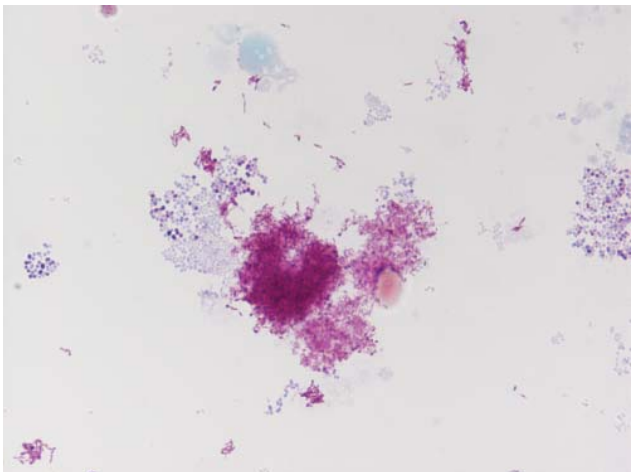

Figure. Gimenez stain of *Bartonella henselae* obtained by the culture in human embryonic lung of the skin biopsy of a patient with cat scratch disease, France, 2010. Original magnification ×100.

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Brucella ceti Infection in Harbor Porpoise (*Phocoena phocoena*)

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We describe *Brucella* sp. infection and associated lesions in a harbor porpoise (*Phocoena phocoena*) found on the coast of Belgium. The infection was diagnosed by immunohistochemistry, transmission electron microscopy, and bacteriology, and the organism was identified as *B. ceti*. The infection's location in the porpoise raises questions of abortion and zoonotic risks.

In cetaceans, *Brucella* spp. infections and related lesions have been found in bottlenose dolphins (*Tursiops truncatus*) (*1*), striped dolphins (*Stenella coeruleoalba*) (*2*–*5*), Atlantic white-sided dolphins (*Lagenorhynchus acutus*) (*6*,*7*), common dolphins (*Delphinus delphi*) (*6*,*8*), harbor porpoises (*Phocoena phocoena*) (*6*,*9*), and a minke whale (*Balaenoptera acutorostrata*) (*6*). Recently, *B. ceti* was described as being the cetacean *Brucella* sp. strain that infects dolphins (*10*). We report a case of *B. ceti* infection and associated lesions in a harbor porpoise found on the coast of Belgium in 2008.

The Study

An adult female harbor porpoise died on the coast of Belgium in 2008, and a necropsy was immediately performed by the Marine Animals Research and Intervention Network (Belgium). The most relevant findings (Table) were emaciation and multiple large skin ulcers (acute to chronic) around the genital split and between flippers (Figure 1). Internally, mild to severe nematode infestation (of the right ventricle, pulmonary blood vessels, airways) was associated with acute pulmonary thrombi and severe acute necrotizing pneumonia. The liver was enlarged and yellowish with multiple 1–2-mm red to dark red spots. The uterus was dilated with a larger left uterine horn and prominent congested blood vessels; a corpus luteum cyst was present in the left ovary. Microscopic examination showed severe, acute, necrotizing pneumonia and interstitial subacute to chronic pneumonitis with arteritis (mostly associated with lungworms); multiple foci of acute coagulative necrosis in the liver; and mild, multifocal, nonsuppurative meningitis. The mammary gland contained numerous small acini with small amounts of milk in the acini and ducts. Infiltrate of mononuclear cells under the endometrium suggested endometritis.

Immunohistochemical investigation, using polyclonal antiserum obtained from a rabbit experimentally infected with *B. melitensis*, showed diffuse intracytoplasmic positive staining for *Brucella* spp., primarily in mononuclear and inflammatory cells on various tissues (spleen [Figure 2], lymph nodes, lung, uterus, liver, pancreas, and brain), in lesions, in lungworms, and in mammary gland acini and milk. By transmission electron microscopy, large numbers of relatively small (diameter 380–450 nm) intracellular coccoid bacteria that suggested *Brucella* spp. were observed in the genital ulcer. A *Brucella* sp. isolate was obtained from brain and lung tissue. The strain grew on *Brucella* agar supplemented with 5% horse serum in the presence of basic fuchsine, thionine, and growth on safranin O. CO_2 was not required for growth, and H_2S was not produced. The isolates showed catalase, oxidase, and urease activity. This biotype profile is in agreement with the strain type profile of *B. ceti* (*10*). Multilocus variable number tandem repeat analysis (MLVA) typing, which used MLVA panel 1 (8 minisatellite loci: bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55, which are useful for species identification), showed that the strains belong to genotype 23 (*11*). MLVA panel 2 was split into 2 groups, panels 2A and 2B, comprising 3 (bruce18, bruce19, bruce21) and 5 (bruce04, bruce07, bruce09, bruce16, bruce30) markers, respectively (*12*). Using panel 2A, we obtained the same profile as the one described for all *B. ceti* strains isolated from porpoises (*11*), whereas panel 2B showed a new genotype (bruce04: 6 repeats, bruce07: 6 repeats, bruce09: 3 repeats, bruce16: 7 repeats, bruce30: 6 repeats), closely related to genotypes ascribed to *B. ceti* strains isolated from porpoises mainly stranded in Scotland (*11*). The new genotype identified by panel 2B is possibly associated with southern North Sea porpoises. However, panel 2B contains the more variable loci, and this panel has been given a lower weight in clustering analysis (*12*).

The results suggest a bacteremia associated with *B. ceti*. The infection was suspected after examination by electron microscopy and confirmed by bacteriologic and immunohistochemical investigations; finally, the bacterium was identified as *B. ceti*. In Europe, most reported

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Table. Postmortem findings in a harbor porpoise infected with *Brucella ceti*, Belgium, 2008*

Sample	Necropsy	Histologic	Immunohistochemistry†	Electron microscopy	Bacteriologic
Brain	NS	Slight subacute meningitis	Glial cells	NT	*Brucella* isolate
Uterus	Congestion and hyperplasia	Slight subacute endometritis	Mononuclear cells under the endormetrial epithelium	NT	NT
Mammary gland	Congestion	Well-developed acini with milk	Mononuclear cells between acini, acinar cells, and milk	NT	NT
Liver	Multifocal red to dark red spots	Coagulative necrosis	Mononuclear cells in portal areas	NT	NT
Lungs	Multifocal acute necrotizing thrombo-embolic pneumonia	Acute purulent pneumonia, severe subacute to chronic interstitial pneumonitis	Mononuclear cells and nematode larvae	NT	*Brucella* isolate
Lymph nodes	Hyperplasia	Lymphoid depletion	Mononuclear cells near the capsule	NT	NT
Skin and genital split	Multiple acute to chronic ulcers	Acute ulcerative dermatitis with ballooning degeneration	Balloon degenerated epithelial cells and inflammatory infiltrate	Intracellular coccoid bacteria (genital ulcer)	NT
Spleen	Hypoplasia	Lymphoid depletion	Mononuclear cells near the splenic capsule and between splenic corpuscles	NT	NT

*NS, not significant; NT, not tested.
†With polyclonal antibody against *B. melitensis.*

cases of cetacean brucellosis have been reported from the coasts of Scotland and England and found in striped dolphins, Atlantic white-sided dolphins, common dolphins, harbor porpoises, and a minke whale (*2*,*3*,*6*,*7*,*9*). Meningoencephalitis associated with *Brucella* spp. infection has been reported for striped dolphins (*2*,*3*) and 1 Atlantic white-sided dolphin (*7*). Necrosis of spleen, liver, and lymph nodes associated with *Brucella* spp. infection has also been reported for Atlantic white-sided dolphins (*6*). In porpoises, *Brucella* spp. have been isolated from different organs without associated pathologic changes other than coagulative necrosis of the spleen (*6*) and a testicular abscess (*9*). Finally, in the minke whale, foci of liver necrosis and inflammation were consistent with lesions caused by *Brucella* spp. (*6*). In our study, the enlarged uterine horn, the corpus luteum cyst, and the presence of milk in mammary acini suggested recent pregnancy, and the positive immunolabeling of the endometrium raised the question of a possible abortion. Indeed, *Brucella* spp. are known to be responsible for abortions in terrestrial mammals, Brucella spp.–induced abortions have been described in 2 bottlenose dolphins with associated placentitis (*1*), and *Brucella* spp. have been isolated from an aborted bottlenose dolphin fetus (*13*). *Brucella* antigens were detected in the placenta of a stranded striped dolphin with a 7-month-old dead fetus (*5*). In addition, vaginal lithiasis suspected to be the result of ossification of aborted fetuses in 2 common dolphins positive for *Brucella* spp. in the uterus has been reported (*8*).

Conclusions

In the present case, a final conclusion cannot be drawn with respect to a possible abortion. Identification of *B. ceti* in milk (as in the present study) and in fetal tissues and secretions of a pregnant dolphin suggest that *B. ceti* has tropism for placental and fetal tissues and that it can be shed externally (*4*). This finding suggests potential vertical and horizontal transmission to newborns (*4*). Nevertheless, indirect transmission through parasites should not be excluded because *Brucella* spp. have been identified from

Figure 1. Longitudinal skin ulcer between flippers of a harbor porpoise (*Phocoena phocoena*) with *Brucella ceti* infection, Belgium, 2008.

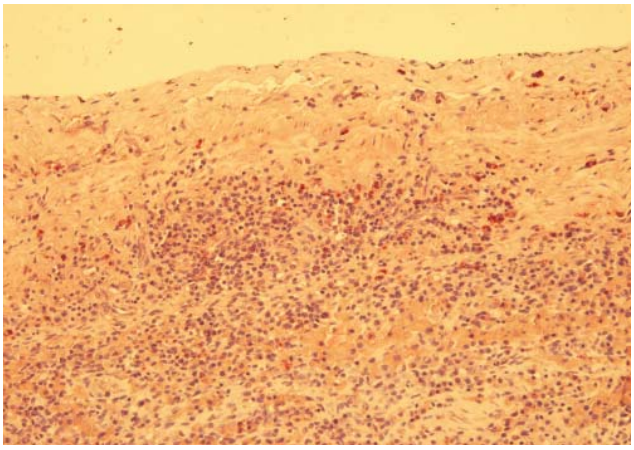

Figure 2. Positive immunohistochemical staining in mononuclear cells below the splenic capsule in a harbor porpoise (*Phocoena phocoena*) with *Brucella ceti* infection, Belgium, 2008. Original magnification ×200.

lungworms (*14*). In addition, the observation of *Brucella* spp. antigens in milk and in skin ulcers may represent routes of bacterial transmission between individual animals and raises the question of the risk for transmission to a person handling the cetacean (e.g., on the beach or in a rehabilitation center). All persons handling wild or captive marine mammals (alive or dead) or samples collected from the mammals should be aware of such risks and take necessary precautions. To date, 4 cases of human infection with *Brucella* spp. from marine mammals are known. One was mild and uncomplicated in a laboratory worker; however, the 3 other cases were severe naturally acquired without direct contact with marine mammals but with a history of eating raw fish or shellfish (*15*).

We emphasize that further investigations are needed to improve knowledge of the prevalence, the impact on individual cetaceans and populations, and the zoonotic potential of marine mammal brucellosis. The zoonotic risk should be taken into account by all persons in contact (direct or indirect) with marine mammals. Finally, the present case confirms the need for careful monitoring and complete postmortem examinations of stranded marine mammals.

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Proportion of Deaths and Clinical Features in Bundibugyo Ebola Virus Infection, Uganda

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The first known Ebola hemorrhagic fever (EHF) outbreak caused by Bundibugyo Ebola virus occurred in Bundibugyo District, Uganda, in 2007. Fifty-six cases of EHF were laboratory confirmed. Although signs and symptoms were largely nonspecific and similar to those of EHF outbreaks caused by Zaire and Sudan Ebola viruses, proportion of deaths among those infected was lower (≈40%).

Ebola hemorrhagic fever (EHF) is a severe disease caused by several species of *Ebolavirus* (EBOV), in the family *Filoviridae*. Before 2007, four species of EBOV had been identified; 2 of these, *Zaire ebolavirus* and *Sudan ebolavirus*, have caused large human outbreaks in Africa, with proportion of deaths ≈80%–90% and 50%, respectively (*1*–*5*). Large outbreaks are associated with person-to-person transmission after the virus is introduced into humans from a zoonotic reservoir. Data suggest that this reservoir may be fruit bats (*6*,*7*). During outbreaks of EHF, the virus is commonly transmitted through direct contact with infected persons or their bodily fluids (*8*–*11*). The onset of EHF is associated with nonspecific signs and symptoms, including fever, myalgias, headache, abdominal pain, nausea, vomiting, and diarrhea; at later stages of disease, overt hemorrhage has been reported in ≈45% of cases (*12*).

Bundibugyo District is located in western Uganda, which borders the Democratic Republic of Congo. After reports of a mysterious illness in Bundibugyo District, the presence of a novel, fifth EBOV virus species, *Bundibugyo ebolavirus* (BEBOV), was identified in diagnostic samples submitted to the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, in November 2007 (*13*). In response to detection of EBOV, an international outbreak response was initiated. In this report, we summarize findings of laboratory-confirmed cases of BEBOV infection.

The Study

Anecdotal reports suggested that human illness consistent with a viral hemorrhagic fever arose in Bundibugyo District as early as August 2007. After EHF was confirmed (*13*), isolation wards were established at 2 medical facilities in the district. Diagnostic samples from hospitalized patients with acute illness and community residents who had febrile illnesses and multiple additional signs, symptoms, or epidemiologic exposures suggestive of EHF, were routinely collected for EBOV testing. These signs and symptoms included headache, vomiting, diarrhea, abdominal pain, conjunctivitis, skin rash, muscle pain, fatigue, difficulty swallowing, difficulty breathing, hiccups, bleeding, unexplained death, or contact with a patient with suspected EHF. A laboratory-confirmed case of EHF was defined as illness in a person whose diagnostic samples were found positive for EBOV infection by any of the following tests: PCR (*13*), virus isolation, antigen detection, or immunoglobulin (Ig) M ELISA (*14*,*15*).

In addition, in a subset of surviving persons who had a history of illness consistent with EHF but no acute-phase blood samples available for testing, a convalescent-phase blood sample was collected for laboratory confirmation by IgG ELISA (*14*, *15*). Laboratory testing was performed at the Uganda Viral Research Institute in Entebbe, and subsequent testing was performed on some samples at CDC, Atlanta. This analysis is limited to laboratory-confirmed EHF cases, although additional suspected cases were identified during the outbreak.

Fifty-six confirmed cases of EHF were identified; 43 of these were diagnosed on the basis of positive test results from acute-phase specimens (Figure). Twenty-six patients had a positive EBOV IgG titer in convalescent-phase serum, including 13 persons who had evidence of EBOV infection in acute-phase and convalescent-phase samples.

The proportion of deaths during this outbreak was calculated for case-patients confirmed on the basis of an acute-phase diagnostic sample (those who only had a convalescent-phase sample are by definition surviving case-patients and represent a biased sample). Of the 43 cases

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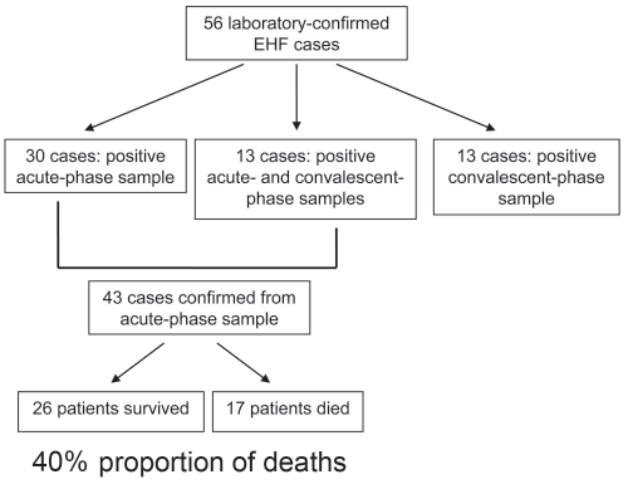

Figure. Number of laboratory-confirmed Ebola hemorrhagic fever (EHF) cases diagnosed on the basis of positive acute-phase or convalescent-phase diagnostic samples and calculation of proportion of deaths among case-patients who had an acute-phase diagnostic sample, Bundibugyo District, Uganda, 2007.

confirmed from acute-phase samples, 17 deaths occurred, for a proportion of 40%. The mean age of those who died (42 years, range 20–70 years) was significantly higher than that of survivors (33 years, range 12–50 years; p = 0.0390). No gender bias was observed between survivors and those who died (Table).

Signs and symptoms were reported by patients on standardized surveillance case-report forms at the time of case identification, and in some instances, were examined further by chart review or follow-up interview. Common symptoms among patients with laboratory-confirmed cases included fever, fatigue, headache, nausea/vomiting, abdominal pain, muscle/joint pain, diarrhea, and anorexia/weight loss (Table). No difference in the proportion of those reporting signs and symptom between those who survived and those who died was noted, except for difficulty swallowing. This symptom was more common among case-patients who died (though marginally significant, p = 0.0851). Bleeding of any type (bleeding from injection site, gums, eyes, nose, vagina; black or bloody stool; bloody vomitus; or hematuria) was reported among 54% of all patients with laboratory-confirmed cases.

As part of the standardized surveillance case-report form, patients were also asked whether they had had contact with a sick person during the 3 weeks before development of illness. A large portion of the laboratory-confirmed case-patients in this outbreak reported direct contact with a specific person, (case X), who died of a severe hemorrhagic febrile illness consistent with EHF (no diagnostic specimens were collected from this person) in November 2007. Using the date of last contact for those reporting contact with case X or reporting contact with another laboratory-confirmed case-patient to the date of symptom onset, we

Table. Demographic characteristics and signs and symptoms for 56 case-patients who had laboratory-confirmed EHF, Bundibugyo District, Uganda, in 2007*

Characteristic	Case-patients confirmed by acute-phase sample, n = 43			Total no. confirmed case-patients, n = 56
	No. survived, n = 26	No. died, n = 17	p value	
Mean age, y (range)	33 (12–50)	42(20–70)	0.039†	37.4 (11–70)
Male sex (%)	16 (62)	8 (47)	>0.100‡	30 (54)
Mean incubation period, d (95% CI)§	5.7 (4.4–7.0)	7.4 (5.4–9.3)	>0.100†	6.3 (5.2–7.3)
Signs and symptoms, no. reporting/no. available (%)¶				
Fever	26/26 (100)	16/16 (100)	>0.100	55/55 (100)
Fatigue	22/23 (96)	14/14 (100)	>0.100	49/50 (98)
Headache	21/25 (84)	14/15 (93)	>0.100	48/53 (91)
Nausea/vomiting	24/26 (92)	13/15 (87)	>0.100	48/54 (89)
Abdominal pain	23/26 (88)	13/14 (93)	>0.100	47/53 (89)
Muscle/joint pain	19/23 (83)	12/14 (86)	>0.100	44/50 (88)
Diarrhea	24/26 (92)	13/15 (87)	>0.100	47/54 (87)
Anorexia/weight loss	19/23 (83)	12/15 (80)	>0.100	43/51 (84)
Difficulty swallowing	10/23 (43)	6/15 (60)	>0.100	27/51 (53)
Rash	9/26 (35)	5/15 (33)	>0.100	25/54 (46)
Difficulty breathing	6/23 (26)	8/14 (57)	0.085	23/50 (46)
Hiccups	4/23 (17)	6/15 (40)	>0.100	16/51 (31)
Bleeding#	11/26 (42)	9/17 (53)	>0.100	30/56 (54)

*EHF, Ebola hemorrhagic fever; CI, confidence interval.
†By 2-sample t-test.
‡By χ^2 test.
§For case-patients reporting contact with a confirmed case-patient, or contact with case-patient X (see text for full description), based on time from last reported contact with case-patient to development of signs and symptoms (n = 16 for case-patients in Survived column; n = 8 for case-patients in Died column; n = 24 for all confirmed case-patients).
¶No. case-patients reporting the sign or symptom/no. case-patients with information available about presence of that the sign or symptom. p value was determined by Fisher exact test in which a comparison was made between the proportion of case-patients who survived vs. those who died.
#At least 1 of the following manifestations: bleeding from injection site, gums, eyes, nose, vagina; black or bloody stool; bloody vomitus; hematuria.

calculated an average incubation period of 6.3 days among laboratory-confirmed EHF case-patients (n = 24). No significant difference was noted in the incubation period between survivors (5.7 days) and those who died (7.4 days).

Conclusions

The 2007 outbreak of EHF in Bundibugyo District, Uganda, was caused by a new EBOV species, *Bundibugyo ebolavirus* (*13*). Previous outbreaks of EHF have resulted in high proportion of deaths, ranging from 50% to 90% (*1*–*5*). The proportion of deaths among case-patients with EHF confirmed by acute diagnostic specimens in this outbreak was 40%, a lower percentage than for other species of EBOV that have caused human outbreaks in Africa. However, we cannot exclude the possibility that the lower proportion of deaths in this outbreak is an artifact of differences in the severity of laboratory-confirmed cases detected through outbreak surveillance or the quality of care received by hospitalized laboratory-confirmed EHF case-patients in Bundibugyo District. Nonetheless, sustained person-to-person transmission was sufficient to result in a sizeable outbreak, and death was clearly not uncommon. Thus, BEBOV should be considered a pathogen of serious public health concern.

As with previously documented EHF outbreaks, older age appeared to be a risk factor for death (*3*). The incubation period of EHF was ≈1 week, and signs and symptoms were largely nonspecific; infections frequently involved fever, fatigue, headache, gastrointestinal involvement, and muscle and joint pain. The nonspecific nature of these signs and symptoms, which may mimic other tropical diseases, made diagnosis of EHF based on clinical characteristics alone particularly challenging and underscores the importance of laboratory-based diagnostics to confirm and monitor control and response efforts for EHF outbreaks. Notably, signs and symptoms described in this report are primarily based on information from patient surveillance case-report forms filled out at the time of triage, and they may not represent the full spectrum of illness experienced by all persons with EHF during this outbreak.

It is apparent that novel emerging infections continue to occur. The outbreak of EHF described in this report involved a previously unidentified EBOV species, with a proportion of deaths of 40%. BEBOV represents the fourth EBOV species–associated disease in humans, and the third species to cause large human outbreaks of EHF. Although proportion of deaths was lower than that documented in previous EHF outbreaks, BEBOV is a severe human pathogen with epidemic potential. These findings demonstrate the need for increased surveillance and diagnostic capabilities, as well as the capacity to respond quickly to emerging human infections.

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Leishmania tropica Infection in Golden Jackals and Red Foxes, Israel

Dalit Talmi-Frank, Noa Kedem-Vaanunu, Roni King, Gila Kahila Bar-Gal, Nir Edery, Charles L. Jaffe, and Gad Baneth

During a survey of wild canids, internal transcribed spacer 1 real-time PCR and high-resolution melt analysis identified *Leishmania tropica* in samples from jackals and foxes. Infection was most prevalent in ear and spleen samples. Jackals and foxes may play a role in the spread of zoonotic *L. tropica*.

Leishmania tropica is a major cause of cutaneous leishmaniasis in the Old World. Although cutaneous leishmaniasis associated with *L. tropica* usually is considered an anthroponotic infection (*1*) in Israel, Jordan, and the Palestinian Authority, it appears to be a zoonosis with a main putative reservoir host, the rock hyrax (*Procavia capensis*) (*2*,*3*). Nevertheless, the possible involvement of other animals in the sylvatic transmission of *L. tropica* infection is not yet fully understood. *L. tropica* has been sporadically reported from domestic dogs from human cutaneous leishmaniasis foci in Iran and Morocco (*4*,*5*) but not from wild canids. Previous studies of leishmaniasis in wild canids, such as red foxes (*Vulpes vulpes*) in southern Italy (*6*) and wolves (*Canis lupus*) in southwestern Europe (*7*), found them to be infected with *L. infantum.* Golden jackals (*Canis aureus*) infected with *L. infantum* were reported in Iraq (*8*) and Kazakhstan (*9*). A seroepidemiologic study of *Leishmania* spp. infection in Israel showed that 7.6% of jackals and 5% of foxes tested were seropositive by using *L. donovani* antigen (*10*). The aim of this study was to identify and characterize *Leishmania* spp. infection in wild canids, including jackals, foxes, and wolves, in Israel by using species-specific real-time PCR.

The Study

Wild golden jackals, red foxes, and gray wolves were trapped at 57 different locations in Israel as part of a survey for oral rabies vaccination conducted by the Israeli Nature and Parks Authority and the Veterinary Services. DNA was extracted from ear pinna, snout, blood, and spleen by using the guanidine thiocyanate technique (*11*); in some animals, samples were not available from all sites. DNA from all tissues was tested for *Leishmania* spp. infection by internal transcribed spacer 1 (ITS1) real-time PCR and high-resolution melt analysis (ITS1-HRM) PCR (*12*). A 265–288-bp fragment, depending on the *Leishmania* species, within the ITS1 region of the leishmanial rRNA was amplified as previously described (*12*). All samples were tested in duplicates and results were compared with those from HRM analysis of positive controls for each assay. These were *L. infantum* (MCAN/IL/2002/Skoshi), *L. tropica* (MHOM/IL/2005/LRC-L1239), and *L. major* (MHOM/TM/1973/5ASKH). Negative controls included samples from jackals born and reared at a zoo in central Israel, as well as from foxes and wolves from areas in which leishmaniasis is not endemic, that were tested by PCR and found negative. All positive PCR products were purified by using ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced at the Center for Genomic Technologies, Hebrew University of Jerusalem. Sequences obtained were compared for similarity to sequences in GenBank by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST). Positive samples also were verified by kDNA PCR as described (*13*).

Sequences were analyzed by using MEGA version 3.0 (www.megasoftware.net). A phylogenetic tree was constructed by using the neighbor-joining method in agreement with maximum-parsimony and maximum-evolution algorithms and by using the Kimura 2-parameter model with uniform rates for transitions and transversions. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1,000 randomly selected samples of the aligned sequence data. Sequences were compared with the following *Leishmania* sequences deposited in GenBank: *L. tropica* FJ595949 and FJ595950 from central Israel and IARA/IL/02/LRC-L910 and ISER/IL/02/LRC-L909 from northern Israel; *L. infantum* (MHOM/TN/1980/IPT1) and *L. major* (MHOM/TM/1973/5ASKH) were used as outgroups.

We examined 208 samples from 113 wild canids by ITS1-HRM PCR: 152 samples from 77 golden jackals, 44 from 25 red foxes, and 12 samples from 11 wolves. None of the animals had clinical signs attributed to leishmaniasis. Seven animals tested positive for *L. tropica*, and 1 was positive for *L. infantum*. The overall *Leishmania* infection rate for jackals was 7.8% (6/77) and for foxes 8% (2/25). All wolves were negative. Fourteen tissue samples (ear, snout, spleen, and blood) tested positive. Five (63%) of the 8 animals positive for *Leishmania* spp. had ≥2 infected tissues (Table). Ears were positive for 6 of 8 infected animals and spleen for 4 of 8 animals. The snout sample was positive

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Table. Sequence similarity obtained for *Leishmania* spp. ITS1-positive tissue samples from jackals and foxes, Israel*

Subject no.	Animal species	Tissue	HRM results	ITS1 sequence length (% similarity†)	GenBank accession nos.	
					Comparison isolate	Identified isolate
918	Jackal	Right ear, left ear	*L. tropica*	236–239 bp (98)	FJ948456	GU591390
922	Jackal	Right ear, left ear	*L. tropica*	235–239 bp (98)	FJ948456	GU591391
1067	Jackal	Right ear, spleen	*L. tropica*	233–237 bp (98)	FJ948456	GU591392
1086	Jackal	Right ear, spleen	*L. tropica*	234–238 bp (98)	FJ948456	GU591393
1380	Jackal	Spleen	*L. tropica*	235–239 bp (98)	FJ948456	GU591394
115	Jackal	Blood	*L. infantum*	221–222 bp (99)	GU045592	GU591395
1084	Fox	Right ear, snout, spleen	*L. tropica*	234–239 bp (98)	FJ948456	GU591396
579916	Fox	Left ear	*L. tropica*	235–239 bp (98)	FJ9484556	GU591397

*ITS1, internal transcribed spacer 1; HRM, high-resolution melt analysis.
†Similarity to comparison isolate by BLAST (www.ncbi.nlm.nih.gov/BLAST).

for another animal for which blood also was positive. Four (15%) of 26 spleens collected were positive.

The ITS1-HRM PCR DNA product size was 265 bp for *L. infantum* and 273 bp for *L. tropica*. Sequencing verified the species specific results. All samples positive by ITS1-HRM PCR were also positive by kDNA PCR and produced a 120-bp kDNA product.

Thirteen sequences from positive DNA products obtained by ITS1-HRM PCR were identified as belonging to *L. tropica*, showing the closest similarity (98%–99%) to *L. tropica* sequences deposited in GenBank (Table). Only 1 sequence was amplified by using DNA extracted from the blood of a jackal for which other tissues were not available. This sequence was closest (99.5% identity over 222 bp) to *L. infantum* (Table). DNA sequences from all the positive tissues belonging to an individual animal were aligned, and consensus sequences representing each animal were created. These consensus sequences were deposited in GenBank under accession nos. GU591390–GU591397 and included in the phylogenetic tree (Figure). By using 3 algorithms, sequences obtained from 7 wild canids clustered with *L. tropica* isolated from hyraxes in central Israel (FJ595949 and FJ595950), and the sample amplified from the jackal blood clustered with *L. infantum* (MHOM/TN/1980/IPT1).

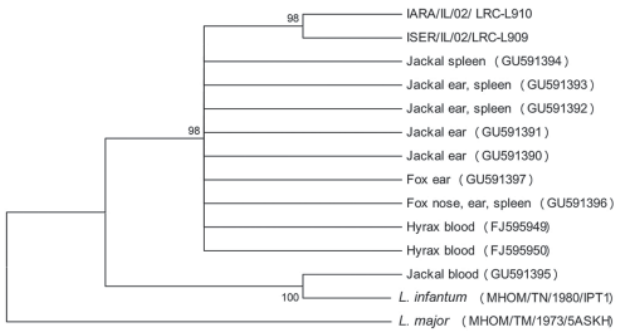

Figure. Neighbor-joining tree phylogram comparing internal transcribed spacer 1 (ITS1) *Leishmania tropica* DNA sequences from wild canids, Israel. The neighbor-joining tree constructed in MEGA version 3.0 (www.megasoftware.net) by the ITS1 HRM PCR sequences (222–239 nt) agrees with the maximum-likelihood algorithm. The tree shown is based on the Kimura 2-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. The analysis provided tree topology only; the lengths of the vertical and horizontal lines are not significant. *L. major* was used as an outgroup. GenBank accession numbers of *L. tropica* from hyraxes deposited from this study are shown in brackets. Numbers on nodes represent bootstrap values. MHOM, human; IARA, *Phlebotomus arabicus* sand fly; ISER, *Phlebotomus sergenti* sandfly.

Conclusions

We report *L. tropica* infections in jackals and foxes from Israel. Sequence analysis (using 3 algorithms) of ITS1 fragments showed perfect correlation with *L. tropica* isolates from hyraxes in central Israel. The finding of *L. tropica* positivity in >1 tissue sample from infected asymptomatic animals implies that wild canid species could be natural hosts for this parasite. Furthermore, the relatively high percentage of infected spleens indicates that this parasite can visceralize in foxes and jackals. Unlike hyraxes, which generally stay close to their burrows in caves or boulders, jackals and foxes travel long distances, potentially transmitting *L. tropica* from 1 area to another, provided that competent sandfly vectors are found. The home range of golden jackals in Israel is adapted to the food resources available. For golden jackals, it was 6.6 km^2 near settlements and 21.2 km^2 in sparsely inhabited settings (*14*). Foxes may foray 5.3 km and less frequently roam 7.8 km–15 km (*15*). Wild canids may transmit *L. tropica* from an area with an infected population of hyraxes to a remote naive hyrax population or be responsible for infecting humans because they tend to live peridomestically and frequently rely on human waste. The involvement of wild canids in the sylvatic life cycle of *L. tropica* could be crucial to understanding disease emergence in Israel, Jordan, and the Palestinian Authority (*2*). Populations of jackals and foxes, which were nearly eliminated in Israel during 1950–1980, have recovered and grown in parallel with the local outbreaks of *L. tropica* in humans (*10*). Further study is required to discern the potential epidemiologic role of wild canids in spreading and transmitting infection.

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Co-detection of Pandemic (H1N1) 2009 Virus and Other Respiratory Pathogens

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From May through October 2009, a total of 10,624 clinical samples from 23 US states were screened for multiple respiratory pathogen gene targets. Of 3,110 (29.3%) samples positive for pandemic (H1N1) 2009 virus, 28% contained ≥1 other pathogen, most commonly *Staphylococcus aureus* (14.7%), *Streptococcus pneumoniae* (10.2%), and *Haemophilus influenzae* (3.5%).

For previous and current influenza A pandemics, postmortem studies have established a strong link between secondary bacterial infections and increased deaths (*1*,*2*). Numerous respiratory pathogens can be detected from a single sample by using a multiplex molecular method called target-enriched multiplex PCR (*3*–*6*). During the 2006 influenza season, this method was used at Vancouver Children and Women's Hospital to study 1,742 patients with acute respiratory infections; >2 pathogens were detected for ≈27% of patients studied (*7*). We used this method to learn more about infections occurring concurrently with pandemic (H1N1) 2009.

The Study

From May through October 2009, a total of 10,624 clinical samples from 23 states throughout the United States were submitted to Diatherix Laboratories (www.diatherix.com; Huntsville, AL, USA) and screened for multiple respiratory pathogen gene targets. Diatherix, a reference laboratory certified by the Clinical Laboratory Improvement Amendments, provides molecular differential detection services based on target-enriched multiplex PCR technology. The respiratory infections panel detects bacterial and viral pathogens associated with respiratory infections and includes targets for the following: adenovirus (types 3, 4, 7, 21), coxsackievirus, echovirus, human metapneumovirus (types A and B), influenza virus (types A and B), parainfluenza virus (types 1–4), respiratory syncytial virus (types A and B), rhinovirus, *Acinetobacter baumannii, Chlamydophila pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Legionella pneumophila, Mycoplasma pneumoniae, Neisseria meningitidis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae,* and *Streptococcus pyogenes* (group A). Additionally, targets specific for pandemic (H1N1) 2009 virus were developed, validated, and approved by the US Food and Drug Administration under Emergency Use Authorization provisions for patient testing.

Of the respiratory specimens shipped by overnight mail from the 23 states, >95% were nasopharyngeal swabs in transfer buffer. High-throughput nucleic acid extraction was performed automatically by using KingFisher 96 instrumentation (Thermo Scientific, Hudson, NH, USA) and MagnetX chemistry (Scigenix, Marietta, GA, USA) according to manufacturers' specifications. Multiplex PCR amplification and Luminex (Austin, TX, USA) liquid suspension detection methods were based on internally validated protocols. Reactions were amplified by using ABI 9700 thermocyclers (Applied BioSystems, Singapore), and the resulting PCR products were detected by using the LiquiChip 200 Workstation (Luminex) according to previously described protocols (*3*,*6*).

Of the 10,624 samples studied, 4,690 (44.1%) were negative for all pathogens detectable with the assay. Among the 7,514 (70.73%) samples negative for pandemic (H1N1) 2009 virus, 3 bacterial pathogens predominated: *S. aureus* (875; 11.65%), *S. pneumoniae* (573; 7.63%), and *H. influenzae* (411; 5.47%) (Table 1). The most common viral pathogens in the pandemic (H1N1) 2009–negative samples were from the family *Picornaviridae*: coxsackie/echovirus (650; 8.65%), and rhinovirus (449; 5.98%) (Table 2).

Of the 10,624 samples studied, 3,110 (29.3%) were positive for pandemic (H1N1) 2009 virus, representing 52.4% of samples positive for any pathogen. Among pandemic (H1N1) 2009 virus–positive samples, ≥1 other pathogen was co-detected for 28% (Figure). The most commonly co-detected pathogens were *S. aureus* (458; 14.73%), *S. pneumoniae* (316; 10.16%), and *H. influenzae* (110; 3.54%) (Table 1).

A significant difference ($t = 25.6$, $p = 0.01$) was found for the age distribution between patients with positive and negative pandemic (H1N1) 2009 virus results. The mean ± SD age was 19.64 (±14.45) years for those who were pandemic (H1N1) 2009 positive and 29.67 (±19.74) years for those who were negative. The median age of the 5 patients for whom 3 other pathogens were co-detected with pandemic (H1N1) 2009 virus (Figure) was 15.5 years. *S. pneumoniae* was detected in all 5 of these samples. For most,

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Table 1. Results of screening of clinical samples from 23 US states for pandemic (H1N1) 2009 virus and bacterial respiratory targets, May–October 2009*

	Bacteria detected, no. (%) samples									
No. (%) samples	***S. aureus***	*P. aeruginosa*	*S. pyogenes*	*N. meningitidis*	***H. influenzae***	*C. pneum*	*K. pneum*	*M. pneum*	***S. pneum***	*A. baumannii*
Pandemic (H1N1) 2009 positive, 3,110 (29.270)	**458 (14.727)**	3 (0.096)	4 (0.129)	7 (0.225)	**110 (3.537)**	1 (0.032)	22 (0.707)	1 (0.032)	**316 (10.161)**	34 (1.093)
Pandemic (H1N1) 2009 negative, 7,514 (70.730)	**875 (11.645)**	28 (0.373)	34 (0.452)	16 (0.213)	**411 (5.470)**	8 (0.106)	44 (0.586)	8 (0.106)	**573 (7.626)**	152 (2.023)
Total, 10,624 (100.000)	**1,333 (12.547)**	31 (0.292)	38 (0.358)	23 (0.216)	**521 (4.904)**	9 (0.085)	66 (0.621)	9 (0.085)	**889 (8.367)**	186 (1.751)

*Screening for *Legionella pneumophilia* detected no bacteria in any samples. *S. aureus, Staphylococcus aureus; P. aeruginosa, Pseudomonas aeruginosa; S. pyogenes, Streptococcus pyogenes; N. meningitidis, Neisseria meningitidis; H. influenzae, Haemophilus influenzae; C. pneum, Chlamydophila pneumoniae; K. pneum, Klebsiella pneumoniae; M. pneum*, *Mycoplasma pneumoniae;* S. *pneum, Streptococcus pneumoniae*; *A. baumannii*, *Acinetobacter baumannii*. **Boldface** indicates predominant pathogens.

the other 2 pathogens were bacteria; for only 1, a virus (parainfluenza) was detected. Of the 96 samples in which pandemic (H1N1) 2009 virus and 2 other pathogens were co-detected (Figure), 30 (31.25%) contained *S. pneumoniae* and *H. influenzae*. The median age of these 30 patients was 4.25 years, whereas the median age of all 96 patients was 8.2 years. The median age for the 28% of patients for whom ≥1 other target was detected was 11.8 years.

Conclusions

The main finding of this large-scale clinical study was the co-detection of multiple pathogens with the pandemic influenza virus strain. In 44% of samples, no pathogens were detected, which may represent infection with common pathogens not detected by the assay. For example, bocavirus and all coronavirus groups not detected by the assay account for ≈12% and 5%–10%, respectively (*8*,*9*), of respiratory infections. An expanded test menu may improve the detection rate for such pathogens.

This study raises 2 questions. First, does co-detection equal co-infection? Second, and more practical, does co-detection change the clinical outcome? We chose the word co-detection rather than co-infection or co-colonization because co-infection means all identified microorganisms contributed to the pathogenic effect, and co-colonization may not indicate the causative agent. Co-detection indicates that ≥1 other pathogen was detected in a sample. The differences among the definitions have etiologic meaning, but the data presented here cannot be used directly to address etiology.

Most samples in this study were nasal swabs rather than upper or lower respiratory tract samples. Nasal swab samples have greater potential for contamination with normal flora, particularly *S. aureus.* No data on asymptomatic carriers were available because these persons rarely seek healthcare. However, these findings raise questions about the effectiveness of the single-agent etiology approach toward infectious diseases. Pandemic (H1N1) 2009 virus and multiple other pathogens are often detected during autopsy (*1*,*2*), indicating that co-infection may play a major role in the disease process. In addition, detection of multiple pathogens is associated with increased critical illness in children (*7*).

The Centers for Disease Control and Prevention identified "the need for early recognition of bacterial pneumonia in persons with influenza" (*2*). However, no suggestions were provided for meeting this need. Furthermore, the Centers "underscore the importance of managing patients with influenza who also might have bacterial pneumonia with both empiric antibacterial therapy and antiviral medications" (*2*) without identifying measures that would make this task tangible. Current practices of clinical diagnosis based on signs and symptoms inherently lack this type of information.

Table 2. Results of screening for of clinical samples from 23 US states for pandemic (H1N1) 2009 virus and respiratory pathogen gene targets, May–October 2009*

	Viruses detected, no. (%) samples							
No. (%) samples	Adeno	Coxackie/echo	Metapneumo	Influenza A	Influenza B	Parainfluenza	RS	Rhino
Pandemic (H1N1) 2009 positive, 3,110 (29.270)	1 (0.032)	13 (0.418)	0	0	0	3 (0.096)	0	7 (0.225)
Pandemic (H1N1) 2009 negative, 7,514 (70.730)	17 (0.226)	**650 (8.651)**	14 (0.186)	3 (0.040)	2 (0.027)	173 (2.302)	3 (0.040)	**449 (5.976)**
Total, 10,624 (100.000)	18 (0.169)	**663 (6.240)**	14 (0.132)	3 (0.028)	2 (0.019)	176 (1.656)	3 0.(028)	**456 (4.292)**

*RS, respiratory syncytial. **Boldface** indicates predominant pathogens.

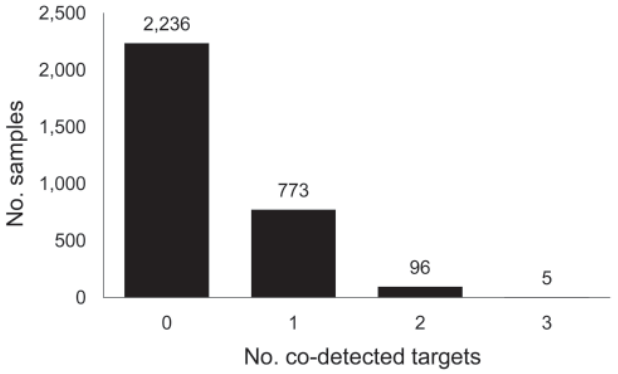

Figure. Respiratory pathogens co-detected with pandemic (H1N1) 2009 virus in clinical samples from 23 US states, May–October 2009.

The true value of a multiplex molecular method of screening for infectious respiratory agents depends on the clinical relevance. Among the samples with ≥1 positive results, 53% had positive results for viral pathogens without co-detection of bacterial pathogens. For these patients, prescription of antimicrobial drugs on the basis of clinical findings alone could serve to spread drug resistance through selective pressure on normal flora. Furthermore, limited secondary treatment resources, such as oseltamivir administration during a pandemic, could be prioritized on the basis of screening results. Of the 10,624 samples studied, 70.7% were negative for the pandemic (H1N1) 2009 virus strain.

Our findings suggest that multiplex screening for respiratory pathogens is useful for providing rapid surveillance information to inform physicians who would otherwise base decisions on clinical signs and symptoms alone. Electronic reporting of empirical laboratory respiratory pathogen detection provided by a Clinical Laboratory Improvement Amendments–approved laboratory can greatly enhance surveillance data collection (*10*). Because most states have the authority to collect data of public health relevance (*10*), the screening service provided by the Diatherix Laboratories could facilitate reporting of notifiable diseases.

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Alkhurma Hemorrhagic Fever in Travelers Returning from Egypt, 2010

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Two travelers returning to Italy from southern Egypt were hospitalized with a fever of unknown origin. Test results showed infection with Alkhurma virus. The geographic distribution of this virus could be broader than previously thought.

Alkhurma virus (ALKV) is a recently described member of the tick-borne hemorrhagic fever group of the genus *Flavivirus*. It was initially isolated in the late 1990s (*1*,*2*) and is today considered a variant of the Kyasanur Forest disease virus, sharing 89% nt sequence homology (*3*,*4*). This emerging pathogen causes signs and symptoms such as fever, headache, joint pain, muscle pain, vomiting, and thrombocytopenia; severe cases may have hemorrhagic manifestations (epistaxis, ecchymoses, petechiae, hematemesis) and encephalitis, which can result in death (reported case-fatality rate as high as 25%) (*5*–*8*). Camels and sheep are thought to be the natural hosts of ALKV, but whether other mammals are also involved in its life cycle remains unknown. ALKV RNA was recently detected in an *Ornithodoros savignyi* tick collected near Jeddah, Saudi Arabia (*9*); on the Arabian Peninsula, these ticks have been associated with camels and their resting places and can be found where cases of ALKV infection in humans have been reported. These ticks seek multiple hosts, are nocturnal and cryptic, and commonly attack humans and other animals resting under trees (*10*). The hypothesis that mosquitoes could also be vectors has been suggested by 2 studies (*6*,*7*); despite the absence of data to substantiate it, this possibility cannot be excluded.

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Evidence suggests that ALKV infects humans either transcutaneously (by contamination of a skin wound with the blood of an infected vertebrate or through the bite of an infected tick) or orally through consumption of unpasteurized contaminated milk. Transmission to humans has been associated with butchering of sheep and camels. No human-to-human transmission has been reported. ALKV is classified in different countries as a BioSafety Level 3 or 4 agent.

ALKV has been detected only in Saudi Arabia, but the closely related Kyasanur Forest disease virus has spread as far as India and the People's Republic of China (4). We describe 2 cases of Alkhurma hemorrhagic fever in 2 travelers who returned to Italy from Egypt in 2010.

The Cases

The first patient, a 64-year-old man from Italy, spent 1 week (April 25–May 1, 2010) in a touristic village in southern Egypt, near the Sudan border. While visiting a camel and dromedary market in Shalatin on April 29, he was bitten on the foot by an unidentified arthropod (although not formally identified, was described as tick shaped). Soon after, a small, papular lesion developed. During his return flight to Italy, ≈48 hours after the bite, the patient experienced high fever, shaking chills, anorexia, malaise, nausea and vomiting, and blurred vision. During the next 5 days, these signs and symptoms worsened, and the man was admitted to the "Ospedali Riuniti di Bergamo" in northern Italy. His medical history was unremarkable, but he frequently traveled abroad and had been vaccinated against yellow fever in 1998.

Laboratory test results showed leukopenia (2,250 cells/mm^3), thrombocytopenia (67,000 platelets/mm^3), and increased liver enzymes (aspartate transaminase 469 U/L, reference 3–46 U/L; alanine transaminase 406 U/L, reference 3–46 U/L). The patient was given acetaminophen, and fever and general malaise progressively decreased over the next 5 days. He was discharged 11 days later, on May 17, in good general condition despite persistence of asthenia.

Acute-phase and convalescent-phase serum samples (collected on May 10 and 27, respectively) were sent to the virology laboratory of the "Lazzaro Spallanzani" National Institute for Infectious Diseases in Rome to be tested for dengue and West Nile viruses. Immunoglobulins (Ig) G and M for both viruses were detected by immunofluorescence of both samples; for each virus, IgG titer was ≥640 and IgM titer was ≥20. No evidence of rising antibody titers was found in the convalescent-phase specimen, raising suspicion of cross-reactivity to a previous *Flavivirus* infection or yellow fever vaccination. A genus-specific reverse transcription–PCR selective for the nonstructural protein (NS) 5 gene of flaviviruses (*11*) was positive for the acute-phase and negative for the convalescent-phase

samples. Sequence analysis of the amplicon (GenBank accession no. HM629507) showed high similarity with ALKV sequences in GenBank (BLAST [www.ncbi.nlm.nih.gov/blast/Blast.cgi] submission showed 97% identity with AF331718). This unexpected result called for further investigations to confirm the diagnosis of an ALKV infection. Thus, an ALKV-specific nested reverse transcription–PCR selective for a wider region of a different gene (E) was designed by using the following primers: outer forward 5′-TGGAACCCCACACGGGTGACT-3′; outer reverse 5′-ATGCCCACTGTCGGTTGGCG-3′; inner forward 5′-CCCACAGCAATCGAAAAACGGCATC-3′; inner reverse 5′- GCCCACATCACAGGTGACATGACC-3′.

All residual biological samples collected during the patient's hospital stay were sent to the virology laboratory of the Spallanzani Hospital (Italy's national reference laboratory for viral hemorrhagic fever viruses, BioSafety Level 4) in compliance with biosafety procedures. The new ALKV PCR result was positive, and the sequence of the amplicon (GenBank accession no. HM629508) showed high homology with ALKV (99% identity with AF331718). The phylogenetic trees based on partial sequences of NS5 (Figure 1) and E (Figure 2) genes confirmed the diagnosis of ALKV infection.

After submitting this article, we detected ALKV infection in a second patient. This patient had traveled to the same area ≈1 month later, visited the same camel market, and was affected by a milder disease. NS5 (HQ218942) and E (HQ218941) gene sequences obtained from this patient have been included in the phylogenetic tree, showing that they cluster together with those from the first patient (Figures 1, 2).

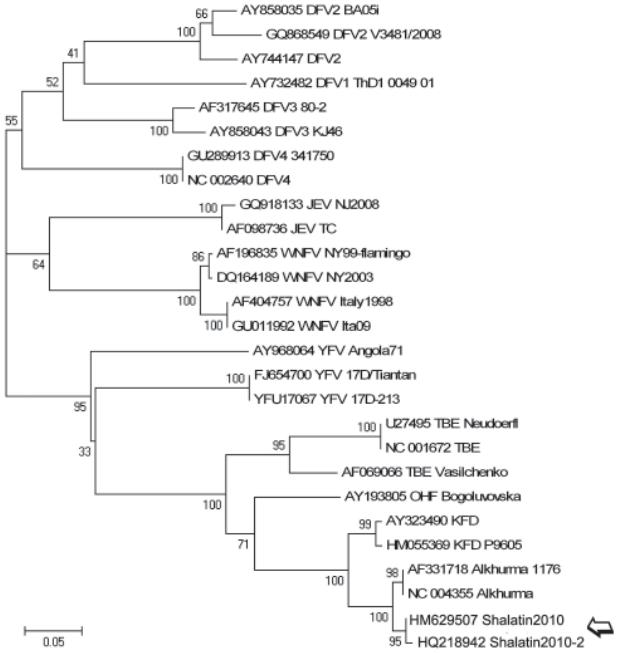

Figure 1. Phylogenetic tree based on sequences of the amplicon produced by the flavivirus nonstructural protein (NS) 5 gene reverse transcription–PCR (amplicon size, 208 bp; position in reference AF331718, nt 9077–9275), performed on the acute-phase serum samples of 2 travelers returning to Italy from Egypt (open arrow) showing relationship with other flaviviruses. Sequences are identified by name and GenBank accession number. Multiple alignment of other flavivirus sequences available in GenBank was generated by use of the ClustalW 1.7 software (www.clustal.org) included in the Bioedit package (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The phylogenetic tree was constructed by nucleotide alignment, the Kimura 2-parameter algorithm, and the neighbor-joining method implemented in MEGA 4.1 software (www.megasoftware.net). The robustness of branching patterns was tested by 1,000 bootstrap pseudo-replications. Scale bar indicates nucleotide substitutions per site. DFV, dengue fever virus; JEV, Japanese encephalitis virus; WNFV, West Nile fever virus; TBEV, tick-borne encephalitis virus; OHFV, Omsk hemorrhagic fever virus; KFDV, Kyasanur Forest disease virus.

Conclusions

The 2 patients had traveled to an area of the world where ALKV had not been previously reported. Although viremia was demonstrated 10 days after symptom onset, and we can reliably suppose that it started when fever and chills appeared, the probability of a susceptible vector in Europe is small, and the infection seems not to be transmissible from human to human.

Laboratory diagnosis of this infection is not easy to obtain and requires a specialized laboratory because of antibody cross-reactivity with other members of the family *Flaviviridae* and because of the absence of commercially available serologic tests and reference biologic materials for their development. However, surveillance of travelers returning from areas where highly dangerous infectious diseases are endemic should be improved and should include ALKV. The finding that the distribution of this virus is wider than previously thought and that it includes the African continent is in line with the hypothesis that tick-borne flaviviruses originated in Africa (*12*). The low genetic distance between the Egypt and Saudi Arabia sequences supports the hypothesis of a recent divergence from Kyasanur Forest disease virus, i.e., the closest flavivirus (*5*), and a slow microevolution of ALKV, as for other tick-borne flaviviruses (*13*). The higher genetic divergence in the NS5 gene than in the E gene of ALKV strains confirms previous observations for viruses isolated from human samples after inoculation of suckling mice (*5*) and deserves more detailed evolutionary analysis.

The detection of 2 independent infection events for travelers who visited the same area in a restricted period strongly supports the hypothesis of sustained local ALKV circulation. Further veterinary and entomologic investigations are needed to expand understanding of the geographic distribution of ALKV and to assess the danger for local populations and visitors. It would be advisable to inform

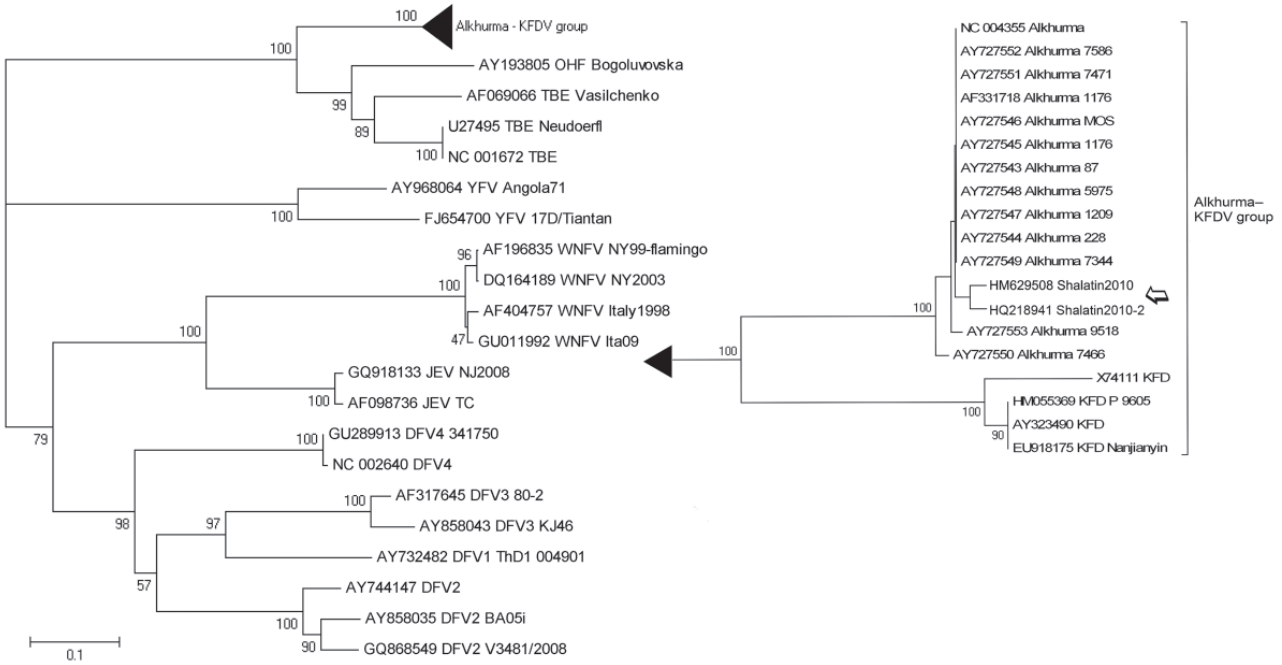

Figure 2. Phylogenetic tree based on the sequences of Alkhurma hemorrhagic fever virus E gene amplicon (amplicon size, 516 bp; position in reference AF331718, nt 1398–1913),obtained from acute-phase serum samples from a patient who had traveled to Egypt (open arrow) with respect to other flaviviruses. Sequences are identified by name and GenBank accession number. The phylogenetic tree was constructed by nucleotide alignment, the Kimura 2-parameter algorithm, and the neighbor-joining method implemented in MEGA 4.1 software (www.megasoftware.net). The robustness of branching patterns was tested by 1,000 bootstrap pseudo-replications. Scale bar indicates nucleotide substitutions per site. DFV, dengue fever virus; JEV, Japanese encephalitis virus; WNFV, West Nile fever virus; TBEV, tick-borne encephalitis virus; OHFV, Omsk hemorrhagic fever virus; KFDV, Kyasanur Forest disease virus. The relevant part of the tree is enlarged at right.

travelers about the danger of coming into contact with infected animals in areas where the virus has been reported. Avoidance of or minimization of exposure to infected ticks should be recommended as the most effective prevention measure.

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Multispacer Typing of *Bartonella henselae* Isolates from Humans and Cats, Japan

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To determine genotypic distribution of and relationship between human and cat strains of *Bartonella henselae,* we characterized 56 specimens using multispacer typing (MST). Of 13 MST genotypes identified, 12 were grouped into cluster 1. In Japan, human infections can be caused by *B. henselae* strains in cluster 1.

The causative agent of cat-scratch disease (CSD), *Bartonella henselae*, is a gram-negative bacterium associated with cats. Human infection usually occurs through scratches or bites by infected cats and typically is seen with localized lymphadenopathy. Occasionally, the infection may have an atypical manifestation, such as endocarditis, encephalopathy, neuroretinitis, or systemic CSD with hepatic and splenic granuloma (*1*).

B. henselae strains are classified into two 16S rRNA genotypes, 16S type I/Houston-1 and 16S type II/Marseille. Although both genotypes are present worldwide, 16S type II appears to be dominant in the cat population of Europe, whereas 16S type I is more common in Asia, including Japan (*2*,*3*).

Multispacer typing (MST) is a nucleotide sequencing-based genotyping method that uses highly variable intergenic spacers as typing markers. It is the most suitable genotyping procedure for evaluating the population structure of closely related strains of *B. henselae* (*4*). Previously, 50 MST genotypes from 201 *B. henselae* strains were phylogenetically organized into 4 lineages, and human strains mostly grouped within 2 of these lineages, Houston-1 and Marseille (*5*,*6*). Because genotypic data on *B. henselae* from Asian countries are limited, we applied MST to 56 human and cat specimens to determine the genotypic distribution and relationship of human and cat strains of *B. henselae* in Japan.

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The Study

During 1997 through 2008, we collected 56 *B. henselae* specimens from western Japan, mainly from Yamaguchi prefecture; the specimens included 1 *B. henselae* isolate from a patient with endocarditis (*7*), 24 clinical specimens from CSD patients who had test results positive for *B. henselae* DNA, and 31 *B. henselae* isolates from domestic cats (*8*). The 24 clinical specimens included 5 lymph node specimens and 16 pus specimens from patients with typical CSD, 1 blood specimen from a patient with bacteremia, 1 liver specimen from a patient with hepatic granuloma, and 1 spleen specimen from a patient with splenic granuloma. Total genomic DNA was extracted from the specimens by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). *B. henselae* DNA was detected by using PCR with specific primers for the 16S–23S rRNA intergenic spacer (*9*) and the *htrA* gene (*10*), and the 16S rRNA genotype was confirmed by partial sequencing of the 16S rRNA gene (*11*).

In addition, previously described MST primers were used to amplify and sequence the 9 intergenic spacers in *B. henselae* DNA (*6*). Locus-specific PCR was performed for spacer S1; direct sequencing was unsuccessful because of an unusual number of variable number tandem repeats (VNTR) (*8*). Spacer sequences were assigned according to published data (*5*,*6*,*8*). For each of the 9 spacers (S1–S9), we identified 8, 2, 3, 4, 3, 2, 3, 2, and 3 genotypes, respectively (Table). Three novel spacer sequences were deposited in the DNA Data Bank of Japan with these accession numbers: AB558532, S2 genotype 9; AB558533, S4 genotype 7; and AB558534, S4 genotype 8. We identified 13 different MST genotypes among the 56 specimens (Table). Of these 13 MST genotypes, 7 were novel (types 51–57). Six MST genotypes belonged to human and cat strains, including 2 predominant MST genotypes (14 and 35). All MST data were deposited in the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

Subsequently, we analyzed the phylogenetic relationships of the 7 novel MST genotypes identified in this study with the 50 previously identified genotypes. Multiple sequence alignment of the concatenated spacer sequences was performed by using ClustalW (www.ebi.ac.uk/clustalw). Finally, a phylogenetic tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA4 (*12*). This phylogenetic tree is grouped into 4 clusters (Figure). Of the 13 MST genotypes identified in this study, 12 genotypes belonged to cluster 1, but one genotype (MST genotype 52) belonged to cluster 4.

Conclusions

This study showed that MST genotypes in Japan were mainly grouped into 1 lineage (cluster 1), which was composed of Asian and American strains of *B. henselae*, and

Table. Multispacer typing of 56 *Bartonella henselae* strains isolated from humans and cats, Japan*

B. henselae source		Genotypes										16S rRNA
No. human	No. cat	S1	S2	S3	S4	S5	S6	S7	S8	S9	MST	genotype
1	1	7	2	5	4	1	2	1	1	3	7	I
8	12	4	2	5	4	1	2	1	1	3	14	I
1	0	3	2	6	5	2	2	2	1	1	21	I
1	0	8	2	5	4	1	2	2	1	3	32	I
2†	1	4	2	5	4	1	2	2	1	3	33	I
9‡§	4	5	2	6	5	2	2	2	1	1	35	I
0	1	11	2	6	4	1	2	1	1	3	51	I
0	2	12	9	2	7	5	4	4	3	2	52	II
1	2	5	2	6	8	2	2	2	1	1	53	I
1¶	1	4	2	5	5	1	2	1	1	3	54	I
0	4	5	2	6	5	1	2	2	1	1	55	I
0	3	7 + 4#	2	5	4	1	2	1	1	3	56	I
1	0	4	2	5	4	2	2	1	1	3	57	I

*MST, multispacer typing; I, 16S type I; II, 16S type II.
†Includes a clinical specimen isolated from a patient with hepatic granuloma.
‡Includes *B. henselae* strain isolated from a patient with endocarditis.
§Includes a clinical specimen isolated from a patient with bacteremia.
¶Includes a clinical specimen isolated from a patient with splenic granuloma.
#Strain with 2 different copies of intergenic spacer S1 in its genome.

that the genotypic distribution of human strains coincided with that of cat strains. Although only 1 human strain from the West Indies belonged to cluster 1 before this study (*6*), we discovered that all 25 of our human strains from Japan were grouped into cluster 1. These results demonstrate that human infections can be caused by *B. henselae* strains in cluster 1, which differed from clusters corresponding to the Houston-1 and Marseille type strains.

In a previous study, MST genotype 35 was the most common genotype in cluster 1, and 4/6 (67%) of Japanese cat strains belonged to this genotype (*5*). In this study, MST genotypes 14 (36%; 20/56) and 35 (23%; 13/56) were predominant genotypes. Additionally, most human strains (88%; 15/17) belonging to these genotypes were isolated from patients with typical CSD; 2 strains with MST genotype 35 were isolated from patients with endocarditis and bacteremia (Table).

The genotypic distribution of the human strains in this study differed from that reported by Li et al. (*6*) because their strains isolated in France were grouped under 2 lineages (Houston-1 and Marseille). However, we found that the lineages of human strains matched those of cat strains in each country. These results are consistent with the role of cats as the major reservoir of *B. henselae* (*13*).

In this study, we identified 2 cat strains that were classified into cluster 4. These strains belonged to 16S type II, which is rare in Japan (*3*). In previous MST studies, strains in cluster 4 were isolated from cats and belonged to 16S type II (*5*,*6*). Intriguingly, similar lineages consisting of 16S type II isolates from cats were observed in other genotyping studies involving the use of multilocus sequence typing (MLST) (*14*) and multiple locus variable number tandem repeat analysis (MLVA) (*15*). Thus, these lineages may be less pathogenic for humans. However, further studies are needed to investigate this hypothesis.

When we characterized the strains in this study by MLST we found that almost all of them shared the same sequence type as Houston-1 (*8*). In contrast, we identified 13 MST genotypes that belonged to different clusters than Houston-1. The lower resolving power of MLST is mostly likely due to sequence conservation in the 8 housekeeping genes selected for the method. MST has a higher resolving power because the spacers used in this method are more variable than MLST markers. As a result, MST is better suited for evaluating the population structure of closely related *B. henselae* strains.

We conclude that the MST genotypes in Japan are mainly grouped into cluster 1 and that the genotypic distribution of human strains coincides with that of cat strains. In Japan, human infections can be caused by *B. henselae* strains in cluster 1, distinct from clusters containing the Houston-1 and Marseille type strains. These results improve our understanding of the population structure of and geographic relationship between human and cat strains of *B. henselae*.

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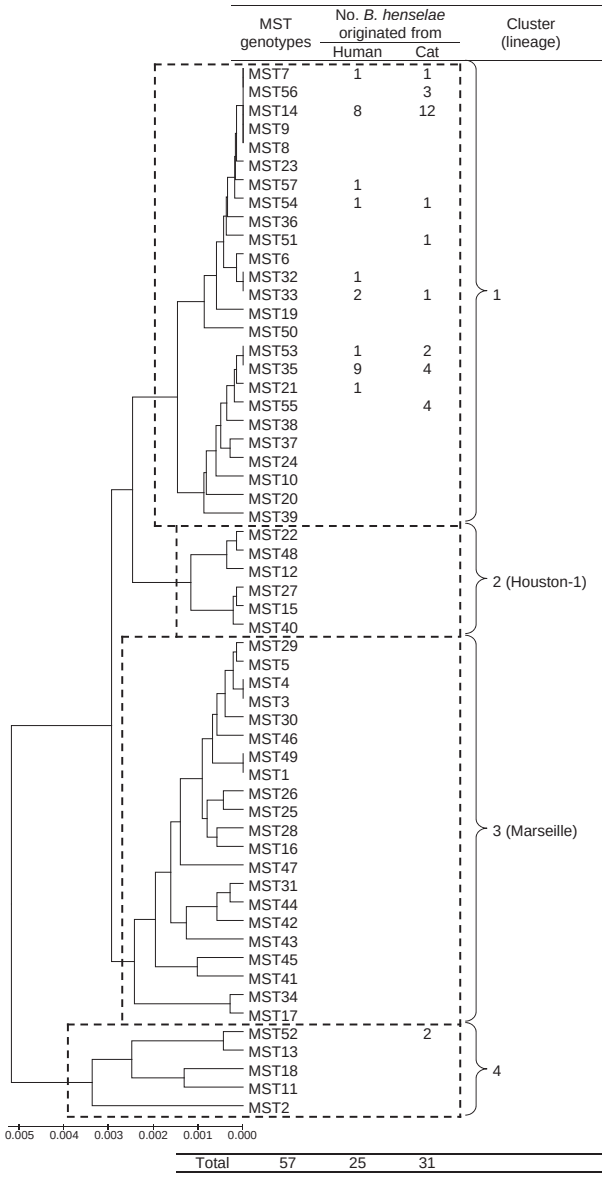

Figure. Phylogeny and clusters of multispacer typing (MST) genotypes of *Bartonella henselae* isolates from humans and cats, Japan, based on 9 concatenated intergenic spacer sequences in 57 MST genotypes. The unweighted pair-group method with arithmetic mean method in MEGA4 software (*12*) was used for phylogenetic analysis. Dotted rectangles show 4 clusters of MST genotypes, 2 of which correspond to the *B. henselae* Houston-1 and Marseille type strains. Scale bar indicates nucleotide substitutions per site.

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Pandemic (H1N1) 2009 Outbreak at Canadian Forces Cadet Camp

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We conducted a case–control study to describe the clinical and epidemiologic characteristics of an outbreak of pandemic (H1N1) 2009 at a Canadian military cadet training center. We found that asthma and obesity confer greater risk for infection. Viral shedding was detected by PCR up to 18 days after symptom onset.

On July 29, 2009, the Public Health Agency of Canada was notified of an outbreak of pandemic (H1N1) 2009 at the Army Cadet Summer Training Centre Argonaut at Canadian Forces Base, Gagetown, New Brunswick. The Cadet Summer Training Centre camp opened in early July and ran sessions lasting 2–6 weeks. The camp setting was semiclosed, with limited movement on and off camp. A case–control study was conducted to describe transmission, clinical characteristics, viral shedding, and risk factors for infection.

The Study

Approximately 506 cadets, 12–18 years of age, and 322 staff cadets, officers, and support staff lived on camp premises. All persons at the camp were invited to participate. This study received expedited approval from the Health Canada Research Ethics Board. Participants were interviewed in person at the camp or by telephone; swab specimens were collected by on-site nurses. Samples were sent to the National Microbiology Laboratory for testing using reverse transcription–PCR and primer sets developed by the US Centers for Disease Control and Prevention (*1*). Specimens were cultured in primary CMK cells (Viromed Laboratories, Inc., Minnetonka, MN, USA) and the hemagglutinin titer was checked at days 6 and 10.

A modified case definition for pandemic (H1N1) 2009 infection was developed based on Canada's surveillance case definition for influenza-like illness. Symptom onset was defined as earliest date of onset of self-reported history of fever or cough. The case definition is outlined in Table 1.

During August 3–27, 2009, we conducted 144 face-to-face and 21 phone interviews. Approximately 20% of cadets and 20% of staff cadets, officers, and support staff participated. Of the 165 participants, 56 were classified as confirmed cases, 24 as suspected cases, and 85 as controls. Participant age ranged from 13 to 43 years; 88% were 13–18 years of age, and 55% were male. No statistically significant demographic differences ($p<0.05$) were observed between confirmed cases, suspected cases, or controls.

The epidemic curve (Figure) summarizes the outbreak among those with known symptom onset date for cases (n = 54), suspected cases (n = 21), and 27 additional cases of fever and cough identified by the camp Health Care Centre (HCC) but not included in the study. The minimum camp attack rate for cases/suspected cases and HCC cases not in the study was 13.5% (112/828), 14.0% among cadets (71/506) and 12.7% among staff cadets and officers (41/322).

The outbreak was identified on July 17. In response, respiratory etiquette and hand hygiene were emphasized; camp residents were encouraged to seek care if ill. Those having fever and cough were isolated for 7 days, until parents came, or until laboratory results returned negative. Group outdoor activities stopped on July 23; all group activities were cancelled as of July 25. Some cadets were fast-tracked to graduate early, and arrival of new cadets

Table 1. Case definitions for pandemic (H1N1) 2009 infection, Army Cadet Summer Training Centre Argonaut at Canadian Forces Base, Gagetown, New Brunswick, Canada, 2009

Confirmed cases
Persons who
1. Had laboratory-confirmed pandemic (H1N1) 2009 influenza infection OR
2. Reported fever AND cough while at camp, excluding those who had negative PCR results for pandemic (H1N1) 2009 within 5 days after symptom onset
Suspected cases
Persons who
1. Reported fever OR cough with ≥2 of the following symptoms: sore throat, nausea, nasal congestion, chills OR
2. Reported fever and cough and had negative PCR results for pandemic (H1N1) 2009 within 5 days after symptom onset
Controls
Persons who
1. Did not report fever or cough OR
2. Reported fever or cough but without ≥2 of the following symptoms: sore throat, nausea, nasal congestion, chills

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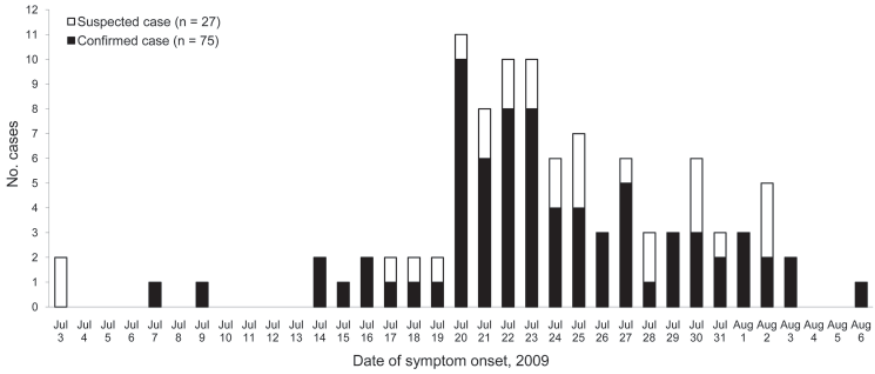

Figure. Epidemic curve of 54 confirmed and 21 suspected cases of pandemic (H1N1) 2009 infection and of 27 additional cases of fever and cough identified by the camp Health Care Centre, Army Cadet Summer Training Centre Argonaut at Canadian Forces Base, Gagetown, New Brunswick, Canada, 2009.

was delayed 2 weeks. Mass screening for fever and cough was undertaken on August 6, before the arrival of new cadets; all the new cadets were screened on arrival.

No activity or exposure was linked to increased risk for illness (data not shown). All but 1 person with a suspected or confirmed case reported symptoms; 58/85 (68.2%) of controls also reported symptoms during the outbreak period. Odds of experiencing shortness of breath, chest pain, sputum production, vomiting, rhinorrhea, nose bleeds, or change in level of awareness were all >5× higher for those with cases/suspected cases than for controls (Table 2). The mean number of symptoms among those with symptomatic cases/suspected cases was greater than among symptomatic controls (8.7 vs. 3.4; p<0.001). This relationship held true when comparing those with cases/suspected cases with symptomatic controls who had negative PCR results.

Of the 78 persons with symptomatic/suspected cases for whom complete information was available, 25 (32.1%) had recovered by the interview; median symptom duration was 7 and 9 days, respectively. Symptom duration ≥10 days was reported by 40% of persons with cases/suspected cases whose symptoms had resolved and 47% of those with unresolved symptoms. Median time from symptom onset to illness peak was 2 days (range 1–14 days). With the exception of cough, sputum production, and malaise, symptoms peaked rapidly (24–48 hours) after onset.

Overall, 86.1% of persons with cases/suspected cases accessed the HCC; none were hospitalized. Oseltamivir was given to 2 persons with confirmed cases who had comorbid conditions (asthma and kidney disease). Forty-four persons with cases/suspected cases (55.7%) were not isolated because they did not seek treatment at the HCC or not while both fever and cough were present.

Eight persons had positive PCR results for pandemic (H1N1) 2009 7–18 days after symptom onset, and live virus was detected up to 14 days after symptom onset. All but 1 of these persons were capable of transmitting virus given upper respiratory symptoms, and 2 reported diarrhea and vomiting on the day the swab sample was obtained. Four persons had live virus detected after day 7 of illness (up to 14 days); 2 of these reported comorbid conditions.

Persons with confirmed and suspected cases did not differ with regard to comorbidity or risk factors, except for seasonal influenza vaccination; 6/48 (12.5%) of persons with confirmed cases reported having received the seasonal influenza vaccine in the year of the study versus 8/22 (36.4%) of those with suspected cases (odds ratio 4.0; p<0.05). No difference was found in the proportion of those with cases/suspected cases and controls reporting seasonal influenza vaccination during the current year or past 2 years.

The odds of reporting ≥1 comorbidity was >2.7× higher for persons with case/suspected cases than for controls (p<0.05) and for asthma >3.9× higher (p < 0.05). The odds of being obese were >3× higher for persons with cases/suspected cases (odds ratio 3.4, 95% confidence interval 1.0–10.9).

Conclusions

In accordance with national recommendations (*2*), antiviral drugs were not used for control; transmission appeared to be reduced through nonpharmaceutical measures. Multiple index cases could not be ruled out. No individual activity or exposure was linked to increased risk for illness.

High rates of obesity have been noted among hospitalized patients with pandemic (H1N1) 2009 (*3*–*6*). This study suggests obesity is a risk factor for infection or clinical illness and given low prevalence of comorbid conditions may stand alone as a risk factor. Consistent with international studies (*7*), vaccination for seasonal influenza was neither protective nor a risk factor for acquiring pandemic (H1N1) 2009. One third of case-patients reported change in level of awareness, which suggests the potential for mild neurologic sequelae. Neurologic complications of influenza infection have been reported in hospitalized children (*8*,*9*).

Seven of 8 participants who had positive PCR results for pandemic (H1N1) 2009 ≥7 days after symptom onset

Table 2. Frequency of reported symptoms of pandemic (H1N1) 2009 infection, Army Cadet Summer Training Centre Argonaut at Canadian Forces Base, Gagetown, New Brunswick, Canada, 2009*

Symptoms	No. persons reporting symptom/no. persons reporting (%)			
	All participants, n = 165	Persons with confirmed/ suspected cases,† n = 80	Controls, n = 85	OR (95% CI)‡
None	28/165 (17.0)	1/80 (1.3)	27/85 (31.8)	
Systemic				
Fever	56/164 (34.1)	55/80 (68.8)	1/84 (1.2)	
Chills	44/165 (26.7)	41/80 (51.2)	3/85 (3.5)	
Headache	58/165 (35.2)	39/80 (48.8)	19/85 (22.4)	3.3 (1.7–6.5)§
Prostration	52/164 (31.7)	37/79 (46.8)	15/85 (17.6)	4.1 (2.0–8.4)¶
Malaise	67/165 (40.6)	47/80 (58.8)	20/85 (23.5)	4.6 (2.4–9.0)¶
Arthralgia	19/165 (11.5)	13/80 (16.2)	6/85 (7.1)	2.6 (0.9–7.1)
Myalgia	26/165 (15.8)	20/80 (25.0)	6/85 (7.1)	4.4 (1.7–11.6)§
Lower respiratory				
Cough	97/165 (58.8)	76/80 (95.0)	21/85 (24.7)	
Sputum production	33/164 (20.1)	26/79 (32.9)	7/85 (8.2)	5.5 (2.2–13.5)¶
Shortness of breath	30/165 (18.2)	26/80 (32.5)	4/85 (4.7)	9.8 (3.2–29.5)¶
Chest pain	14/165 (8.5)	13/80 (16.2)	1/85 (1.2)	16.3 (2.1–127.8)§
Upper respiratory				
Sore throat	76/164 (46.3)	56/80 (70.0)	20/84 (23.8)	
Nasal congestion	76/164 (46.3)	55/79 (69.6)	21/85 (24.7)	
Sneezing	27/164 (16.5)	20/79 (25.3)	7/85 (8.2)	3.8 (1.5–9.5)§
Runny nose	41/159 (25.8)	32/75 (42.7)	9/84 (10.7)	6.2 (2.7–14.2)¶
Nosebleeds	10/165 (6.1)	9/80 (11.2)	1/85 (1.2)	10.6 (1.3–86.1)#
Gastrointestinal				
Nausea	50/165 (30.3)	42/80 (52.5)	8/85 (9.4)	
Abdominal pain	28/165 (17.0)	17/80 (21.2)	11/85 (12.9)	1.8 (0.8–4.2)
Diarrhea	26/165 (15.8)	19/80 (23.8)	7/85 (8.2)	3.5 (1.4–8.8)#
Vomiting	24/165 (14.5)	20/80 (25.0)	4/85 (4.7)	6.8 (2.2–20.8)¶
Neurologic				
Seizures	0/164 (0.0)	0/80 (0.0)	0/85 (0.0)	NA
Change in awareness	29/165 (17.6)	25/80 (31.2)	4/85 (4.7)	9.2 (3.0–27.9)¶
Other				
Conjunctivitis	2/164 (1.2)	1/79 (1.3)	1/85 (1.2)	1.0 (0.1–17.5)
Other**	16/165 (9.7)	14/80 (17.5)	2/85 (2.4)	8.8 (2.0–40.1)§
Mean no. symptoms	6.5	8.7	3.4	

*One person reported no symptoms but was PCR and culture positive. OR, odds ratio; CI, confidence interval; NA, not applicable.
†Fever and nausea reported by a greater proportion of cases than suspected cases ($p<0.001$ and $p = 0.02$, respectively). Cases and suspected cases did not differ significantly in frequency of symptoms not in the case definition or median number of symptoms and have therefore been combined here.
‡Odds ratios were not calculated for symptoms included in the case definition (fever, chills, cough, sore throat, congestion, nausea).
§$p<0.01$.
¶$p<0.001$.
#$p<0.05$.
**Other symptoms reported: bilateral ear infection, bruising, ear pain, ear popping, eye pain, hot flashes, jaw pain, loss of appetite, loss of voice, rib pain, swelling of eyes, swollen tonsils, toothache (1 person each); dizziness (3 persons). All persons who reported symptoms in this category had ≥1 other reported symptom.

were capable of transmitting virus, given their upper respiratory symptoms on the day the swab sample was obtained. Studies of seasonal influenza indicate median viral shedding of 7–8 days after illness onset, with titers low or undetectable by day 5, although prolonged shedding has been reported (*10–13*). In this study, test results for pandemic (H1N1) 2009 were positive by culture up to 14 days and by PCR up to 18 days after symptom onset. This and other studies describe longer shedding periods for pandemic (H1N1) 2009 as compared with seasonal influenza (*10*,*13–15*).

Study limitations should be acknowledged. The case definition included self-reported fever and cough. Therefore, misclassification of persons with illnesses other than pandemic (H1N1) 2009 may have occurred. Convenience sampling was used, so participants may differ from nonparticipants; complete demographics for the camp were not available for comparison. A small proportion of phone interviews were conducted ≤6 weeks after symptom onset, raising the possibility of recall bias.

Infection control procedures likely contributed to the control of transmission in the absence of antiviral drug use or early treatment for contacts. Shedding in otherwise healthy adolescents and young adults may be longer than shedding of seasonal influenza viruses, which may have implications for public health planning.

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At the time this work was conducted, Ms Kropp was an epidemiologist and policy advisor with the Public Health Agency of Canada. Currently, she is the director of the Travel Health Division in the same agency. Her research interests include the epidemiology, prevention, and control of infectious diseases.

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Characterization of Nipah Virus from Naturally Infected *Pteropus vampyrus* Bats, Malaysia

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We isolated and characterized Nipah virus (NiV) from *Pteropus vampyrus* bats, the putative reservoir for the 1998 outbreak in Malaysia, and provide evidence of viral recrudescence. This isolate is monophyletic with previous NiVs in combined analysis, and the nucleocapsid gene phylogeny suggests that similar strains of NiV are co-circulating in sympatric reservoir species.

Nipah virus (NiV) first emerged in Malaysia in 1998 (*1*), with subsequent human cases reported in Bangladesh (*2*) and India (*3*). Serologic and virologic evidence support the hypothesis that *Pteropus* spp. bats are the reservoir hosts for henipaviruses (*4*,*5*). However, the mechanisms of transmission between individual bats and of viral maintenance in a colony are poorly understood. We report isolation of NiV from *Pteropus vampyrus* bats, the putative reservoir for the 1998 outbreak in humans and pigs, and present evidence that these bats can harbor latent infections that recrudesce. Finally, we characterized this isolate and compared its phylogenetic position with all other known henipavirus sequences.

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The Study

We conducted a prospective cohort study from June 2004 through June 2005 on a group of 17 *P. vampyrus* flying foxes captured in 2 locations, using a nonrandom sampling method. Fourteen bats (73%) were from Lenggong (5°07′01.1″N, 100°58′32.7″E), and 3 bats (27%) were from Kampung Gajah (4°10′35″N, 100°55′37″E), Malaysia. This project was approved by the Wildlife Trust Institutional Animal Care and Use Committee, New York, New York, USA, and Department of Wildlife and National Park Malaysia research committee.

Because bats were included in the study in a staggered manner, each bat was monitored for antibody titer against NiV and virus excretion for 5 to 12 months. Bats were quarantined at Taiping Zoo (4°54′N, 100°45′E), Taiping, Malaysia, in a wire net (1 inch square) enclosure, 5 m long × 4 m wide × 3 m high; with a roof and cement floor. Inactivated bat serum specimens were screened for NiV antibodies by using a serum neutralization test. A titer of ≥8 was considered positive for specific antibodies against NiV, because bat serum is frequently toxic to Vero cells at higher concentrations (i.e., 1:2 or 1:4). A 4-fold increase in antibody titer was interpreted as an indication of an acute or recent infection (*6*). Seroreaction of juvenile bats was considered to represent NiV maternal antibody remnants.

During the study, 544 samples were cultured for virus isolation (272 throat and 272 urine or urogenital swab specimens). Samples were added to Vero cells (CRL 81; American Type Culture Collection, Manasssas VA, USA) and observed for characteristic syncytial type of cytopathic effects (*1*). NiV was isolated from only 1 sample, the urine of an adult female bat (no. 24). The antibody profile of this bat showed an antibody titer of 8 when tested on entry to the study, which later waned to negative (titer <4) on the second sampling. The bat remained antibody negative for 11 months, after which the bat again become seropositive; the titer rose from <4 to 32 over a 3-week period.

Virus isolation corresponded to the time when the antibody titer of the bat was on the verge of rising (<4 to 4). Two weeks later, 2 seronegative male bats (nos. 38 and 48) converted to a titer of 32; however, no virus was isolated. Details from our longitudinal serologic testing of these 3 bats are shown in Table 1. The isolation from bat no. 24 was confirmed as NiV as described (*7*). Serum neutralization test and virus isolation were performed in a BioSafety Level 3 Laboratory at Veterinary Research Institute, Ipoh, Malaysia.

The sequence of NiV *P. vampyrus* (GenBank accession no. FN869553) and the alignment analysis show that NiV *P. vampyrus* differs from all known isolates from Malaysia

[1]Members of the Henipavirus Ecology Research Group are listed on the group's website (www.henipavirus.org/staff/Staff.htm).

Table 1. Longitudinal serologic test results from 3 *Pteropus vampyrus* bats that seroconverted while in captivity, June 2004–June 2005, Malaysia*

Date of test	Bat no. 24 (type AF)	Bat no. 38 (type JM)	Bat no. 48 (type JM)
2004 Jun 12	NE	16	NE
2004 Jul 8	8	<4	NE
2004 Jul 28	<4	<4	NE
2004 Aug 30	<4	<4	NE
2004 Oct 20	<4	<4	NE
2005 Jan 5	<4	<4	NE
2005 Mar 1	<4	<4	<4
2005 Mar 29	<4	<4	<4
2005 Apr 4	<4	<4	<4
2005 Apr 12	<4	<4	<4
2005 May 18	<4	<4	<4
2005 May 26	<4	<4	<4
2005 May 3	<4	<4	<4
2005 May 10	<4	<4	<4
2005 May 17	<4	<4	<4
2005 May 24	4	<4	<4
2005 May 31	16	<4	<4
2005 Jun 8	32	16	32

*Serum neutralization test titer of <4, negative; 4, inconclusive; 8–32, seroconverted. A, adult; F, female; J, juvenile; M, male; NE, animal not yet enrolled.

at 98 nt positions; these nucleotide changes translated into amino acid changes at 44 positions. Subsequent analysis of the deduced amino acid sequences of the open reading frames of the nucleocapsid, phosphoprotein, matrix, fusion, attachment, and polymerase genes showed high sequence similarities (98%–99%) between nucleocapsid, matrix, fusion, attachment, and polymerase proteins of NiV *P. vampyrus* and other previously sequenced NiV isolates from Malyasia. However, phosphoprotein shares the lowest homology (96%). Table 2 shows a summary of the specific deduced amino acid changes compared with other NiV sequences.

Phylogenetic analyses were generated by using maximum-likelihood methods (*8*). Sequences were analyzed with henipavirus sequences available in GenBank. The analysis of the combined nucleotide dataset shows that NiV *P. vampyrus* forms a monophyletic clade with other NiV isolates from Malaysia, yet it differs from human, pig, and *P. hypomelanus* bat isolates. NiV from humans in Bangladesh is more distantly related and basal to all NiV sequences from Malaysia (Figure 1). This relationship is further supported by the polymerase gene analysis (data not shown). When the nucleocapsid gene alone was analyzed, including 56 NiV sequences from *P. lylei* bats in Thailand, NiV *P. vampyrus* phylogenetically grouped most closely with NiV *P. lylei* (AY858110), and the monophyly of NiV sequences from Malysia was lost (Figure 2). This sister relationship between NiV *P. lylei* and NiV *P. vampryus* is also evident in analysis of the atttachment gene (data not shown).

Table 2. Summary of deduced amino acid changes in the N, P, M, F, G, and L proteins of NiV from *Pteropus vampyrus* bats compared with other NiV isolates*

Isolate†	N, aa position			P, aa position							
NiV isolate	429	432	457	41	140	195	295	304	309	408	410
P. vampyrus	**V**	**E**	**D**	**R**	**A**	**P**	**S**	**A**	**T**	**G**	**T**
Human-CDC	I	G	N	Q	T	L	N	T	A	T	A
P. hypomelanus	I	G	N	Q	T	L	N	T	A	T	A
Pig-Tambun	I	G	N	Q	T	L	N	A	A	T	A

	P, aa position										
NiV isolate	412	419	420	425	427	430	437	438	439	440	454
P. vampyrus	**N**	**K**	**M**	**R**	**G**	**P**	**P**	**P**	**Q**	**S**	**P**
Human-CDC	Y	N	V	S	A	H	Y	Q	E	G	T
P. hypomelanus	Y	N	V	S	A	H	Y	Q	E	G	T
Pig-Tambun	Y	N	V	S	A	H	Y	Q	E	G	T

	P, aa position						M, aa position			F, aa position	
NiV isolate	463	464	467	468	471	664	147	234	331	11	63
P. vampyrus	**K**	**I**	**P**	**H**	**N**	**V**	**G**	**Y**	**V**	**S**	**A**
Human-CDC	E	V	V	D	D	I	S	S	I	C	P
P. hypomelanus	E	V	V	D	D	I	S	S	I	C	P
Pig-Tambun	E	V	V	D	D	I	G	S	I	C	P

	F, aa position	G, aa position					L, aa position			
NiV isolate	460	20	186	426	444	470	481	223	1645	1753	2039
P. vampyrus	**K**	**N**	**D**	**I**	**V**	**Q**	**D**	**N**	**F**	**V**	**N**
Human-CDC	I	I	N	V	I	L	N	T	S	M	H
P. hypomelanus	I	I	N	V	I	L	N	T	F	M	H
Pig-Tambun	I	N	N	V	I	L	N	N	F	V	N

*N, nucleocapsid; P, phosphoprotein; M, matrix; F, fusion, G, attachment; L, polymerase; NiV, Nipah virus; CDC, Centers for Disease Control and Prevention. **Boldface** indicates amino acid changes.
†GenBank accession nos.: NiV Human-CDC , AF212302; NiV *P. hypomelanus,* AF376747; NiV Pig-Tambun, AJ627196.

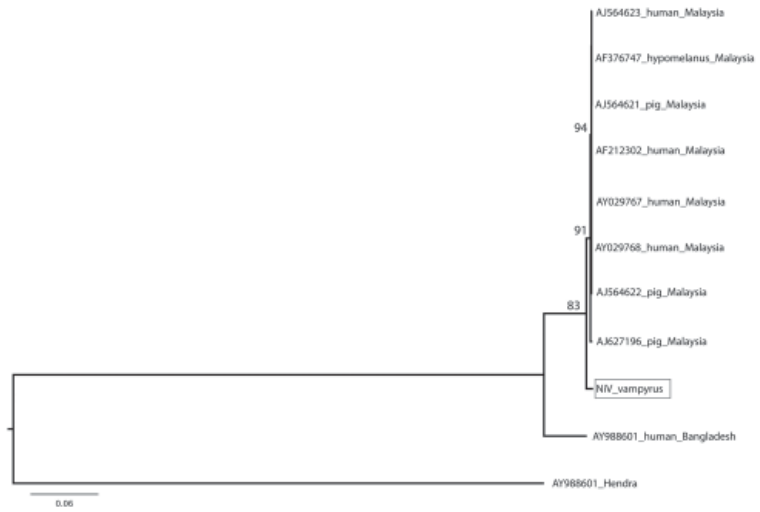

Figure 1. Phylogenetic position of Nipah virus (NiV) isolate from *Pteropus vampyrus* bats (box) in combined analysis of nucleocapsid, phosphoprotein, matrix, fusion, and attachment gene open reading frames (8.3 kb). Maximum likelihood tree, general time reversible + Γ model, 1,000 bootstrap replicates. NiV *P. vampyrus* is distinct but forms a clade with other NiV sequences from Malaysia, and the isolate from Bangladesh is more distantly related and basal to this group. GenBank accession numbers are shown for all comparison isolates; the polymerase gene is missing for AF376747 and thus that isolate is excluded from analysis. Scale bar indicates nucleotide substitutions per site.

Conclusions

Our evidence suggests that NiV can recrudesce in previously infected adult bats, thus providing a new potential mechanism for maintenance in natural hosts. We isolated NiV from a seropositive adult bat at the time of capture, and therefore it was unlikely to have had remnant maternal antibodies. Also, it is unlikely that infection could have been introduced by other wild bats because other bats could not access the enclosure where the study bats were kept. The antibodies waned during the bat's captivity, and subsequent seroconversion correlated with our finding of NiV in the individual bat's urine. The bats were isolated from contact with wild bats, and all other bats placed in the colony were negative for henipavirus by culture; only bats 24, 38, and 48 subsequently seroconverted. Recrudescence of NiV infection in bats is not completely unexpected because NiV infection has resulted in (fatal) relapsing illness in humans, several months to 4 years after initial exposure (*9*). Other paramyxoviruses, including canine distemper (*10*) and measles virus (*11*), can persist in tissues for some years.

The seroconversion of the 3 bats in this colony is consistent with recent viral challenge (*6*) and a scenario in which bat 24 underwent recrudescence of a latent infection. The seroconversion supports the conclusion that bats 38 and 48 were infected through exposure to urine, feces, or saliva from bat 24.

NiV was not isolated from the 2 male bats, which may have been because of a low amount of virus excreted, a very narrow time frame for excretion, or both; however, these findings suggest that they did not undergo recrudescence. Evidence for NiV recrudescence adds to our understanding of henipavirus ecology and transmission dynamics. Repeated shedding of NiV through recrudescence may enhance viral maintenance in isolated colonies without the boom and bust dynamics, typical of acute viral infections with long-term immunity and reduce the necessity of intercolony migration for maintenance.

Our phylogenetic analyses help address some longstanding questions regarding the natural history of henipaviruses (*12*). Close homology between NiV *P. vampyrus* and a NiV *P. lylei* isolates and evidence from nucleocapsid and polymerase gene analysis suggest that NiV is naturally transmitted between these 2 species, which roost together in Thailand and parts of Cambodia. Furthermore, NiV diversity in isolates obtained from *P. lylei* bats demonstrates that multiple strains co-circulate within populations and that the ecology and sympatry of *Pteropus* spp., not coevolutionary patterns, determine NiV strain diversity in reservoir hosts.

During the 1998 outbreak, NiV isolates from *P. hypomelanus* bats was found to be nearly identical to those from pigs and humans (56 nt changes) (*13*,*14*). However, this species is only found on offshore islands, has limited dispersal, and does not overlap with the index farms. Thus, *P. vampyrus* bats are likely the putative spillover hosts in Malaysia, not *P. hypomelanus* bats; nonetheless, our isolate differed markedly from others in the outbreak. Because laboratory contamination of the NiV *P. hypomelanus* isolate seems unlikely (*14*), the co-circulation of multiple strains in *P. vampyrus* bats is probable. Alternatively, the differences we observed in NiV *P. vampyrus* may be the result of rapid RNA virus evolution during the 7-year period between sampling of NiV *P. vampyrus* (2005) and sampling of the other isolates from Malaysia (1998–1999). Our data support this hypothesis. Assuming a known henipavirus genome length of ≈18,000 nt (*15*), the average substitution rate for *Paramyxoviridae* of 0.50×10^{-3} substitutions/site/year, and a constant molecular clock, the 98-nt changes observed correspond broadly to the time frame (≈7 years) between sampling of these isolates.

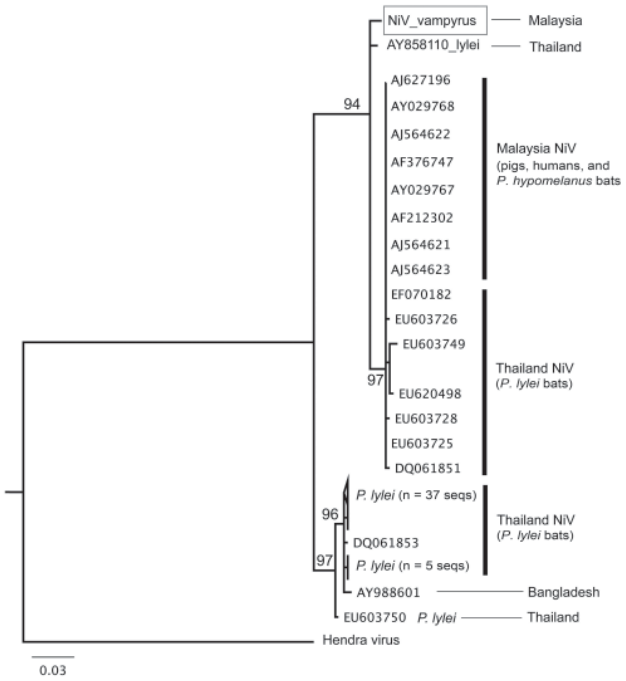

Figure 2. Partial nucleocapsid gene (358 bp) maximum-likelihood tree for all available Nipah virus (NiV) sequences (seqs) in GenBank, showing a high level of NiV sequence diversity in *Pteropus lylei* bat isolates from Thailand. NiV *P. vampyrus* (box) is most closely related to AY858110 from *P. lylei* bats and forms a large clade that includes other *P. lylei* bat isolates and all NiV sequences from Malaysia. GenBank accession numbers are given for NiV isolates from pigs in Malaysia (AJ627196, Tambun; AJ564621, Sg. Buloh; and AJ564622, Seremban), humans in Malaysia (AJ564623, AF212302, AY029767, and AY029768), humans in Bangladesh (AY988601), *P. hypomelanus* bats in Malaysia (AF376747), *P. lylei* bats in Cambodia (nucleocapsid gene-AY858110, DQ061851–58, EF070182–90, EU603724–58, EU620498, and EU624735–37), and Hendra virus from Australia (AF017149). Scale bar indicates nucleotide substitutions per site.

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Increasing Contact with Hepatitis E Virus in Red Deer, Spain

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To describe the epidemiology of hepatitis E virus (HEV) in red deer in mainland Spain, we tested red deer for HEV RNA and antibodies. Overall, 10.4% and 13.6% of serum samples were positive by ELISA and reverse transcription–PCR, respectively. The increasing prevalence suggests a potential risk for humans.

Hepatitis E virus (HEV) is the only member of the *Hepeviridae* family (*1*). Four major genotypes of HEV have been recognized: genotypes 1 and 2 are restricted to humans and associated with epidemics in developing countries; genotypes 3 and 4 are zoonotic in developing and industrialized countries. Wild and domestic animals are being identified as potential HEV reservoirs (*1–3*).

Studies on wild sika deer (*Cervus nippon*) have detected low prevalence rates for HEV, which suggests that sika deer are accidental hosts for the virus (*4*,*5*), despite the transmission link discovered between them and HEV in Japan (*3*) that raised awareness of the possibility that game animals transmit HEV (*2*). In Europe, information about HEV infection in wild ruminants is limited to reports suggesting that roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) can act as HEV hosts (*6–8*). Except for these limited studies, no large-scale surveys have been conducted of HEV epidemiology in wild cervids. In Spain, the relatively high HEV seroprevalence detected in domestic pigs and wild boar suggests that HEV infection is probably widespread (*9*).

Red deer density, distribution, and hunting harvest are increasing throughout Europe (*10*). In Spain, the high densities recorded (*11*) indicate that red deer are an important source of game meat. This scenario emphasizes the need for a better understanding of the epidemiology of HEV in game populations in Spain.

Our goals were to describe the epidemiology and time trends of HEV in red deer in peninsular (mainland) Spain by serologic testing and PCR. On the basis of previous results on wild boar (*9*), we hypothesized that red deer would show widespread contact with HEV in Spain.

The Study

Serum samples from 968 Iberian red deer were collected during 2000–2009. These samples came from hunter-harvested red deer in 21 wild or semifree ranging populations (892 deer) and from 2 farms (76 deer). Sampling sites were representative of a variety of habitats and climates, which can be simplified into 5 different bioregions (Figure) (*12*). Sampling sites were grouped into 7 areas and 2 red deer farms (Table; Figure). Sex and age of deer were recorded. Management conditions of red deer were classified as open (no fencing and no management, 9 sites), fenced (fencing and artificial feeding, 12 sites), and farmed (livestock-like management, 2 farms). To analyze time trends, we classified samples collected during 2000–2005 as time 1 and those collected during 2006–2009 as time 2. Only sites where sampling occurred in both periods and with comparable sampling sizes were included in the time-trend analysis.

Serum samples were tested for HEV immunoglobulin (Ig) G by using ELISA as described (*4*,*13*), except for including protein G horseradish peroxidase (Sigma Chemical, St. Louis, MO, USA) as a conjugate, as in previous

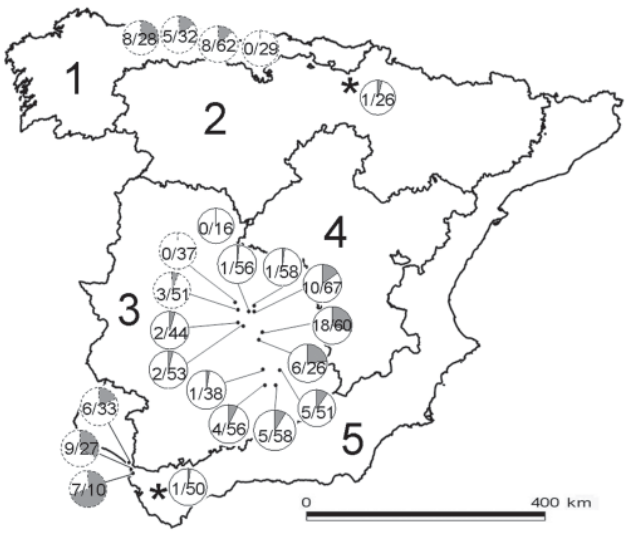

Figure. Five peninsular bioregions (nos. 1–5) and the 21 sampling sites, Spain. Pie charts indicate local prevalence (in gray). Numbers indicate positive animals/sampled animals. Broken line borders indicate open sites; solid lines indicate fenced estates; asterisks indicate the 2 red deer farms.

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Table. IgG serologic results for HEV and RT-PCR results in different regions and 2 red deer farms, Spain*

Region	No. sites	No. samples	No. seropositive	Prevalence, % (95% CI)	RT-PCR
Cantábrico Occidental	3	122	21	17.2 (11.4–24.9)	2/14
Cantábrico Oriental	1	29	0	0.0 (0.0–11.5)	0
Sistema Central	1	16	0	0.0 (0.0–20.8)	0
Montes de Toledo	7	366	19	5.2 (3.2–8.0)	2/18
Valle del Guadiana	2	86	22	25.6 (17.3–35.9)	5/13
Sierra Morena	4	203	15	7.4 (4.3–11.9)	1/14
Doñana	3	70	22	31.4 (21.3–43.5)	1/21
Cádiz†	1	50	1	2.0 (0.1–10.6)	0
Navarra†	1	26	1	3.8 (0.2–18.8)	0
Total	23	968	101	10.4 (8.62–12.53)	11/81

*Ig, immunoglobulin; HEV, hepatitis E virus; CI, confidence interval; RT-PCR, reverse transcription–PCR.
†Red deer farms.

studies of red deer (*12*). Anti-HEV–positive serum was obtained from domestic swine that were positive for HEV by ELISA and reverse transcription–PCR (RT-PCR). Anti-HEV–negative serum was obtained from previous studies (*14*) and negative controls from HEV-negative cattle (*13*). Results were expressed as the percentage of optical density (% OD) by using the formula [% OD = 100 × sample OD/sum of negative controls OD]. Serum samples with % OD values >100% were considered positive.

For the RT-PCR, 81 serum samples were randomly selected and analyzed. Viral RNA was extracted from 150 mL of serum with Nucleospin RNA virus kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions. HEV was detected by using a seminested RT-PCR as described (*14*). In each run, negative and positive controls were added.

Eight HEV RT-PCR–positive samples were sequenced. HEV sequences were identified by using the BLAST algorithm (www.ncbi.org) against HEV sequences available in GenBank (on January 25, 2010). Sequences were deposited in the GenBank database under accession nos. HM113373 and HM113374.

Sterne exact method was used to estimate apparent prevalence confidence intervals (CIs). χ^2 tests were used to analyze the association of age, sex, sampling site, and management conditions with serologic and RT-PCR results. Association between seropositivity and HEV RNA in the serum was also analyzed by using Pearson χ^2 test. Differences were considered statistically significant at $p<0.05$.

Overall, 101 (10.4%, 95% CI 8.6–12.5) serum samples were positive for IgG (Table). HEV seroprevalence did not differ significantly between sex ($\chi^2 = 0.894$, 1 df, $p>0.05$) and age classes ($\chi^2 = 12.436$, 3 df, $p>0.05$). Seroprevalence in time 2 (12.2%, 95% CI 9.8–15.0) was significantly higher than seroprevalence in time 1 (7.5%, 95% CI 5.1–10.8) ($\chi^2 = 5.181$, 1 df, $p<0.05$). Local IgG seroprevalences ranged from 0 (95% CI 0–20.8) to 31.4% (95% CI 21.3–43.5; Figure). IgG seroprevalence differed significantly by management types (χ^2 6.876, 2 df, $p<0.05$), with higher values in open (14.9%, 95% CI 11.3–19.4) than in fenced (9.1%, 95% CI 7.0–11.7) and farmed (2.6%, 95% CI 0.5–9.0) areas.

Eleven (13.6%, 95% CI 7.4–22.7) of 81 samples were RT-PCR positive. Local viral RNA prevalence ranged from 4.5% (95% CI 2.4–22.21 to 38.5% (95% CI 16.6–65.8; Table). HEV prevalence did not differ significantly by geographic area and management type.

Sequence analysis indicated that all deer sequences from this study belonged to genotype 3. Seven samples belonging to sequence HM113374 shared 99% nucleotide identity with domestic swine strains from Spain. One sample, sequence HM113373, showed similarity (91%) with a strain from acute hepatitis E in a person in Marseille, France, according to GenBank.

Conclusions

Our findings of HEV infection confirm that HEV circulates actively among red deer in the Iberian Peninsula, as described for wild boar (*9*). Red deer can be infected with HEV (*7*,*8*), and the results of our large serosurvey in this species in Europe show an increasing prevalence trend in the last decade.

de Deus et al. found higher IgG seroprevalences in estates with higher wild boar densities (*9*). However, in the present study, mean seroprevalence rates were lowest in red deer farms, where densities were the highest and red deer had no contact with wild boar or domestic swine. In contrast, the highest seroprevalence rates were reported in open areas where contact with suids may have occurred. However, wild boar densities also are high in fenced hunting estates (*15*), and HEV antibody prevalence rate was intermediate in deer from these sites. These differences could indicate that red deer may need a source of infection and thus act as spillover hosts more frequently than as true reservoirs.

Presence of HEV RNA in 13% of deer serum implies that deer represent a risk for zoonotic transmission, and consequently, handling of live animals and carcasses is a risk activity. Red deer are infected with HEV at lower rates than are wild boar and domestic pigs but may act as a po-

tential source of HEV infection in humans. Further studies are needed to fully elucidate the epidemiology of HEV in wildlife and the foodborne zoonotic transmission risks.

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Ms Boadella is a PhD student at the Instituto de Investigación en Recursos Cinegéticos. Her research deals with various aspects of shared wildlife diseases, including time trends and risk factors in diseases of wild ungulates.

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Prevalence of Henipavirus and Rubulavirus Antibodies in Pteropid Bats, Papua New Guinea

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To determine seroprevalence of viruses in bats in Papua New Guinea, we sampled 66 bats at 3 locations. We found a seroprevalence of 55% for henipavirus (Hendra or Nipah virus) and 56% for rubulavirus (Tioman or Menangle virus). Notably, 36% of bats surveyed contained antibodies to both types of viruses, indicating concurrent or consecutive infection.

The genus *Henipavirus* in the family *Paramyxoviridae* contains 2 highly lethal viruses, Hendra virus (HeV) and Nipah virus (NiV), both of which use pteropid bats as their main natural reservoir (*1*). The discovery of HeV in Australian flying foxes in 1996 (*2*) marked the beginning of a new wave of research activities, which led to the association of bats with some of the most notable viral pathogens to emerge in recent history, including NiV (*1*), severe acute respiratory syndrome–like coronaviruses (*3*), Ebola virus (*4*), and Marburg virus (*5*). In addition to the henipaviruses, 2 novel paramyxoviruses in the genus *Rubulavirus* were discovered in Australia and Malaysia. Menangle virus (MenPV) was isolated in Australia during a disease outbreak in pigs, with epidemiologic evidence suggesting the involvement of human patients as a result of pig-to-human transmission (*6*). Tioman virus (TioPV) was isolated from bat urine collected on Tioman Island in Malaysia and, although anti-TioPV antibodies were detected in residents of the island, its potential to cause disease in humans is unknown (*7*).

Several orthoreoviruses were also isolated from bats in Australia and Malaysia. Pulau virus was isolated from bat urine collected on Tioman Island in 2000; it is closely related to the Nelson Bay virus (NBV) isolated from a pteropid bat in 1968 in Australia (*8*). More recently, Melaka virus and Kampar virus, both closely related to viruses in the NBV species group, were isolated from human patients with respiratory symptoms; epidemiologic investigations strongly suggested they were the causative agents (*9*,*10*). Broome virus (BroV), a new orthoreovirus species, was isolated from a sick little red flying fox (*Pteropus scapulatus*) in 2002 in Australia, but its disease-causing potential is unknown (*11*). This study was conducted in June 2008 to survey bats in Papua New Guinea to determine the presence of various known bat viruses and to assess the potential of these viruses to be transmitted to the bat populations in Australia.

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The Study

A total of 66 bats were caught at 3 locations in Papua New Guinea (Figure; online Appendix Table, www.cdc.gov/EID/content/16/12/1997-appT.htm). They were anesthetized by using a combination of ketamine and medetomidine at doses similar to those stated in a previous study (*12*). Blood samples were held at room temperature for 24–48 hours and then serum separated by using centrifugation as required. Serum samples were held at 4°C until they were shipped to the Australian Animal Health Laboratory.

Virus-specific antibodies were detected by using a variety of assays previously developed by our group at the Australian Animal Health Laboratory. For the henipaviruses, the Luminex-based binding and inhibition assays (*13*) were used for initial screening, and positive samples were confirmed by virus neutralization test (VNT). Only those with positive results in all 3 assays are shown in the online Appendix Table. For TioPV and MenPV, initial screening was conducted by using an ELISA with purified TioPV virion as antigen. ELISA-positive samples were then confirmed by VNT against each virus. For viruses in the NBV species group, a mixture of purified recombinant sigma C proteins from NBV, Pulau virus, Melaka virus, and Kampar virus was used as ELISA antigen for initial screening. Positive samples were then confirmed by Western blot against each of the 4 recombinant proteins. For BroV, initial screening was conducted by ELISA using purified virion as antigen; positive samples were confirmed by immunofluorescent antibody test on cells infected with BroV.

A summary of the results is presented in the online Appendix Table. Seroprevalence for HeV was 50% (33/66); for NiV, 55% (36/66); TioPV, 38% (25/66); MenPV, 56% (37/66); NBV-like viruses, 17% (13/66); and BroV, 6% (4/66). The seroprevalence of the 2 types of paramyxoviruses, HeV/NiV at 55% and TioPV/MenPV at 56%, is high. The most striking finding is the presence of antibodies to both groups of viruses in 36% (24/66) of the samples. Considering that VNT is a more specific and less sensitive

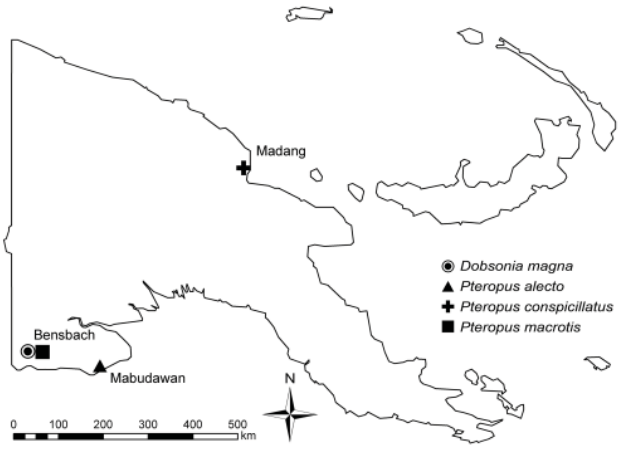

Figure. Location and species of bats collected for study of henipavirus and rubulavirus antibodies in pteropid bats, Papua New Guinea, June 2008.

assay, the actual positive rate could be >36%. Compared with results of the study conducted in Madgascar (*14*), in which 1/427 serum samples contained VNT-positive antibodies to both henipavirus and TioPV, our finding suggests extremely different paramyxovirus infection dynamics in bats in Papua New Guinea. Whether these data suggest concurrent or consecutive infection is not clear. We anticipate that the use of pteropid immunoglobulin M–specific reagent (currently in development in our group) will clarify this question in the future.

The positive rate for both groups of paramyxoviruses is much higher in *P. conspicillatus* bats (spectacled flying fox) from Madang than *Dobsonia magna* bats (bare-backed fruit bat) from Bensbach. We believe this difference has more to do with the bat species than the geographic location. For henipaviruses, seroprevalence is slightly higher for NiV than for HeV, which is also consistent with the Luminex inhibition assay showing a trend of slightly higher inhibition for NiV than for HeV (data not shown). This higher seroprevalence may suggest that henipaviruses circulating in Papua New Guinea are more NiV-like than HeV-like. However, in the absence of genetic sequence data, serologic findings are inconclusive.

In addition, for the 2 rubulaviruses, seroprevalence is higher for MenPV than for TioPV. This finding is noteworthy because our previous data indicated a one-way cross-neutralization, with MenPV antibodies failing to neutralize TioPV (G. Crameri and J. Barr, unpub. data). The results from this study suggest either that the main strain of bat rubulavirus(es) circulating in bats in Papua New Guinea is more closely related to MenPV or that there are different strains circulating and the MenPV-like are more dominant.

Although the overall seroprevalence for orthoreoviruses was much lower, the results nevertheless produced some useful information. First, the prevalence of 18% (2/11) of the NBV group viruses in *D. magna* bats at Bensbach is not much different from the 20% (11/54) in *P. conspicillatus* bats at Madang, indicating the NBV group of orthoreoviruses is present in bats of different species in Papua New Guinea. Second, none of the 66 serum samples was positive for both NBV and BroV, which supports our previous conclusion that BroV is a new species in the genus *Orthoreovirus* and that no significant cross-reactivity occurs between BroV and orthoreoviruses of other species groups (*11*).

Conclusions

In this study, a serologic survey was conducted for 4 groups of viruses, 2 from the family *Paramyxoviridae* and 2 from the genus *Orthoreovirus,* family *Reoviridae*. The surprising finding of a high prevalence of antibodies to both henipaviruses and TioPV/MenPV in individual pteropid bats highlights the need for more structured studies to investigate the infection dynamics of zoonotic viruses in different bat populations across the world. It is not clear at this stage what factors are responsible for the vast difference in prevalence of antibodies to paramyxoviruses and orthoreoviruses. In the context of potential incursion of exotic bat viruses into Australia, it is noteworthy that the bat rubulavirus(es) circulating in Papua New Guinea are more MenPV-like (Australia), whereas the Papua New Guinea henipavirus(es) seem to be more NiV-like (Asia). These findings are consistent with those in Indonesia (*15*) and call for further molecular epidemiologic investigation to better assess the risk of NiV entry into Australia.

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Fatal 1918 Pneumonia Case Complicated by Erythrocyte Sickling

To the Editor: The year 2010 marks the 100th anniversary of Herrick's original case description of what is now known as sickle cell anemia (*1*). Additional case reports followed in 1911 and 1915; in 1922, Mason described a fourth case and coined the term sickle cell anemia (*2*). In 1949, Pauling et al. published an important study that identified differences in the electrophoretic mobilities of normal and sickled erythrocytes (*3*). The inheritance pattern of sickle cell anemia was determined in 1949; in 1957, Ingram identified the single amino acid change in hemoglobin S (*4*).

Patients with sickle cell anemia are at markedly increased risk for infections with several bacteria, including *Streptococcus pneumoniae*, and emerging viral infections such as pandemic influenza. We report a retrospectively identified case of fatal bacterial pneumonia complicated by abundant erythrocyte sickling in a patient bearing the sickle cell trait. The patient's illness occurred in July 1918, representing one of the first identified symptomatic cases of sickle cell trait.

The patient, a 21-year-old African American male, was a US Army private admitted to the post hospital in Fort Riley (Camp Funston), Kansas, USA, on July 11, 1918, >1 month before the first recognized cases of the fall wave of the influenza pandemic in the United States. The patient had a 2-day history of fever, headache, chest pain, and a dry, hacking, nonproductive cough. Medical history consisted only of frequent headaches. Admission temperature was 105.4°F. Physical examination found indistinct breath sounds over the entire right lung. Lobar pneumonia was diagnosed in the patient. On July 15, his leukocyte count was 7,600 cells/mm^3, and physical examination found crepitant rales over the right lung and tubular breathing over the right upper lobe. On July 19, his condition was grave. Probably because most US military camps had experienced epidemics of measles with fatal streptococcal pneumonia during winter 1917–spring 1918, he was given 100 mL anti-streptococcus antiserum intravenously. He died July 20.

Postmortem examination on July 21 indicated marked consolidation of both the right middle and lower lung lobes, which appeared hemorrhagic. Large areas of right upper lobe necrosis were mixed with areas corresponding grossly to lobar pneumonia. The right pleural cavity was obliterated by fibrinous adhesions. Postmortem cultures from the right upper and lower lung lobes were positive for *S. pneumoniae* type II. Pleural cavity and heart blood cultures were negative. Notable findings included an enlarged hemorrhagic and necrotic spleen and numerous small hemorrhages in the medullary regions of the kidneys. Microscopic results were absent from the available postmortem examination record.

Two hematoxylin and eosin–stained lung sections from this patient, examined by using material from the archives of the Armed Forces Institute of Pathology (*5*) as part of a review of possible 1918 influenza virus pneumonia cases, showed acute pneumonia with extensive necrosis (online Technical Appendix Figure, panel A, www.cdc.gov/EID/content/16/12/2000-Techapp.pdf). Brown and Hopps tissue Gram stain revealed abundant gram-positive cocci (online Technical Appendix Figure, panel B). Histologic examination found abundant sickled erythrocytes in small pulmonary vessels (online Technical Appendix Figure, panels C, D). Results of real-time reverse transcription–PCR for influenza virus matrix 1 gene (*5*) were negative, as were immunohistochemical examination results for influenza viral antigen (online Technical Appendix Figure, panel F); control immunohistochemical examination results for cytokeratins were positive (online Technical Appendix Figure, panel E).

DNA was extracted from 1 of the formalin-fixed, paraffin-embedded lung blocks. Partial sequence of the hemoglobin beta gene was performed with PCR primer sets designed to span portions of the open reading frame (primers available upon request). Sequence of multiple clones across the gene showed the patient to be heterozygous for the Glu6Val hemoglobin S mutation (*6*), with 1 wild type and 1 mutant allele (online Technical Appendix Figure, panels G and H), indicative of sickle cell trait. Sequence analyses for the mutations associated with hemoglobin C (Glu6Lys), hemoglobin D (Glu121Gln), and hemoglobin O (Glu121Lys) showed only wild-type sequence (data not shown).

Although the material had been examined for possible influenza infection, the timing of the illness makes influenza an unlikely cofactor because epidemiologic records show no evidence of influenza or excess deaths from respiratory disease at Fort Riley in July 1918 (*7*). We found no evidence of influenza A viral RNA by reverse transcription–PCR or viral antigen by immunohistochemical examination.

Sickle cell trait has been occasionally associated with debilitating illness and death. Pulmonary complications associated with sickle cell trait include venous thromboembolic disease, sickle chest syndrome (*8*), and pulmonary infarction (*9*), which recently prompted US college officials to screen athletes for sickle cell trait (*10*). We speculate that the clinical severity and rapid development of acute bacterial pneumonia in the patient reported here led to profound terminal hypoxemia, which led in turn to erythrocyte sickling. The postmortem gross evidence of necrotic areas in the lung and spleen and hemorrhages in

the kidneys is clearly consistent with sickled erythrocytes causing vascular congestion and infarction, thus contributing to the patient's death.

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Human Brucellosis, Inner Mongolia, China

To the Editor: Brucellosis is one of the most common zoonotic diseases worldwide (*1*). The disease is caused by *Brucella* spp. and mainly transmitted from its animal reservoirs to humans by direct contact with infected animals or through the ingestion of raw milk or unpasteurized cheese (*2*). Human brucellosis has a wide spectrum of clinical manifestations, which can vary from subclinical infection with seroconversion to a full-blown clinical picture of fever; osteoarticular involvement; sweating; constitutional symptoms; and hepatic, cardiac, central nervous system, or ocular involvement (*2–4*). Although controlled in many industrialized countries, the disease remains endemic to many parts of the world, including Spain, Latin America, the Middle East, parts of Africa, and Asia (*5*). In the People's Republic of China, human brucellosis was highly endemic from the mid-1950s well into the 1970s, but then incidence decreased until the mid-1990s. However, incidence has increased sharply in China since 1995 (*6*), and the Inner Mongolia Autonomous Region is the most severe endemic focus; most reports of the disease occurred during 1999–2008. National and local public health authorities are concerned about the increasing incidence of the disease in this province. Here we report the epidemic characteristics that existed in this region during 1999–2008.

Human brucellosis is a reportable disease in China; suspected or confirmed cases must be reported to local and provincial Centers for Disease Control and Prevention (CDC) and then to Chinese CDC (CCDC) through the National Notifiable Disease Surveillance System. To meet case definitions, disease in persons must be accompanied by clinical signs and must be confirmed by serologic tests or isolation in accordance with the case definition of the World Health Organization (*1*,*7*).

We obtained the National Notifiable Disease Surveillance System data that were confirmed by the Chinese CDC from Inner Mongolia CDC. A total of 43,623 cases were reported during 1999–2008, of which 70.7% occurred in male patients; the difference in incidence between sexes was significant by χ^2 test ($\chi^2 = 581.9$, $p<0.00001$). A total of 28,237 (64.7%) reported cases occurred in persons 30–59 years of age, male (70.2%) and female (29.8%). However, 658 patients (396 boys) were <10 years of age, and 497 patients (333 men) were >70 years of age. The number of cases peaked in 2008, with 7,645 and 3,460 cases in male and female patients, respectively. The epidemic peaked in March–August, with 74.8% reported cases during the study period. The number of reported cases in 2008 was 25.6× the number reported in 1999. The highest proportion of cases (55.9%) occurred among persons engaged in agricultural activities (planting, animal husbandry) in rural areas; the next highest proportion was in shepherds (29.2%), who depend only on their herds to satisfy their nutritional needs. The number of cases sharply increased from 37 and 16 in 2001 to 315 and 308

in 2008 among housekeepers and students, respectively. In this province, *B. melitensis* was the most common pathogen, although *B. abortus* prevailed in certain regions. During our epidemiologic investigation, the number of agriculture workers who were inexperienced in animal husbandry increased suddenly and quickly; thus the trade and transportation of unquarantined and unvaccinated animals rose sharply. This situation most likely led to easier transmission to humans by direct contact with infected animals than had occurred previously. The results of our investigation indicate that the main risk factors associated with this outbreak were occupation (agriculture worker, shepherd, butcher, slaughterhouse worker, and cattle dealer) and risky practices (handling of ruminant abortions, skinning of stillborn lambs and kids, and crushing the umbilical cord of newborn lambs and kids with teeth) and certain dietary preferences (consuming unpasteurized and unboiled milk and fresh cheese) (W. Guo, pers. comm.).

Our results show that the annual incidence of the disease varied greatly from 0 to 818.52/100,000 at county levels during the study period (Figure). The largest incidence of the disease occurred in Abaga County in the center of Inner Mongolia. The spatial distribution of the disease clustered in the northeastern (Hulunbeir) and central (Xilinguole) districts. Hence, future public health planning and resource allocation should focus on Hulunbeir and Xilinguole, and active surveillance should be strengthened in these high-risk districts.

We report the epidemic features of human brucellosis in a province in China. This information will be helpful to establish strategies for prevention, surveillance, and management of human brucellosis in China and in other countries where the disease is endemic.

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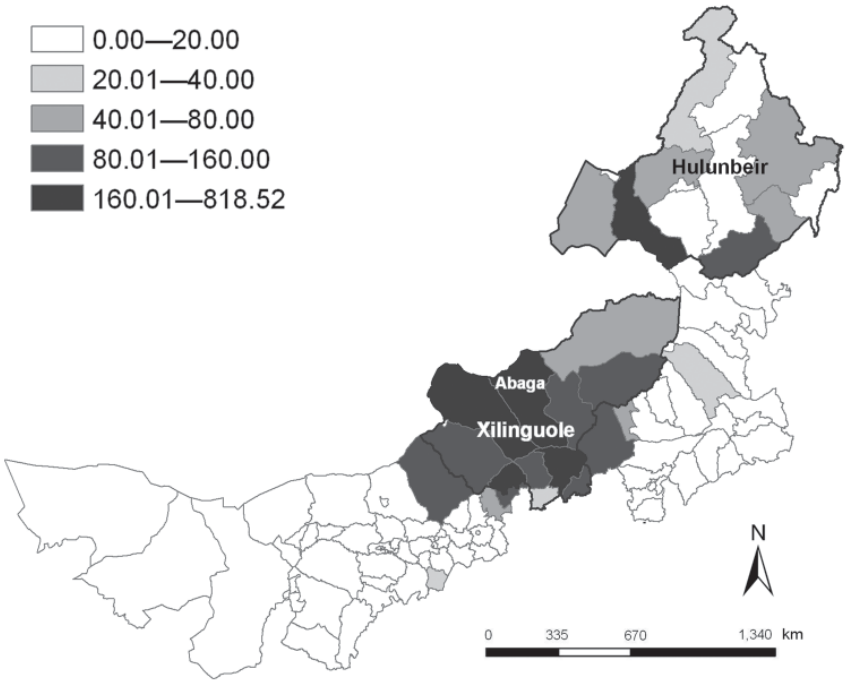

Figure. Annualized average incidence of human brucellosis, Inner Mongolia Autonomous Region, People's Republic of China, 1999–2008.

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Multiple Serotypes of Bluetongue Virus in Sheep and Cattle, Israel

To the Editor: In September 2008, the Israeli Veterinary Field Services were notified of uncharacteristic disease on a dairy farm near the border with Lebanon in Rosh Ha Nikra, (online Appendix Figure, www.cdc.gov/EID/content/16/12/2003-appF.htm). In November, blood samples were obtained from 5 cows, 4 of which were recovering from signs of infection with bluetongue virus (BTV). Virus isolation was conducted at the Kimron Veterinary Institute, Bet Dagan, Israel. One isolate (ISR2008/03) was sent to the World Animal Health Organisation Bluetongue Reference Laboratory at the Institute for Animal Health (IAH), Pirbright, UK, for further characterization. BTV-16 was identified by using serotype-specific real-time reverse transcription–PCR (RT-PCR) for genome segment 2 (Seg-2). BTV-16 has been detected in Israel and is considered endemic, along with BTV serotypes 2, 4, 6, and 10 (*1*).

Ten additional blood samples and 1 spleen sample subsequently obtained from affected cattle on the farm were sent to IAH. All samples were tested for BTV by serogroup-specific real-time RT-PCR specific for Seg-1. Six samples (including 1 from the spleen) were positive for BTV. Serotype-specific real-time RT-PCR showed that 2 blood samples contained BTV-16 and 1 blood sample contained BTV-4 and BTV-16. The amount of BTV RNA in the remaining 3 RT-PCR–positive samples was low, and attempts to identify serotype were unsuccessful. Virus from the spleen was isolated in an insect cell line (KC cells from *Culicoides sonorensis* midge embryos, CRL 1660; American Type Culture Collection, Manassas, VA, USA), and the virus was serotyped as BTV-8 by RT-PCR.

BTV-4 was isolated from bovine blood obtained in October 2008 from a farm in Zde Eliahu, 100 km east of Rosh Ha Nikra. However, this animal was co-infected with BTV-24, which has been found at numerous sites in Israel (online Appendix Figure). BTV-24 was isolated at IAH from samples obtained from sheep and cattle showing clinical signs of disease. BTV-4, BTV-16, and BTV-24 all reemerged in Israel during 2009, the mortality rate was up to 80% on 1 sheep farm infected with BTV-24 (*2*). An outbreak in Hatzafon in November 2009 was confirmed as BTV-5 by serotype-specific real-time RT-PCR.

To determine origins of BTV strains causing these outbreaks, we sequenced Seg-2 of the BTV-4 (Zde Eliahu) and BTV-8 and BTV-16 (ISR2008/02, ISR2008/13, and ISR2008/03) isolates from Israel. BTV-16 ISR2008/03 had >99% nt sequence identity (2,935 bp) with BTV-16 (GRE1999/13) isolated in Greece in 1999 but was distinct from BTV-16 (OMN2009/02) recently isolated in Oman. BTV-8 isolate ISR2008/13 had >99% nt sequence identity (2,939 bp) with the northern European strain of BTV-8 (NET2006/04). This finding indicates that the BTV-8 isolate from Israel (ISR2008/13) belongs to the same lineage as BTV-8 from northern Europe (NET2006/04) and may have been introduced into Israel during importation of BTV-8–positive animals from northern Europe.

BTV-4 isolate ISR2008/02 had >99% nt sequence identity (2,926 bp) with BTV-4 (DQ191279) isolated in Israel in 2001, which suggests that this serotype has either continued to circulate or has reemerged. BTV-24 (ISR2008/05) belongs to a western topotype. However, few nucleotide sequences are available for comparison of BTV-24 Seg-2 regions. BTV-5 has not been isolated; therefore, no sequence data are available.

Although BTV-2, BTV-4, BTV-6, BTV-10, and BTV-16 are considered endemic to Israel, clinical signs of disease are uncommon. We report clinical signs of infection in cattle in Israel caused by BTV-8 and BTV-24. We also report active circulation of 5 BTV serotypes (BTV-4, BVT-5, BTV-8, BTV-16, and BTV-24) during 2008–2009. Multiple serotypes were isolated on 3 farms containing sheep that had clinical signs of BT (farm 1: BTV-4 and BTV-24, farm 2: BTV-8 and BTV-24, and farm 3: BTV-4, BTV-8, and BTV-24). BTV-4, BTV-8, and BTV-16 were also isolated from cattle at Rosh Ha Nikra. Identification of multiple cocirculating BTV serotypes increases the likelihood of genome segment reassortment, which could potentially lead to increased virulence. Whole genome sequencing of isolates from these farms is in progress to determine whether any of these isolates are reassortants, as has been observed in Italy (*3*)

Our study indicates that BTV-8 strains from Israel and northern Europe (*4*–*6*) are closely related and share a recent common origin. The strain from Israel may represent an extension of the outbreak in Europe. Use of inactivated virus vaccines has dramatically decreased the number of cases caused by virulent BTV-8 in Europe (*7*), which suggests that a similar campaign might be effective in Israel. However, the BTV-24 strain from Israel appears to be highly virulent in

cattle and sheep, and absence of a live or inactivated vaccine against this serotype could lead to its reemergence and to severe economic losses. In the absence of an appropriate vaccine and control strategy, the virus could potentially spread to neighboring countries and pose an additional risk to Europe.

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Rabies Virus RNA in Naturally Infected Vampire Bats, Northeastern Brazil

To the Editor: Rabies is a major zoonotic disease and causes ≥55,000 human deaths annually worldwide (*1*). The predominant infection route for humans is by canids but zoonotic transmission from bats has been reported (*2*,*3*). Of >1,000 bat species, the only 3 species that feed on blood (*Desmodus rotundus*, *Diphylla ecaudata*, and *Diaemus youngi*) are found exclusively in Latin America (*4*). Rabies outbreaks caused by *D. rotundus* vampire bats have resulted in human deaths in Latin America and estimated livestock losses of $6 million annually (*4*).

To study rabies virus (RABV) prevalence and transmission in bat populations, we sampled 199 suborder Microchiroptera bats (mostly from families Phyllostomidae [86.4%] and Molossidae [11.1%]) in Bahia, northeastern Brazil, during 2008–2010. Areas where vampire bat activity or rabid livestock were reported were visited by members of the Bahia State Agency for Agriculture and Livestock Defence to identify bat roosts. All sampling was approved by the Brazilian Institute of the Environment and Natural Renewable Resources.

Bats were caught at roosts by using mist nets, killed with ether, and transported on ice to our laboratory. In accordance with rabies control program policies in Brazil, only vampire bats that were physically impaired (e.g., poor flight ability) or found dead could be sampled.

Thirty milligrams of brain or medulla oblongata per animal was homogenized and purified by using the RNEasy Kit (QIAGEN, Hilden, Germany). RNA was detected by using nested reverse transcription–PCR (RT-PCR) specific for viral nucleoprotein gene (*5*). RABV RNA was detected in 8 (27.6%) of 29 *D. rotundus* bats.

The 8 bats originated from 6 of 9 sampled roosts located in an area of ≈7,200 km^2. Nucleotide sequencing of PCR amplicons confirmed close phylogenetic relationships with vampire bat RABV (GenBank accession nos. HM171529–HM171536), which is consistent with reported absence of other *Lyssavirus* species in the Americas (*4*). Conventional RABV diagnostic tests (direct immunofluorescent test and infection of suckling mice) confirmed presence of RABV in central nervous system specimens from all 8 bats.

Viruses were quantified by using strain-specific real-time RT-PCR with the OneStep RT-PCR Kit (QIAGEN) and primers BRDesrot-Fwd, 5′-CGTACTGATGTGGAAGGGAATTG-3′; BRDesrot-Probe, 5′-FAMACAAGGGACCCTACTGTTTCAGAGCATGC-3′-Black Hole Quencher 1; and BRDesrot-Rev, 5′-AAACTCAAGAGAAGGCCAACCA-3′. Absolute quantification was performed by using in vitro–transcribed cRNA for the specific region.

Muscle, interscapular brown fat, tongue, and reproductive, thoracic, abdominal, and retroperitoneal organs from all 8 RABV-positive bats were tested. RNA concentrations were consistently highest in central nervous system specimens (median

$10^{10.91}$ genome copies/g tissue) (online Appendix Figure, www.cdc.gov/EID/content/16/12/2004-appF.htm). Tongue specimens (containing salivary glands) also showed high concentrations (median $10^{8.66}$ copies/g tissue). High concentrations in heart and lung were compatible with anterograde virus secretion through the vegetative system, similar to that in other mammals (*6*). Not all spleen samples were RABV positive, which suggested no specific involvement of RABV with the lymphatic system. Two of 5 female RABV-positive vampire bats were pregnant at the time of sampling. Virus was detected in both placentas and in 3 of 4 uterus specimens tested (median $10^{6.55}$ copies/g tissue). However, the 2 fetuses were too immature for analysis. Testicle specimens were available for 2 of 3 male bats; 1 bat was positive ($10^{6.76}$ copies/g tissue).

Modes of RABV transmission and pathobiology in bat populations are unclear. RABV infection at high doses leads to death. High seroprevalence rates in populations of apparently healthy animals suggest that bats may be capable of controlling natural infection, in contrast to other mammals (*6*). Nevertheless, these findings and those of another study (*7*) demonstrated that bats may also die from natural RABV infection. However, because only moribund vampire bats were sampled in our study, the proportion of bats that die of natural infection is unknown.

Although regurgitation of blood for feeding offspring or roost mates was suggested to be a route of infection in vampire bats (*8*), the organ distribution of RABV in our study suggests secretion from salivary glands after spread from the central nervous system, which is compatible with virus transmission in other mammals. Our finding is also consistent with those of a study on tissue distribution of European bat Lyssavirus 2 in *Myotis daubentonii* bats (*9*).

Increased virus concentrations in placentas and reproductive organs suggest vertical transmission, supporting previous findings of RABV in reproductive organs of a deceased *Eidolon helvum* bat (*7*). However, whether similar observations can be made in healthy bats is unknown. Sporadic detection of virus and low virus concentrations in bladder and intestine make RABV transmission by excreta less likely (*7*,*9*). Whether RABV infection was the primary cause of disease in our RABV-positive bats is unknown. Distribution of RABV in organs of moribund vampire bats was similar to that observed in autopsy specimens from humans (*10*). Thus, if we assume that the patterns of organ distribution we observed are representative for free-ranging vampire bats, transmission patterns may be similar to those seen in other mammals.

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Wildlife-associated *Cryptosporidium fayeri* in Human, Australia

To the Editor: Molecular tools are essential for *Cryptosporidium* spp. identification, taxonomy, and epidemiology because of morphologic similarities between species within this genus. Molecular analyses have now identified 22 *Cryptosporidium* spp. and >40 cryptic species (i.e., genotypes) across all vertebrate classes (*1*). The myriad of potential *Cryptosporidium* spp. hosts, in conjunction with the robustness of the infectious stage (oocyst), means diverse *Cryptosporidium* spp. constantly circulate through the environment. This circulation increases the potential for disease from a diversity of contamination sources.

Human cryptosporidiosis is a global problem causing illness in young, elderly, immunocompromised, and immunocompetent persons in both industrialized and developing nations. The 2 most common etiologic agents, responsible for 90% of reported human infections, are *C. hominis* and *C. parvum* (*2*,*3*). Additional species identified as human pathogens are *C. meleagridis*, *C. canis*, *C. felis*, and the *Cryptosporidium* rabbit genotype (*4*). Each of these species was once thought to be specific for turkeys, dogs, cats, and rabbits, respectively. Incidental findings of *C. muris*, *C. andersoni*, *C. suis*, *C. hominis* monkey genotype, *C. parvum* mouse genotype, and *Cryptosporidium* cervine (W4), chipmunk I (W17), skunk, and horse genotypes have also been reported in humans (*4*). The pathogenicity of these zoonotic species and genotypes to humans remains unclear.

In July 2009, a 29-year-old woman who sought care because of prolonged gastrointestinal illness had a fecal test positive for *Cryptosporidium* spp. by the Remel ProSpecT *Giardia*/*Cryptosporidium* microplate assay (Thermo Fisher Scientific, Lenexa, KS, USA). Oocysts were purified from the specimen (*5*) and stained with the *Cryptosporidium* spp.–specific antibody CRY104 labeled with fluorescein isothiocyanate (Biotech Frontiers, North Ryde, Australia) for enumeration. A parasite load of 1.34 × 10^6 oocysts/g feces was determined by using epifluorescence microscopy at 400× magnification.

To identify *Cryptosporidium* spp., DNA was extracted (*5*), and a diagnostic fragment of the small subunit (SSU) rRNA) was amplified (*6*). Clones were screened to identify species and determine the possibility of mixed infection. Plasmids from 50 clones were recovered and digested with the enzyme *SspI* (New England Biolabs, Beverly, MA, USA) (*6*). Two different restriction profiles were visualized. The sequence from each of the restriction types was determined; profile 1 contained *SspI* fragment sizes of 33, 109, 247, and 441 bp; profile 2 had fragments of 33, 254, and 540bp. A BLAST search (www.ncbi.nlm.nih.gov/blast) confirmed the sequences as *C. fayeri* type 1 and type 2. These 2 sequences correspond to known heterogeneity within the SSU rRNA of *C. fayeri* (*7*).

The identification of *C. fayeri* by SSU rRNA was confirmed by the sequence of the actin gene (*8*), showing 99.8% similarity to *C. fayeri* (GenBank accession no. AF112570). Further analysis at the 60-kDa glycoprotein (gp60) locus was used to determine the *Cryptosporidium* subtype family (*5*). The MQ1022 gp60 sequence was 98% similar to *C. fayeri* subtype family IVa (*9*). Analysis of the microsatellite region further characterized isolate MQ1022 to *C. fayeri* subtype IVaA9G4T1R1. The nucleotide sequences generated in this study were submitted to GenBank under accession nos. HQ008932–HQ008934.

Because the patient was imunocompetent, the disease was believed to be self-limiting, and she was lost to follow-up. The patient resided in a national forest on the east coast of New South Wales, Australia, an area where marsupials are abundant. She had frequent contact with partially domesticated marsupials. Notably, *C. fayeri* has been identified in 6 Australian marsupial species. Identification of *C. fayeri* in a human patient is a concern for water catchment authorities in the Sydney region. The main water supply for Sydney, Warragamba Dam, covers 9,050 km^2 and is surrounded by national forest inhabited by diverse and abundant marsupials. A previous study that investigated *Cryptosporidium* spp. in a wild eastern gray kangaroo (*Macropus giganteus*) population reported a prevalence of 6.7% (*10*). Oocyst shedding ranged from 20/g feces to 2.0 × 10^6/g feces (*10*). Subtype IVaA9G4T1R1 identified from the patient in this study has been characterized from eastern gray kangaroos in Warragamba Dam (*9*). Throughout the year, large groups of eastern gray kangaroos graze within riparian zones in the catchment. Such close proximity to the water presents a high possibility that the dam's water is contaminated with oocysts from these animals.

The *Cryptosporidium* genus is diverse, both in species and suitable hosts. The mechanisms of host specificity remain unknown, but the frequency of *Cryptosporidium* spp. crossing the host barrier and becoming

zoonoses is increasing. This increase indicates that *Cryptosporidium* spp. host specificity is not as stringent as previously thought.

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Canine Distemper Epizootic among Red Foxes, Italy, 2009

To the Editor: Canine distemper virus (CDV) is an enveloped, single-stranded, negative RNA virus of the family *Paramyxoviridae*, genus *Morbillivirus* (*1*). The host range for CDV is broad, and infection has been found in several mammalian species of the families Canidae, Mustelidae, Procyonidae, Ursidae, and Viverridae (*2*).

Stelvio National Park (SNP) encompasses 1,333 km^2 of protected land in Italy and covers 2 regions (Lombardia and Trentino Alto Adige); the Lombardia section of the park covers the northern part of Sondrio Province (Valtellina). SNP is surrounded by other parks (Schweitzer National Park, Adamello, and Adamello-Brenta) to form a large protected area (2,500 km^2) in the heart of the Alps mountains. Within SNP, the terrestrial mammals that are susceptible to CDV include red foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*), pine martens (*Martes martes*), beech martens (*Martes foina*), badgers (*Meles meles*), and bears (*Ursus arctos*).

In August 2009, three young red foxes were captured in Valtellina (Sondrio), Lombardia, Italy, within the southwestern borders of SNP. The animals showed canine distemper–like signs (e.g., prostration, altered behavior, and conjunctivitis), and CDV infection was confirmed by quantitative reverse transcription–PCR of pooled organs (*3*). In September and October 2009, another 2 young foxes were captured and found to be positive for CDV. From September on, at least 30 foxes with altered behavior were seen near human habitations and facilities in SNP; 10 were captured. In the same period, infected foxes were also reported from Engadina, Switzerland, at the northern and western borders of SNP. In February 2010, two symptomatic foxes were euthanized in Grosotto, 50 km south of where the initial cases were identified. The epizootic appeared to have originated from the eastern regions of Italy (Trentino Alto Adige, and Veneto), where CDV infection had been reported in red foxes and badgers since August 2006 (*4*) (Figure). A large CDV epidemic in foxes in southern Bavaria in 2008 has also been described, thus suggesting spread of the virus throughout the Alps area (*5*).

Reverse transcription–PCR genotyping of the hemagglutinin (H) gene (*6*) identified 15 CDV strains, which were analyzed and characterized as European genotypes. The full-length H gene of the CDV strains was determined (GenBank accession no. HM120874). Sequence analysis of the H gene indicated that the fox CDV

strains were highly related to each other (>99.9% nt and 100% aa identity), to the CDV strains identified in foxes in southern Bavaria 2008 (>99.7% nt and 99.3% aa identity; accession nos. FI416336–FI416338), and to a canine strain identified in Hungary during 2005–2006 (99.4% nt and 99.2% aa identity; accession no. DQ889177).

During the CDV epizootic in SNP in 2009, cases of CDV in 3 domestic dogs living within the borders of the park were also reported. Because vaccination against CDV is a core recommendation for dogs, most dogs are expected to be vaccinated and protected; population immunity is high enough to keep CDV infection under control, and only sporadic cases occur (*7*). Accordingly, the reported CDV cases in dogs were more likely a spillover event caused by the high pressure of CDV infection in SNP wildlife. In addition to foxes, badgers in the same area were also reported to have canine distemper–like disease. These findings are consistent with spread of a multihost epizootic, in which foxes likely played a major role in CDV amplification and diffusion because of their social behavior during reproductive season and because of the wide geographic range over which juveniles migrate during the dispersion period.

Serologic investigations for CDV in some fox populations in Europe have identified antibody prevalence rates of 4%–26.4% (*8*), suggesting that CDV circulates in foxes in Europe, but these investigations did not examine spatial and temporal variations in CDV activity. Clues for understanding the pattern of CDV disease in wildlife have been provided by structured surveillance of wild canids living in Yellowstone National Park, USA. Yearly fluctuations in CDV seroprevalence with evidence of multihost outbreaks in distinct years, contemporaneously affecting different animal species, have been noted. Cycles of CDV epizootics that swept through the animals in the park were associated with low pup survival rates (*9*).

In SNP, most foxes captured during the epizootic were juveniles. We have no information on the prevalence rates of CDV-specific antibodies in SNP foxes before the epizootic. However, CDV disease had not been reported in the SNP for at least 10 years, and no animal with CDV infection had been identified in a 2004–2005 survey of red foxes in SNP (*10*). Similarly, no evidence for CDV infection had been found in carnivores in Trentino Alto Adige during 2001–2002 (*10*). Accordingly, one can assume that the population immunity in SNP foxes (and in other susceptible hosts) was low.

Adequately controlling CDV infection in wildlife in Europe is difficult. It requires concerted transnational actions, including effective surveillance and prompt gathering and dissemination of information.

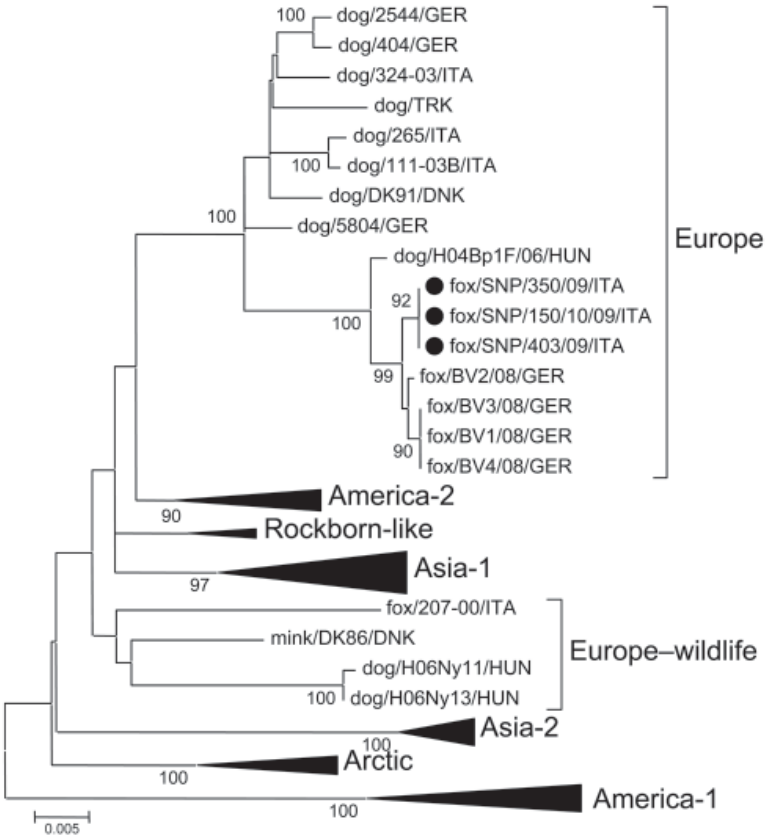

Figure. Phylogenetic tree showing the genetic relationships among selected canine distemper virus strains of various lineages and generated by using the full-length nucleotide sequence of the hemagglutinin gene. The tree branches including viruses not from Europe were collapsed (triangles). Full circles indicate the canine distemper strains identified in foxes from Stelvio National Park, Italy. The neighbor-joining tree was generated by using the Kimura 2-parameter distance correction, and statistical support was provided by bootstrapping >1,000 replicates, using the software package MEGA4 (www.megasoftware.net). Scale bar indicates nucleotide substitutions per site.

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Ribavirin for Lassa Fever Postexposure Prophylaxis

To the Editor: Lassa fever is an acute, viral, hemorrhagic illness endemic to West Africa. Intravenous ribavirin drastically reduces deaths from Lassa fever (*1*). During outbreaks, oral ribavirin is often considered for postexposure prophylaxis (PEP), but no systematically collected data exist for this indication of drug use (*1–5*). We therefore conducted a retrospective follow-up study to examine adherence and adverse effects associated with oral ribavirin given as PEP during an outbreak of Lassa fever in Sierra Leone in 2004 (*6*). During this outbreak, family members and some healthcare workers who had direct contact with patients did not use personal protective equipment and were subsequently prescribed oral ribavirin as PEP (200 mg capsules; Schering-Plough Corporation, Kenilworth, NJ, USA).

Approximately 3 months after the possible exposures, we surveyed 23 (92%) of 25 persons known to have been prescribed ribavirin PEP. Respondents were asked about demographics, medical history, details of possible exposure to Lassa virus (LASV), dosage and duration of ribavirin prescribed and taken, and use of concomitant medications. When possible, serum was obtained and tested by ELISA for LASV-specific immunoglobulin (Ig) M and IgG (*7*).

The mean age of the 23 respondents was 38 years (range 23–73 years); 14 (61%) were male, 17 (74%) had been exposed at home (during bathing, cleaning, and feeding of family members with Lassa fever), and 6 (26%) had had in-hospital contact with blood and bodily fluids. No needle-stick injuries were reported.

All respondents had begun taking oral ribavirin within 2 days after exposure. The drug was prescribed at a mean dose of 800 mg 1×/d (most often as 400 mg 2×/d) for 10 days; however, respondents reported actually taking 400–1,200 mg/d. Only 10 (43%) completed the full 10 days of therapy; mean duration of therapy was 8 days (range 1–14 days). No correlation was found between the prescribed daily dose of ribavirin and the likelihood of completing therapy ($p = 0.60$).

Therapy was completed by 6 (38%) of the 16 (70%) respondents who reported having experienced minor adverse effects and by 4 (57%) of the 7 who reported not having experienced adverse effects (Figure). Many respondents reported having had symptoms even before beginning ribavirin, suggesting at least a partial psychosomatic or other etiology. No correlation was found between likelihood of adverse effects and age ($p = 0.18$), sex ($p = 0.16$), or place of exposure ($p = 0.63$). Six (26%) respondents reported having premorbid health conditions (gastric ulcers, $n = 3$; gastroesophageal reflux disease, $n = 2$; hypertension, $n = 1$), and 15 (65%) took medications in addition to ribavirin during the postexposure

period, including paracetamol, folic acid, multivitamins, iron, antacids, antimalarial drugs, antimicrobial drugs, and nonsteroidal anti-inflammatory drugs.

Minor adverse effects from oral ribavirin PEP, either biologic or psychosomatic, were frequently noted and decreased adherence. Many of the same adverse effects have been reported (*8*). Because interviews in our study were conducted months after medication had been taken, recall bias may have occurred. However, 11 (85%) of the 13 repondents who reported not completing therapy could show the interviewer their leftover ribavirin capsules, thus validating their reports. The observational nature of our study prevented us from establishing a causal association between taking ribavirin and the reported adverse effects. Other factors, especially the anxiety often associated with possible LASV exposure, likely contributed to the noted symptoms.

Although we cannot exclude the possibility of asymptomatic infection, we found no evidence of secondary transmission of LASV among the respondents. One person reported having fever and malaise after exposure, but test results for LASV were negative. Only 8 (35%) persons consented to follow-up laboratory testing, probably because most did not think it was necessary because of lack of symptoms; all 8 were LASV IgM negative. The duration of IgM after LASV infection has not been well characterized, and antibodies could have cleared in the 3 months between exposure and testing (*7*). Another possibility is that swift administration of ribavirin blunted the antibody response. Although not studied in humans, total Ig titers in LASV-infected, ribavirin-treated monkeys eventually reached titers similar to those in untreated monkeys (*9*). Three persons were LASV IgG positive, indicating past exposure. All 3 had other risk factors for infection in addition to their recent exposure, including residence in a Lassa fever– hyperendemic area (all 3) and occupation as healthcare workers (2 of 3).

The limitations inherent in our study are its small sample size and retrospective, uncontrolled design. Considering the relatively low secondary attack rate, the restriction of LASV endemicity to remote areas of West Africa, and the infrequency of high-risk exposures, controlled trials for ribavirin PEP in Lassa fever will probably never be possible. Experiences in the field must therefore be used to inform future decisions with regard to use of ribavirin for this indication. Use of oral ribavirin PEP for Lassa fever is likely to be challenging because of poor adherence and adverse effects.

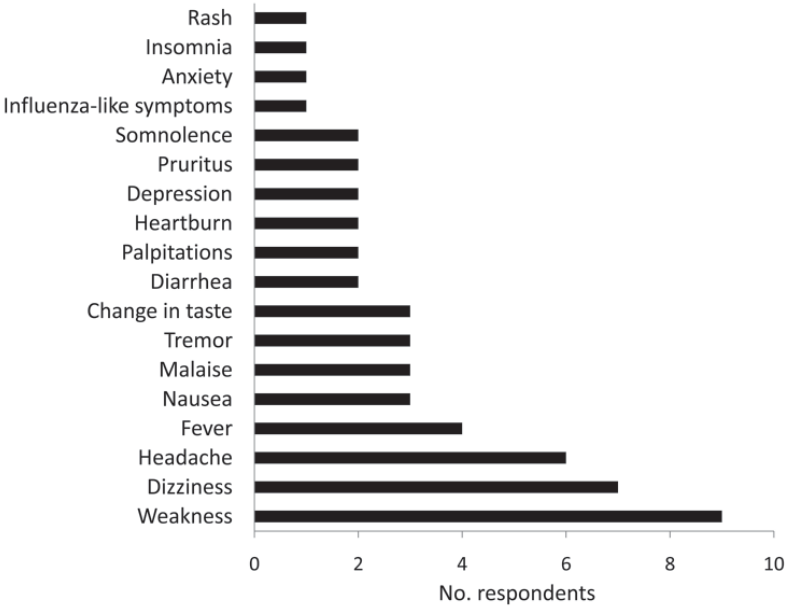

Figure. Adverse effects reported by 23 persons who took oral ribavirin prophylactically after potential exposure to Lassa virus, Sierra Leone, 2004.

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Transmission of Ovine Herpesvirus 2 from Asymptomatic Boars to Sows

To the Editor: Malignant catarrhal fever (MCF) is an often lethal viral disease of susceptible biungulates from the Bovidae, Cervidae, and Suidae subfamilies. MCF in pigs has been associated with direct or indirect contact with sheep, which are the main reservoir of ovine herpesvirus 2 (OvHV-2) (*1*). A recent report detected infected but asymptomatic swine in the absence of known exposure to sheep or goats (*2*). Porcine MCF is difficult to diagnose because of its nonspecific clinical signs and sporadic nature; however, an outbreak involving 41 swine has been described (*3*). Pigs are terminal hosts and are not believed to spread the virus. Here we describe OvHV-2 DNA in the blood and semen of asymptomatic boars and from the brain of symptomatic sows and gilts with MCF that was probably transmitted by artificial insemination.

The MCF cases occurred on two 3-site commercial farms with 2,700 and 1,670 sows in 2 different counties in southwestern Brazil. No MCF losses previously had been recorded in the region, and the animals had no known direct or indirect contact with sheep. The 2 farms had high biosecurity. The first case was recorded in September 2004, and the number of cases increased in July 2006. Twenty-eight sows and gilts, 20 of them pregnant and at ≥25 days' gestation, died during January 2007–March 2008, when the last case was observed.

Clinical features in sows and gilts were depression followed by abortion, fever (41°C), and anorexia. After the onset of clinical signs, neurologic symptoms developed such as ataxia, tremors, convulsions, and aggressive behavior. Animals that survived longer showed forelimb paralysis, stood in a dog-sit position, and gnawed with abundant salivation on pen bars.

Specimens from randomly selected dead sows and gilts from the outbreaks during 2004–2008 were obtained for histopathologic examination, immunofluorescence testing for rabies virus, viral and bacterial isolation, and PCR. No bacterial or viral growth was detected, and direct immunofluorescence for rabies virus was negative. Microscopic examination showed high-grade nonpurulent meningoencephalitis characterized by lymphocytic cuffings with vasculitis in the brain hemisphere, the brainstem, the spinal cord, and, to a lesser extent, the cerebellum. Multifocal areas of edema, fibrinoid necrosis, and lymphocytic infiltration also were observed (Figure). OvHV-2 DNA was detected by using a specific PCR (*4*) in 5 of 7 paraffinized sections of the brainstem (*5*). To analyze the possible presence of other porcine lymphotropic herpesviruses in samples that reacted positively for OvHV-2, a nested PCR with degenerate primers (*6*,*7*) was applied. None of the OvHV-2–positive samples reacted positively for porcine lymphotropic herpesviruses. To confirm that the virus was a member of the MCFV group, we purified 1 amplicon and submitted it for automated sequencing. This nucleotide sequence was deposited in GenBank under accession no. HQ223415, and it showed 99% identity with previously deposited OvHV-2 sequences.

To find possible carriers of the virus, blood samples were collected from 9 pregnant sows, 10 nonpregnant sows, and 30 breeding boars and analyzed for OvHV-2 DNA. Samples from 3 boars were positive. Nasal swabs and semen samples were collected from these infected boars to investigate the potential mode of OvHV-2 transmission, and OvHV-2 DNA was detected only in semen samples. Two of the 3 semen samples had >350 copies/2 μg of total DNA, suggesting that these

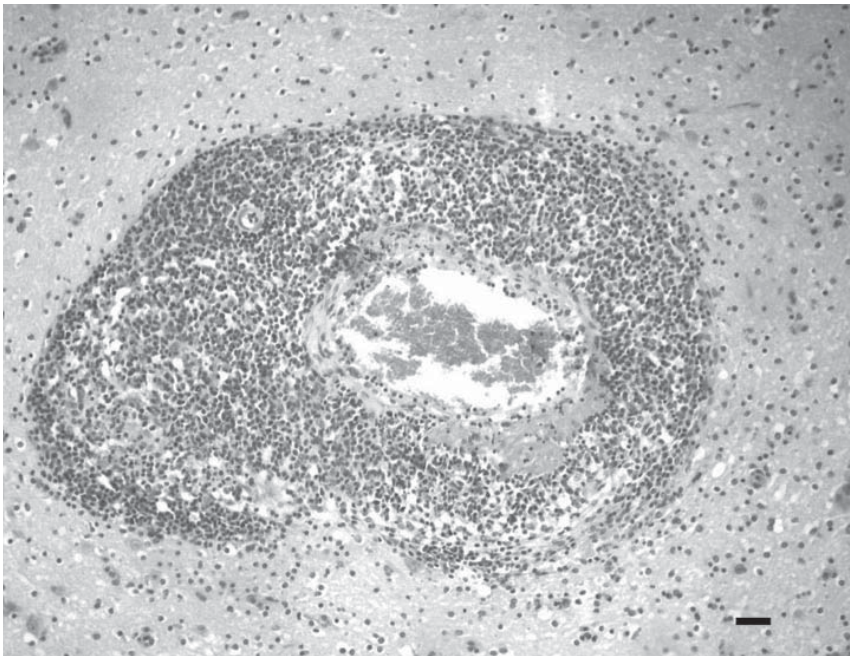

Figure. Brainstem of a sow with vasculitis that was associated with ovine herpesvirus 2, showing edema, necrosis (fibrinoid necrosis), and infiltration of lymphocytes in the adventitia of the artery. Severe perivascular (Virchow-Robin space) lymphocytic cuffing was observed. Hematoxylin and eosin stain; scale bar = 50 μm.

animals shed virus (*8*,*9*). During this period, all infected boars remained clinically healthy.

In Brazil, porcine MCF has been found primarily in pregnant sows and gilts. Our findings of OvHV-2 DNA in the semen of asymptomatic boars suggest that the OvHV-2 in the sows and gilts originated from asymptomatic boars that were responsible for maintaining the virus in the herd. Whether virus shedding in the semen was temporary or lasted for a long period is not known.

Emergence of OvHV-2 in boars that had no known contact with sheep was surprising, especially given the possibility of venereal transmission through contaminated semen. The occurrence of OvHV-2 infection in other specific pathogen–free farms is unknown, and it is not possible to suggest a strategy to guarantee OvHV-2-free herds.

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Molecular Detection of *Bartonella alsatica* in Rabbit Fleas, France

To the Editor: *Bartonella alsatica* was first isolated from the blood of wild rabbits from the Alsace region in France (*1*). This bacterium is now considered an emerging infectious disease zoonotic agent in persons in close contact with rabbits; at least 2 human cases of endocarditis and 1 human case of lymphadenitis have been reported (*2*–*4*). In this study, we report the molecular detection of *B. alsatica* in fleas (*Spilopsyllus cuniculi*) collected from rabbits in southern France.

During January and February 2008, a total of 60 fleas were collected from wild rabbits (*Oryctolagus cuniculus)* from 3 regions in southern France: Canohes (42°38′N, 2°51′E), Pollestres (42°38′N, 2°52′E), and Toreilles (42°45′N, 2°58′E). The fleas were collected and identified phenotypically, kept in ethanol, and sent to Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes in September 2009.

DNA from these fleas, as well as negative controls from uninfected lice maintained as colonies in our laboratory, were extracted by using a QIAmp Tissue Kit (QIAGEN, Hilden, Germany), as described (*5*). Identification of flea species at the molecular level was achieved by PCR amplification and sequencing of partial siphonapteran 18S rDNA gene (1.95 kbp) as described (*5*). Sequences were assembled in Sequencher 4.2 (GeneCodes Corporation, Ann Arbor, MI, USA). DNA was used as templates in a real-time quantitative PCR specific for a portion of the *Bartonella* genus 16S–23S intergenic spacer (ITS) performed in a Smart cycler instrument (Cepheid, Sunnyvale, CA, USA), as described (*2*). Positive samples at the genus level were confirmed by PCR amplification and sequencing of the *Bartonella* ITS region, as described (*2*). Finally, *B. alsatica* amplification and specific identification was confirmed by using 2 new specific PCRs with primers and TaqMan probes (Applied Biosystems, Courtaboeuf, France) specific for a portion of the heat shock protein 60 (*hsp60*) and the DNA gyrase subunit B (*gyrB*) genes of *B. alsatica* (Table). Specificity of these 2 PCRs was verified in silico (computer simulation) and by using a panel of 14 *Bartonella* species available in our laboratory (data not shown).

All fleas were morphologically identified as *S. cuniculi* by using current taxonomic criteria (*6*). Moreover, the 18S rRNA gene amplified and sequenced as described (*6*) from fleas gave a sequence with 100% similarity with the sequence of *S. cuniculi* fleas deposited in GenBank (accession no. EU336097). *B. alsatica* was detected by ITS reverse transcription–PCR in 8 (13.3%) of 60 fleas: 6 from Toreilles (17.6%, 6/34) region, 2 from Canohes (10.5%; 2/19), and none from Pollestres (0/7). Sequences obtained after PCR amplification and sequencing of partial ITS showed 96.6% identity with *B. alsatica* (GenBank accession no. HM060955). Using our 2 new PCRs specific for partial *hsp60* and *gyrB* genes from *B. alsatica,* we identified all *Bartonella* spp.–positive fleas, which had cycle threshold values ranging from 12.15 to <32.35 and 13.21 to <36.99 for *hsp60* and *gyr*B genes, respectively.

We report the specific detection of *B. alsatica* in *S. cuniculi* rabbit fleas from southern France using 4 different PCRs and sequencing, including 2 new reverse transcription PCRs described in this study. There is 1 report of molecular detection of *B. alsatica* from *S. cuniculi* fleas from a European wildcat (*Felis silvestris silvestris)* in Andalusia, Spain (*7*). Although *S. cuniculi* fleas are rare on cats, this study demonstrates that cats in contact with rabbits may be infected by these fleas and consequently become a potential source for *B. alsatica* transmission to humans. Márquez has also recently reported the molecular detection of *B. alsatica* in blood from 48/279 (17.2%) of wild rabbits (*O. cuniculus*) in Andalusia, Spain (*8*).

In conclusion, further research is needed to better understand the mode of transmission of *B. alsatica* in humans and mammals and the role of rabbit fleas for potential transmission for these bacteria. The recent description of *B. alsatica* as a human pathogen and the discovery of rabbit fleas as a potential vector reemphasize the emergence potential of this bacterium in humans who have close contact with rabbits.

Table. Oligonucleotide primers and TaqMan* fluorescent probe sequences of *hsp*60 and *gyr*B genes used for reverse transcription PCRs of *Bartonella alsatica*†

Gene	Oligonucleotide	Sequence (5′ → 3′)	Length, bp	Amplicon size, bp
hsp60	B_alsa_hsp60_F	TGCTAACGCTATGGAAAAAGTTG	23	108
	B_alsa_hsp60_R	CCACGATCAAACTGCATTCC	20	
	B_alsa_hsp60_P	6FAM-TGTCGAAGAAGCAAAAACGGCTGAAACC-TAMRA	28	
gyrB	B_alsa_gyrB_F	CGAAGCAAAACTTCTTATTAGTAAGGT	27	126
	B_alsa_gyrB_R	GCAAGCTTTCCTGGCAGAG	19	
	B_alsa_gyrB_P	6FAM-ATAGAGGCTGCTGCGGCGCG-TAMRA	20	

*Applied Biosystems, Courtaboeuf, France.
†*hsp60*, heat shock protein 60; gyrB, DNA gyrase subunit B.

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Cutaneous Myiasis Caused by *Chrysomya bezziana* Larvae, Mexico

To the Editor: We report a case of cutaneous myiasis caused by *Chrysomya bezziana* larvae in a 62-year-old woman who had a complex vascular cutaneous anomaly in her lower right extremity for 8 years. On physical examination, in September 2009, she had a nonlimping walk with pink and painful feet and an ulcerative lesion on the internal surface of the right leg above the internal malleolus. This ulcer was large, clean, without evidence of infection, and had tissue in the process of granulation. Adjacent to the upper edge of this lesion, we observed a second, crater-like ulcer ≈2.5 cm in diameter from which drained an abundant, highly purulent, serohematic material (Figure, panel A).

Approximately 10 days earlier, the patient had detected discharge of worms from the second lesion, motivating her to seek medical consultation. We performed surgical cleaning and manual removal of worms (Figure, panel B) and referred the patient for external consultation to control vascular, metabolic, and parasitologic evolution and for instruction in proper hygiene. The worms were identified as *C. bezziana* larvae by the Parasitology Laboratory of the Microbiology and Parasitology Department, Faculty of Medicine, National Autonomous University of Mexico.

Myiasis, a zoonotic disease, is defined as invasion of human living tissue by eggs or larvae from flies of the order Diptera. Among the diverse types of human myiasis that can occur in tropical regions, those in skin tissue are the most frequent, especially those generated by flies of the family *Calliphoridae*, of which the predominant species are *Cordylobia anthropophaga* (tumbu fly); *C. bezziana*, and *Oestrus ovis* in Africa (*1*) and *Dermatobia hominis* (American warble fly) in Central and South America.

Myiases have become increasingly relevant, particularly when human activity is carried out in environments with poor hygiene or in close proximity to domestic and peridomestic animals, such as dogs and rats (*2*). Human myiases generally are present in cavities or wounds but also can affect tissue, such as the skin, eyes, oral cavity, intestines, or urogenital area. *C. bezziana* larvae can usually be found infecting wounds or cutaneous ulcers but are occasionally found in normal skin (*3*–*5*).

Tegumentary and exposed-cavity myiases are relatively easy to diagnose because the source larvae can be observed directly. As a result of the taxonomic study of the larvae based on their morphologic characteristics (*6*), we searched the Medline, PubMed, Scielo, and Lilacs databases for articles describing myiasis caused by the identified species. The published literature showed that no prior cases had been documented in Mexico, and only a few cases had been documented in other regions of North America.

Old World flies, such as *C. bezziana* and *O. ovis*, are the most important producers of myiasis from an economic perspective (*7*). The larvae feed on living tissue causing highly traumatic lesions in a great variety of warm-blooded animals. These

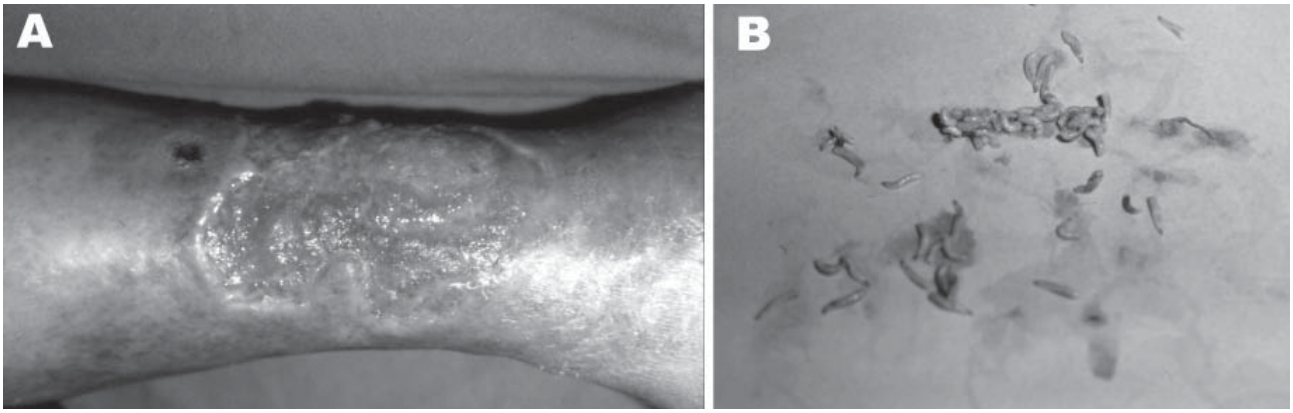

Figure. A) Crater-like ulcer ≈2.5 cm in diameter on internal surface of patient's right leg. B) *Chrysomya bezziana* worms isolated from the ulcer. A color version of this figure is available online (www.cdc.gov/EID/content/16/12/2014-F.htm).

myiases present a great diversity of clinical profiles, depending on the affected sites. Occasionally, even after elimination of the larvae, they may have subsequent effects, such as septic arthritis or even death, particularly in newborns, older persons, or immunosupressed persons (*8*). The spread of this infection to other countries or even across continents is not yet clear, but an overriding factor is the massive population migration, which poses the risk for introduction of new species at different places in different seasons. No reports were found indicating that this infection could be spread in another form (e.g., by food or water). Another probable cause for spread of this infection is the global change of the weather that helps larvae to survive in places where they could not previously survive (*9*).

In the case presented, the myiasis was limited to localized destruction of the tissue and was not associated with hemorrhagic problems or bacterial infections. However, myiasis can affect deeper structures, including striated muscle and eventually bone, causing severe destruction of these tissues. Considering the potential effects of this disease, timely diagnoses are critical to limit the damage, take appropriate hygiene measures, and if necessary, provide adequate treatment (*10*).

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Rabbit Tularemia and Hepatic Coccidiosis in Wild Rabbit

To the Editor: Tularemia is a highly pathogenic zoonosis caused by the gram-negative intracellular bacterium *Francisella tularensis*. *F. tularensis* causes serious septicemia in animals, especially wild rodents and lagomorphs (rabbits and hares), and potentially fatal, multisystemic disease in humans. The human mortality rate can reach 30% in untreated persons (*1*). *F. tularensis* is listed as a category A bioterrorism agent by the Centers for Disease Control and Prevention alongside the causative agents of anthrax, plague, smallpox, botulism, and viral hemorrhagic fevers. Generally, lesions associated with septicemic tularemia include multifocal 1–2-mm, white foci of necrosis in the liver, spleen, lymph nodes, and lungs.

Eimeria stiedae is the causative agent of hepatic coccidiosis, a common disease of wild rabbits (*2*) that can result in severe hepatic injury and death in juveniles and neonates. The gross lesion associated with hepatic coccidiosis is unique and nearly pathognomonic. Because *E. stiedae* causes proliferation of bile duct epithelial cells, affected livers contain multifocal, well-demarcated, linear, occasionally branching, bosselated, yellow to pearl-gray lesions that reflect the course of the biliary tree.

We describe a unique case of tularemia in a rabbit co-infected with *E. stiedae*. This case was initially misdiagnosed as simple *E. stiedae* infection on the basis of the classical gross lesions of hepatic coccidiosis, which overshadowed the more subtle tularemia lesions.

A juvenile wild rabbit was brought to a local veterinary clinic for postmortem examination. The owner, located in southwestern Missouri near the Arkansas–Kentucky border, raises wild-captured rabbits in a 10-acre, fenced area reserved for the training of hunting dogs. Beginning in the summer of 2009, a gradual rabbit die-off occurred, progressing to almost complete depopulation by May 2010. The liver from the dead rabbit was submitted to the University of Missouri Veterinary Medical Diagnostic Laboratory (Columbia, MO, USA). Gross examination showed the liver contained multifocal to coalescing, linear, yellow to gray nodules consistent with the classical appearance of hepatic coccidiosis. Although no gross evidence of tularemia was observed, the specimen was treated as potentially infected with tularemia because the veterinarian requested *F. tularensis* testing. Samples were collected and processed for bacteriologic culture, PCR, and histologic evaluation within the confines of a certified biological hood.

The liver contained 2 distinct microscopic lesions. The first was severe biliary hyperplasia with numerous intraepithelial coccidia, consistent with hepatic coccidiosis, as was anticipated. The second, more surprising lesion was an acute, multifocal, necrotizing hepatitis (Figure). The differential diagnoses for acute, multifocal, necrotizing heptatitis in a rabbit include tularemia, Tyzzer disease, listeriosis, and salmonellosis. In this instance, *F. tularensis* was identified by bacterial culture (*3*) and PCR as previously described (*4*). No other pathogenic bacteria were isolated on culture. These results were reported to the veterinarian, the owner, and public health officials. All remaining biological specimens were immediately discarded following the University of Missouri's select agent protocols, and further analysis was halted, preventing further typing of the isolated *F. tularensis*.

According to the Centers for Disease Control and Prevention, ≈126 cases of tularemia are reported annually in the United States (*5*). During 2000–2008, Missouri had the highest number of reported cases (228) followed by Arkansas (149) (*5*). Two subspecies of *F. tularensis* are endemic to the United States: the highly virulent *F. tularensis* subsp. *tularensis* (type A) and the moderately virulent *F. tularensis* subsp. *holarctica* (type B).

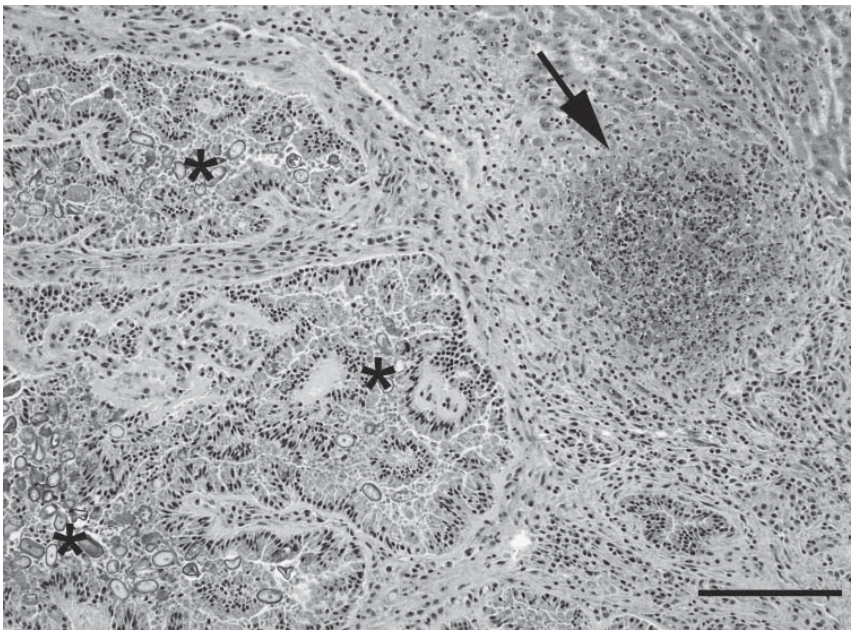

Figure. Liver from a juvenile wild rabbit with numerous oval *Eimeria stiedae* oocysts in the convoluted hyperplastic bile ducts (asterisks) and necrotizing hepatitis (arrow) by *Francisella tularensis*. Hematoxylin and eosin stain; scale bar = 200 μm.

Transmission of the bacterium occurs primarily through bites from arthropods, including the dog tick (*Dermacentor variabilis*), the wood tick (*D. andersoni*), the lone star tick (*Amblyomma americanum*), and the deer fly (*Chrysops* spp.). In addition, contact with infected animals, most commonly rabbits, wild rodents, and cats, is another common route of transmission to humans (*1*,*6*).

Tularemia occurs in various animal species. Lagomorphs, rodents, and sheep are most susceptible; infected animals are frequently found dead or moribund. Carnivores are less susceptible; however, feline tularemia occurs sporadically, and human infections associated with bites and scratches from infected cats have been recognized (*7*). In addition to arthropod bites, contact with infected dead rabbits or their tissues appears to be the most common source of human infection. A wide variety of case reports have been published describing unique incidences of rabbit–human transmission, including a lawn mower aerosolizing rabbit nests along with their occupants (*8*), consumption of undercooked rabbit meat (*9*), and contact with a "lucky" rabbit's foot (*10*).

The purpose of this report is to alert veterinarians, veterinary laboratory personnel, and public health officials that rabbit tularemia can be easily overlooked on gross examination in animals displaying lesions of hepatic coccidiosis, a common disease of the wild rabbit. Therefore, all rabbits submitted for postmortem examinations should be regarded as potentially infected with tularemia, particularly during seasons when vectors are active.

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Imported Leishmaniasis in Dogs, US Military Bases, Japan

To the Editor: Leishmaniasis is found in canids in ≈50 of the 88 countries where leishmaniases are found in humans (*1*). In Japan, 2 cases of imported canine leishmaniasis have been documented in dogs from Spain (*2*,*3*). We report 2 cases of leishmaniasis in dogs in which dermatitis developed mainly on the face. Leishmaniasis was diagnosed from results of a serologic rk39 test, followed by PCR of skin lesion specimens for the *Leishmania* spp.–specific small subunit (SSU) rRNA gene. Because the dogs had lived on a US military base in Sicily, Italy, for 3 years before their owners were transfered to Japan, the animals were likely infected with *L. infantum* in Italy.

Animal 1 was a 6-year-old female dog that had lived in Sicily for 3 years, since 2003, and had been brought to Japan in September 2006. While she lived in Italy, she had exhibited alopecic, pruritic, and crusty skin lesions, mainly around the face and on the forearms and hind legs.

In November 2006, the dog was brought to the US Army Veterinary Command's Zama Veterinary Treatment Facility with dermatitis (online Appendix Figure, panel A, www.cdc.gov/EID/content/16/12/2017-appF.htm) and additional signs of kidney failure. A serum specimen was positive by the rk39 dipstick test for diagnosis of visceral leishmaniasis (Kalazar Detect; InBios, Seattle, WA, USA). A skin punch biopsy specimen was obtained for cultures and PCR for the parasites in December 2006. Cultures of 4 skin specimens were all negative, probably because of cool transportation of the samples for 1.5 days before the cultures were started. The dog's condition was treated with ketoconazole and then allopurinol. The

skin conditions initially improved, but the lesions did not completely resolve (online Apendix Figure, panels B–D). In May 2008, the dog was humanely killed because of central vestibular disease with unknown cause. A necropsy was not performed.

Animal 2 was a 12-year-old male dog that had also lived in Sicily for 3 years since 2000, and was brought to Yokosuka Base in Japan in 2003. In January 2004, the dog was positive for visceral leishmaniasis by the rk39 test; no particular clinical signs were observed.

In March 2007, the dog was referred to Zama Veterinary Treatment Facility with pruritic alopecia on the dorsum and head, and a skin punch biopsy specimen was obtained for histopathologic evaluation. The presence of amastigotes of *Leishmania* species within areas of dermal inflammation was confirmed at the Armed Forces Institute of Pathology (Washington, DC, USA). In April 2007, a second skin punch biopsy specimen was obtained for PCR.

PCR was performed for the *Leishmania*-specific SSU rRNA gene (*4*). For primary PCR, primers R221 (5′-GGTTCCTTTCCTGATTTACG-3′) and R332 (5′-GGCCGGTAAAGGCCGAATAG-3′) were used. For nested PCR, primers R223 (5′-TCCCATCGCAACCTCGGTT-3′) and R333 (5′-AAAGCGGGCGCGGTGCTG-3′) were used. In the primary reaction, the expected PCR products of ≈603 bp were detected in 2 of 4 skin DNA specimens from patient 1 and 1 of 5 skin DNA specimens from patient 2 (Figure, panel A, lanes 2, 3, 9). In the nested reaction, the expected PCR products of ≈359 bp were seen in all 4 specimens from patient 1 and in 4 of 5 specimens from patient 2 (Figure, panel B, lanes 1–4, and 5, 6, 8, 9); some bands were faint. The nucleotide sequences (288 bp) of the nested PCR product of patient 1 were 100% identical to those of patient 2 and sequences of the SSU rRNA gene of *L. infantum* (IPT1 strain, used as a positive control), *L. infantum* (M81429), *L. donovani* (M80295), and *L. chagasi* (M81430).

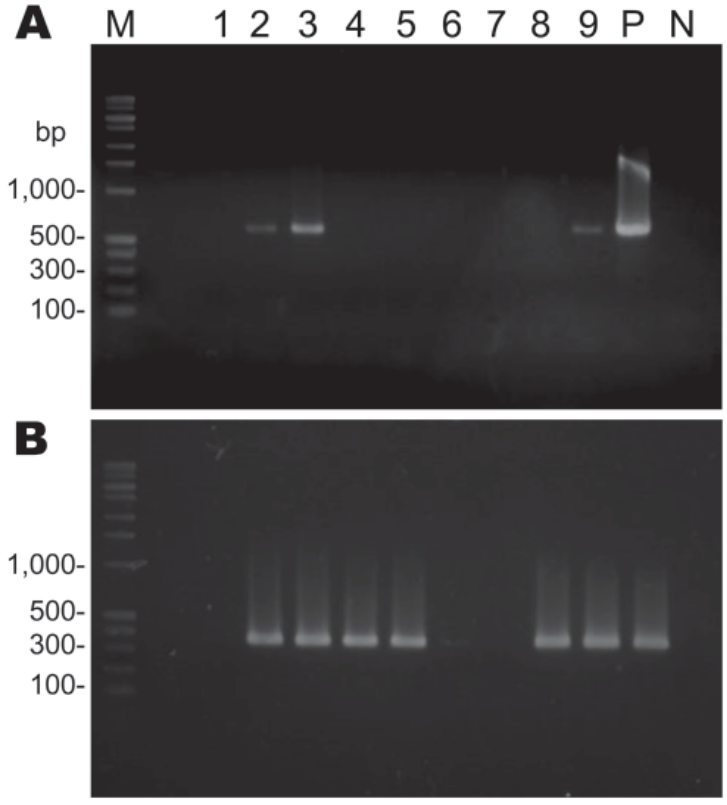

Figure. PCR amplification of the *Leishmania* spp.–specific small subunit rRNA gene from skin biopsy specimens from infected dogs, Japan. DNA samples (100–200 ng) were subjected to primary PCR (A), followed by nested PCR (B). Lanes 1–4, skin DNA samples from patient 1; lanes 5–9, skin DNA samples from patient 2; M, DNA molecular marker; P, positive control; N, negative control.

Global warming, which causes changes in the distribution of the sand fly vectors, and human-produced risk factors, such as travel, migration, and urbanization, may increase the incidence of leishmaniasis (*5*). Military mobility and operations are also a major risk factor for leishmaniasis in humans and canids (*6*). In Japan, of >300 kala-azar (visceral leishmaniasis) patients reported, 218 were soldiers who returned from the People's Republic of China before and after World War II (*7*). In the present study, 2 dogs infected with *L. infantum* had been brought to Japan from Italy by US military families.

Dog-to-dog transmission by direct contact with contaminated blood through biting may explain the recent outbreaks of leishmaniasis in foxhounds in North America (*8*). In Japan, although no sandfly species that could transmit leishmania have been reported (*7*), direct dog-to-dog transmission of leishmaniasis can occur. *Babesia gibsoni* infection is prevalent among fighting dogs in Japan, likely because of the transmission of infected erythrocytes through biting (*9*). Greater sharing of information and of diagnostic procedures is required in Japan because few medical and veterinary practitioners have experience with leishmaniasis patients.

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Serologic Evidence of Pandemic (H1N1) 2009 Infection in Dogs, Italy

To the Editor: Until recently, the general consensus has been that dogs are poorly susceptible to natural infection with influenza A viruses; however, since the recent upsurge of influenza A circulating subtypes H5N1 and H1N1 viruses, cases of natural infection in dogs have apparently increased. Thus, the role of these animals is being reconsidered in the transmission and spread of influenza viruses (*1*–*3*).

In April 2009, the most recent of the human influenza A pandemics, pandemic (H1N1) 2009, was detected in Mexico. The virus rapidly spread worldwide, within weeks of its first isolation. To date, pandemic (H1N1) 2009 has primarily infected humans, although transmission from infected humans to other animals, including pigs, turkeys, ferrets, cats, and dogs has been reported (*4*,*5*).

In Italy (population ≈58 million), the first human cases of pandemic (H1N1) 2009 were reported in May 2009; confirmed cases peaked during the second week of November 2009 (week 46) (*6*). As of May 9, 2010, Italy had recorded an estimated 5,582,000 cases of pandemic (H1N1) 2009. In Italy as well, the population has ≈7 million companion dogs and ≈7.5 million cats (*7*). Because of the close contact between persons and their companion animals, we initiated this serologic study to determine whether evidence of pandemic (H1N1) 2009 transmission could be found in companion animals in Italy.

We tested serum specimens from dogs (n = 964) and cats (n = 97), originally submitted to the Istituto Zooprofilattico Sperimentale delle Venezie in Legnaro, Italy, from October through December 2009 (weeks 41–53), for assessment of rabies vaccine efficacy. An average of 70 samples were tested per week; the highest number of samples (n = 106) was tested for week 51 and the lowest (n = 25) for week 53. Testing for antibody to influenza A nucleoprotein was performed by using a commercially available competitive ELISA (cELISA) (ID Screen Influenza A Antibody Competition Assay; ID Vet, Montpellier, France), according to the manufacturer's instructions. Previous work from our laboratory has assigned a sensitivity of 93.98% and specificity of 98.71% to this cELISA for the testing of canine serum samples (*8*). In total, 29 serum specimens tested at a 1:10 dilution, all from dogs, were positive after a second confirmatory screening. None of the 97 feline serum samples were positive by cELISA.

The cELISA-positive serum specimens were then treated with receptor-destroying enzyme (RDE; Sigma-Aldrich, St. Louis, MO, USA) (1 part serum: 3 parts RDE) for 16 h at 37°C, followed by heat inactivation at 56°C for 30 min. We then tested the specimens by the hemagglutination inhibition (HI) test against the pandemic virus A/Verona/Italy/2810/2009 (H1N1), A/swine/Italy/711/2006 (H1N1), and H3N8 (A/canine/Florida/2004) by using 0.5% chicken erythrocytes and standard methods (*9*). Seven serum samples (nos. 4410, 4438, 4444, 4460, 4517, 4520, 4681) were positive by HI

to A/Verona/Italy/2810/2009 (H1N1) with titers ranging from 16 to 256 (Table), but not for the other viruses, although the samples with the higher titers of 256 (nos. 4460 and 4681) against A/Verona/Italy/2810/2009 also cross-reacted with antigen A/swine/Italy/711/2006 (H1N1) (titers 16 and 32, respectively).

The HI-positive serum specimens were later tested in a microneutralization assay with A/Verona/Italy/2810/2009 (H1N1). Suppression of virus antigen expression was assessed by an ELISA assay as endpoint by using a slight modification of a previously described procedure (*10*). As can be seen from the Table, all 7 serum specimens positive by HI for A/Verona/Italy/2810/09 inhibited infection of MDCK cells by the same virus at dilutions of 1:160 or higher, confirming the presence of anti-H1 antibodies.

To summarize, in this study, 1,061 serum specimens from companion dogs and cats collected during the circulation peak of pandemic (H1N1) 2009 in Italy were screened with 7 (0.7%) of the canine serum specimens showing evidence of exposure to the virus. Notably, the positive samples were collected during the period (weeks 43–45) that almost coincided with the reported peak for human cases of pandemic (H1N1) 2009 in Italy (week 46). Totals of 69, 77, and 56 samples were collected during weeks 43, 44, and 45, respectively, which indicates that the sample group had no bias.

The data thus suggest that transmission occurred, most probably by aerosol or close contact, between pandemic (H1N1) 2009–infected owners and their pets during this peak period of mid-November 2009. How long these animals were infected, whether the infection had clinical manifestations, and whether the dogs were capable of transmitting the virus to other hosts are all questions that remain unanswered. However, on the basis of the low number of positive specimens identified in this study, it would be unrealistic to suggest that dogs are particularly susceptible to pandemic (H1N1) 2009. Nevertheless, as has been seen with infection of dogs with subtype H3N8, influenza A viruses are quite capable of evolving and becoming more host specific. This factor alone would justify the continued surveillance of influenza A viruses in domestic dogs.

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Table. Anagraphic data and HI and MN titers of canine serum specimens against A/Verona/Italy/2810/2009 (H1N1), 2009*

Sample no.	Species	Birth date	Sample origin, Italy	Coordinates	Sample date/wk	HI	MN
4410	English setter	2005 Jun 5	Mercato Saraceno	43°57′0″N,12°12′0″E	Nov 24/43	32	640
4438	Labrador retriever	2005 Oct10	Casale Monferrato	45°8′3″N, 8°27′30″E	Nov 26/44	32	160
4444	Chihuahua	2008 Apr 7	Mantaova	45°10′0″N, 10°48′0″E	Nov 30/44	16	160
4460	Small cross-breed	2005 Jan 20	Milano	45°27′50.98″N, 9°11′25.21″E	Nov 30/44	256	>2,560
4517	Italian Segugio	2007 Jul 4	Giussano	45°42′0″N, 9°13′0″E	Nov 30/44	128	640
4520	Shih tzu	2007 Dec 8	Peschiera del Garda	45°26′0″N, 10°41′0″E	Dec 2/46	64	320
4618	Yorkshire terrier	2008 Dec 21	Garda	45°34′0″N, 10°43′0″E	Dec 9/45	256	>2,560

*HI, hemagglutination inhibition; MN, microneutralization.

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Brucellosis Reactivation after 28 Years

To the Editor: Approximately 10% of patients with brucellosis experience a relapse, 90% of which occur within a year after discontinuation of antimicrobial drug therapy (*1*,*2*). Here we report a patient who had brucellosis in a disease-endemic area, immigrated to a brucellosis-free region, and experienced focal reactivation in her gallbladder 28 years later. To our knowledge, this interval is among the longest reported asymptomatic intervals between a first brucellosis episode and reactivation. The case suggests that physicians should not disregard remote histories of brucellosis and past residence in brucellosis-endemic areas when confronted with possible reactivation disease.

A woman, born in 1955, had prolonged fever without focal symptoms in 1981 and had received a diagnosis of brucellosis while living near Valencia, Spain. The brucellosis was attributed to an episode of eating unpasteurized cheese from the local dairy and was successfully treated. She immigrated to Switzerland in 1990 and was well until March 2009, when malaise and right upper quadrant pain developed, without fever. She was otherwise healthy and did not take any medication. Computed tomography (CT) scan showed a mass contiguous with the gallbladder, extending intrahepatically, with concentric calcifications and multiple gallstones (Figure). Gallbladder cancer was suspected, but when a laparotomy was performed, an acutely inflamed gallbladder with a surrounding inflammatory mass was found and excised. Gallbladder cultures on standard media (Columbia agar with 5% sheep's blood, chocolate agar, *Brucella* blood agar, and brain-heart infusion broth) were discarded when they remained sterile after 5 days of incubation. Histopathologic examination showed granulomatous cholecystitis, and the patient was referred for infectious disease consultation. Formalin-fixed gallbladder tissue was negative for *Mycobacterium tuberculosis* complex DNA but positive for *Brucella melitensis* by PCR (*3*). Blood cultures (BACTEC Plus Aerobic/F and Anaerobic/F [Becton Dickinson, Allschwil, Switzerland], incubated for 10 days) remained sterile. No *Brucella* DNA was detected in blood and serum (*1*,*2*), and a rose bengal serum agglutination test was negative for anti-*Brucella* antibodies. Because of the rarity of the manifestation (late reactivation) and location (gallbladder), plus a residual abscess shown on CT scan 8 weeks after surgery, prolonged treatment with doxycycline and rifampin was administered for 3 months, with gentamicin added during the initial 2 weeks (*4*). Nine months after antimicrobial drug therapy was discontinued, the patient remains well.

Switzerland reported the elimination of animal brucellosis in 1963 (*5*) and has officially been brucellosis-free since 1998, according to article 14.1.2. of the Terrestrial Animal Health Code (www.oie.int/eng/normes/mcode/en_chapitre_1.14.1.htm). The last case of *B. melitensis* infection in a sheep or goat in Switzerland was reported in 1985. The annual number of human brucellosis cases reported in

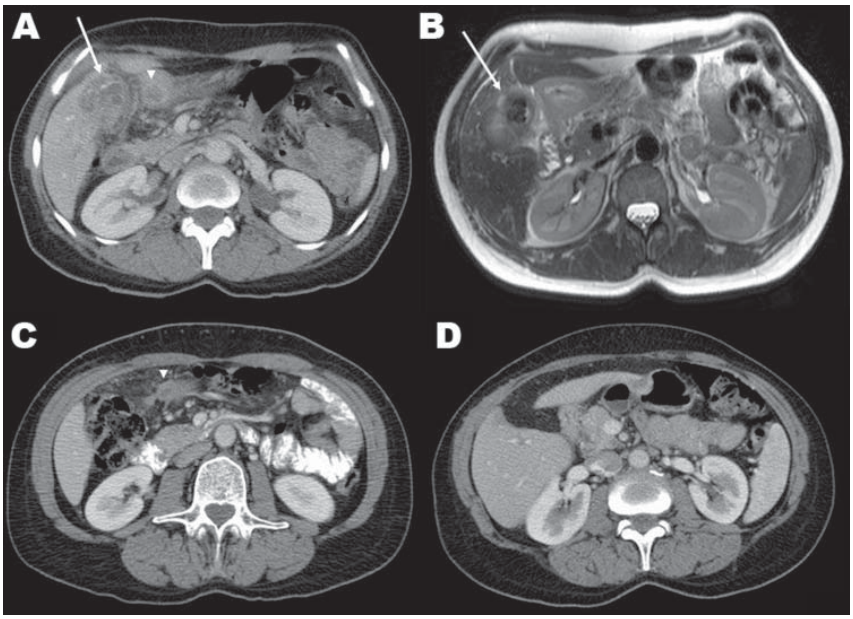

Figure. A) Contrast-enhanced computerized tomography (CT) scan showing a calcified gallbladder wall (arrow), a surrounding, calcified mass located peripherally in the liver, and an abscess in the adjacent fat tissue (arrowhead). B) T2-weighted axial magnetic resonance imaging shows multiple gallstones and a thickened gallbladder wall (arrow), inflammation and edema of the adjacent liver, fat tissue, and proximal duodenum. C) Eight weeks after cholecystectomy, contrast-enhanced CT shows a residual abscess in the adjacent fat tissue (arrowhead). D) Contrast-enhanced CT 5 months after cholecystectomy shows only minimal changes in the gallbladder bed and surrounding tissues, and no residual abscess.

Switzerland remains low but seems to be increasing; 3, 5, and 13 cases were recorded in 2007, 2008, and 2009, respectively (*6*).

Most of these cases were linked to international travel or ingestion of food products imported from disease-endemic areas, e.g., Turkey. Our patient traveled annually to her family's home town in Spain, but she vigorously denied exposure to any dairy or meat products imported from an area with known brucellosis endemicity or to any unpasteurized dairy products in general since she left Spain in 1990. Therefore, the most likely explanation was brucellosis reactivation, 28 years after the initial episode in Spain, where brucellosis was endemic. In the absence of a positive culture, brucellosis remains formally unproven. However, the granulomatous inflammation in the resected gallbladder tissue, the absence of another identified cause (e.g., tuberculosis), and the patient's clinical and radiologic response to specific anti-*Brucella* treatment argue against the positive PCR result being merely attributable to long-term, persistent, but clinically latent, brucellosis.

Ariza (*7*) and Diaz (*8*) have reported patients with chronic hepatosplenic brucellosis after remote episodes of brucellosis (2–40 years previously). Because these patients lived in disease-endemic areas, reinfection cannot be excluded. *Brucella* organisms persist intracellularly, and their DNA has been detected in the peripheral blood of asymptomatic patients, even years after the diagnosis of clinical brucellosis and its clinically successful cure. These facts suggest that the outcome of treated brucellosis may be a chronic, persistent, asymptomatic infection, rather than complete bacterial eradication (*9*).

The pathogenesis of unusually late brucellosis reactivation in our patient remains obscure. Reactivation has only rarely been linked to underlying illnesses or immunosuppression (*2*). Rare cases of reactivation have been reported during pregnancy. *Brucella* persistence has been recorded inside calcified lesions in chronic hepatosplenic brucellosis (*7*), and adherence to foreign bodies such as joint prostheses or prosthetic heart valves and other cardiac devices may occur, perhaps in association with episodes of transient *Brucella* bacteremia (*10*). Unlike the situation with typhoid fever, no evidence links the presence of gallstones to the persistence of *Brucella* organisms in humans.

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In Memoriam: Jocelyn Anne Rankin (1946–2010)

Jocelyn Anne Rankin

Jocelyn Anne Rankin, PhD, chief of the Information Center at the Centers for Disease Control and Prevention (CDC), died on September 19, 2010, at age 63 in her Florida home. Similar to the health professionals in the organization Médecins Sans Frontières, Jocelyn was a humanitarian and a librarian sans frontières. Although some make a difference by what they do, others, like Jocelyn, also make a difference by how they do it. She was a true leader and a mentor to many librarians (and not only to librarians but also to scientists and public health professionals) throughout the nation. These colleagues will remain profoundly grateful for her contributions to the world of information science, and will, just as deeply, cherish memories of how she made her professional mark—quietly, respectfully, and selflessly, yet with clear vision, determination, and passion.

Born in Raleigh, North Carolina, USA, Dr Rankin achieved an education without borders as well: high school in Germany, a diploma in liberal arts from the American College in Paris, a BA cum laude in English from Hollins College in Virginia, an MLn in librarianship from Emory University in Atlanta, and a PhD in educational leadership from Georgia State University in Atlanta. Dr Rankin's career began at the Medical College of Georgia, Augusta, and continued at Georgetown University Medical Center, Washington, DC, and at Mercer University School of Medicine, Macon, Georgia. She designed and led the Georgia Interactive Network for Medical Information, the oldest statewide network of its kind in the nation. At CDC, she led in the creation of a state-of-the-art information center; guided implementation of an integrated, electronic information delivery system; helped create the US Department of Health and Human Services Library Consortium; and most recently, helped build the foundation for CDC's Science Clips, a weekly digest of selected news pertinent to the public health community. Dr Rankin's contribution as book review editor for Emerging Infectious Diseases was also highly valued.

She leaves behind many who feel privileged to have known her. We offer our condolences to her husband, William Rankin; daughters, Stephanie Smith and Kimberley Macdonald; son, William Rankin III; brother, Howell Cobb; and 2 grandchildren.

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Rinderpest and Peste des Petits Ruminants: Virus Plagues of Large and Small Ruminants

Tom Barrett, Paul-Pierre Pastoret, and William P. Taylor, editors

Academic Press, Elsevier, London, UK, 2005
ISBN-13: 978-0120883851
Pages: 341; Price: US $115.00

In Rinderpest and Peste des Petits Ruminants: Virus Plagues of Large and Small Ruminants, Tom Barrett (now deseased), of the Institute for Animal Health, Pirbright Laboratory, Surrey, UK, and his co-editors have subtly presented the main developments in the quest to conquer these diseases. The instructive text, which touches on the key dynamics of both deadly diseases, incorporates considerable historical detail, infection biology, and information on disease diagnosis, control, and eradication. This book consists of high quality scientific and historical research based on the editors' experience with morbilliviruses and collaborations with other researchers worldwide. In total, 22 scientists have contributed their expertise on various infectious diseases to the monograph's 17 chapters.

Throughout, the contributors have tried to maintain an appropriate balance between peste des petits ruminants (PPR) and rinderpest (RP). This hypothesis-based balancing act is important to understand PPR. A future significance of PPR can be realized by the phrase stated by the editors, "If rinderpest becomes a disease of the past, PPR is certainly a disease of the future." The book starts with an historical account of the RP and PPR diseases accompanied by photographs from the 18th century. These photographs are detailed, illustrative, and fascinating. Of particular interest are a condolence letter, written by Emile Roux from Institute Pasteur to the widow of Joseph Hamoir, with whom Emile Roux worked on rinderpest, and a group photo, including Robert Koch, of his visit to the Imperial Veterinary Laboratory (currently Indian Veterinary Research Institute, Mukteshwar, India) in 1897 where he conducted experiments to immunize cattle with the bile taken from an animal that had succumbed in a virulent outbreak of rinderpest.

The book focuses on the following issues: relative position of each member in the genera, comparative molecular biology, pathophysiology of the infectious diseases, global epidemiologic patterns, contribution of countries in the eradication of the disease under the Pan African Rinderpest Campaign Programme and the Pan African Programme for the Control of Epizootics, viral immune suppression, and molecular diagnostic approaches being developed. For all these issues, the importance for clinicians of accurate diagnosis and management and prevention of infectious diseases is highlighted. Because of the current sensitivity about an emergence of PPR and the successful Global Rinderpest Eradication Programme, the contributors believe that PPR can be controlled similarly and its spread prevented. Thus, the last 7 chapters emphasize the traditional prophylactic measures, potency of vaccines and possibility of vaccine use, the history of vaccine improvement, recent advances in vaccine development, implementation of international control campaigns for the eradication of RP and PPR by using vaccine, and a brief overview of the pathogenesis and eradication of measles virus. Finally, the editors assess the real cost and benefits of the Global Rinderpest Eradication Programme campaign and predict that the world will soon be free of RP, at comparatively little cost.

Although the book discusses many aspects of the current situation, such as epidemiological distribution, progress in vaccine development, and advances in the diagnosis of PPR diagnostic procedures, information regarding the most recent developments is lacking; a few chapters were written in ≈2000 before the increase in PPR research. Still, I consider this to be the only book that comprehensively describes PPR. It is suitable not only for academics and researchers, but also for virologists, infectious disease specialists, vaccine researchers, and clinicians. Moreover, this book provides appropriate scientific source material suitable for undergraduate and graduate studies.

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Correction—Vol. 16, No. 8
Table 2 in the article Novel *Mycobacterium tuberculosis* Complex Pathogen, *M. mungi* (K.A. Alexander et al.) contained several errors related to scoring of mycobacterial interspersed repetitive unit–variable number tandem repeats of selected isolates. The article has been corrected online (http://www.cdc.gov/eid/content/16/8/1296.htm).

Correction—Vol. 16, No. 9
The name of author Beth Feingold was misspelled in the letter New Infectious Diseases and Industrial Food Animal Production (E. Silbergeld et al.). The article has been corrected online (http://www.cdc.gov/eid/content/16/9/1503.htm).

Jacopo Bassano (c. 1510–1592) *Allegory of Water* (16th century) Oil on canvas (139.7 cm × 180.3 cm) Bequest of John Ringling, 1936, Collection of The John and Mable Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

Abundant Harvest and Fishing for Trouble

Polyxeni Potter

"Ah, Jacopo, if you had my drawing and I had your color, I would defy the devil himself to enable Titian, Raphael, and the rest to make any show beside us," exclaimed Tintoretto as he discussed contemporary painting with his friend Jacopo da Ponte, known as Bassano for his hometown Bassano del Grappa, near Venice. At the foot of the Alps and surrounded by chestnut woods, this small town once on the crossroads between Italy and Germany, held a special fascination, compounded over the years by aging frescoed walls and other relics of a vibrant arts community preferred by this artist over the opulent court of Emperor Rudolph, where he was invited to live.

Except for traveling to Venice to study under Veronese and later to paint in the Ducal Palace, Bassano lived and worked in Bassano del Grappa all his life, well-loved, especially by the poorest residents, who benefited from his generosity. This attachment to the local scene was perhaps why he was slighted by Vasari, who in his Lives of the Artists, only mentioned him in passing. His paintings were "very beautiful," the famed artist and biographer deigned, "dispersed throughout Venice, and they are held in high esteem—especially the little works with animals of all kinds." Ever so briefly, Vasari captured a specialty of Bassano's thriving Venetian workshop: biblical themes treated as genre in domestic scenes with animals.

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Least known of the 16th-century masters, Jacopo Bassano was the son of one artist and the father of four. He received his early lessons at home from his father, Francesco the Elder, a provincial painter, and retained some attachment to these humble roots, despite his grasp of art developments of his day, among them mannerism and the engravings of Dürer and Parmigianino. He led the Bassano School—a family of artists, including in addition to his sons, his son-in-law, grandson, and great-grandson—with a distinctive style and considerable output. So many used the name Bassano that distinguishing the work of individual members and admirers became a challenge for historians.

"During my voyage to Bassano," wrote Giovanni Tiepolo in the 18th century about *St. Valentine Baptizing St. Lucia*, "I saw a miracle—a black cloak which seemed to be pure white." Tiepolo was taken by the luminosity of color, another hallmark of Bassano's work. Using fewer colors than his contemporaries and keeping them pure, he made them shine "like a beetle's wing," and he created light by deepening the shadows for contrast. His mature style, in the tradition of the High Renaissance in Venice and northern Italy, was influenced by Titian, his older contemporary and friend, whose portrait featured in *Purification of the Temple*. Great mannerist El Greco, who was in Venice in 1567 to 1570, studied the work of Bassano, as well as Tintoretto and Veronese.

Well-known in Europe during his lifetime, Bassano embraced change. In his later years, he expanded on Titian's influence to create his personal bent—a rustic brushwork and dramatically lit night scenes. In painting *An Al-*

legory of Water, on this month's cover, as was the practice in the Bassano workshop, Jacopo was probably assisted by one of his sons, likely Francesco. This type of pastoral scene, filled with still-life details and figures, usually animals around peasants engaged in everyday activities, was a novelty invented by the father–son Bassano team.

Part of a series, this painting masterfully invokes a popular subject, the physical elements water, fire, earth, and air, believed since antiquity to make up the world. Symbolism associated with them and their correspondence to internal human states and emotions, the humors, held great fascination then, as they do today, even though science has added many more elements to the original four.

Allegories, a form of metaphor common in literary works, are also an artistic device used for visual symbolic representation. By 1600, Venice was a major center of the print industry, and the rediscovery of classical texts provided an inexhaustible supply of subjects from ancient philosophy, history, and literature. *An Allegory of Water* shows Neptune, none other than Poseidon, in his chariot, gliding above the proceedings—a take from Greek mythology used to elucidate the virtues of one of the elements.

Water is the element associated with unconsciousness; darkness of night; and the moon's monthly cycles, which control ocean tides. This element's unique property is that, although it does not have its own specific form, it can take that of what surrounds it. Through its humidity and fluidity, water symbolizes the dissolution of form into a mass of possibilities.

Bassano uses a fish market to show the abundance that comes from the sea. The market is being set up on the waterfront just before dawn, an opportunity for a nocturnal presentation. The vendors, expertly highlighted, display a variety of seafood, while other activities involving water are taking place around them. On the left, two men are negotiating the cost of fresh catch. On the right, a woman is drinking at the well; a second one carries a bucket; while two more, near the water banks, are laundering clothes. In the background, a boat takes off.

The iconography, a mélange of realistic, biblical, and mythologic subjects, is animated by the give and take of ordinary people. The rich harvest of fish and brisk activity so early in the day, the backdrop of classical ruins, and divine presence signal that all is well in this community so heavily reliant on water. As long as Neptune is not angry and the waters flow calm, the abundant catch will feed the multitudes.

So it was, 400 years before overfishing, globalization, and aquaculture altered the bounties of the sea. Bassano's water idyll and its harvest benefiting the local populace is not just a Renaissance tribute to the physical elements and their allegorical presence in human lives. It is also a record of a local industry long gone. Even in Bassano's day, "local" had expanded its reach. His work was popular well beyond his hometown, certainly in Venice. And the Pearl of the Adriatic, a cluster of islands at the head of the namesake sea, was then still approachable only by water. In the 15th century, the city expanded her territory into the mainland, the *terraferma*, which included Bassano del Grappa, a source of food supply.

Tiny Venice and its astonishing success due largely to the power of water and its capacity not only to provide food but to move people and things foreshadowed today's aquatic and periaquatic regions also heavily reliant on water harvest for ever-growing markets. Demand now as then, far exceeds local supply, prompting lucrative aquaculture in many parts of the world. But like water itself, water management is formless and dependent on local parameters.

With Neptune out of the picture, climatic and biologic tempests—among them emerging infectious diseases of fish, such as cyprinid herpesvirus 3, a highly contagious pathogen causing severe financial losses in koi and common carp culture industries worldwide—as well as foodborne and occupational zoonoses, are unleashed. Moreover, manipulation of local water resources can expose larger groups to higher levels of contagion, as in northern Vietnam, where fish-borne zoonotic trematodes have infected an estimated one million residents of the Red River Delta. Research into the basic biology of cyprinid herpesvirus 3 may lead to new treatments and control strategies for a full range of pathogenic herpesviruses. Public health research that identifies reservoirs and mechanisms of transmission of zoonotic trematodes provides the management framework for effective and efficient interventions without which fishing for the masses can overflow the boundaries of food safety.

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Sherif Zaki
Dante Zarlenga
Marcus Zervos
Hongxin Zhao
Xiao-Nong Zhou
Matteo Zignol
Marya D. Zilberberg
James Zingeser
Alice Zwerling

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Foodborne Illness Acquired in the United States—Major Pathogens

Foodborne Illness Acquired in the United States—Unspecified Agents

Public Health Implications of Cysticercosis Acquired in the United States

Molecular Typing of Transmissible Spongiform Encephalopathy in Small Ruminants, France, 2002–2009

Seroprevalence of African Swine Fever, Senegal, 2006

Long-term Maintenance and Re-emergence of Dengue Virus Type 2, Puerto Rico

Completeness of Communicable Disease Reporting, North Carolina, 1995–1997 and 2000–2006

Hepatitis E Virus Infection without Reactivation in Solid-Organ Transplant Recipients, France

Concurrent Conditions and Human Listeriosis, England and Wales, 1999–2009

European Babesiosis in Immunocompetent Patients

Experimental Approach to Reduce Prevalence of Raccoon Roundworm

Antimicrobial Drug–Resistant *Streptococcus pneumoniae* in Children, Palestinian Territories, West Bank

Emergence of *Rickettsia africae* in Oceania

Enterovirus 75 Encephalitis in Children, India

Echinostoma revolutum Infection in Schoolchildren, Pursat Area, Cambodia

Foreign Travel and Decreased Ciprofloxacin Susceptibility in *Salmonella enterica* Infections

New Delhi Metallo-β-Lactamase Infection, Canada

CTX-M-producing Isolates of Non-Typhi *Salmonella enterica* from Humans, United States

Streptococcus pneumoniae in the Urinary Tract

Complete list of articles in the Janusry 2011 issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

December 14–15, 2010
Institute of Medicine Meeting
Fungal Diseases–An Emerging Challenge to Human, Animal, and Plant Health
Washington, DC, USA
http://www.iom.edu/Activities/PublicHealth/MicrobialThreats/2010-DEC-14.aspx

2011
February 27–March 2, 2011
CROI 2011: 18th Conference on Retroviruses and Opportunistic Infections
Boston, Massachusetts, USA
http://www.retroconference.org/2011

April 1–4, 2011
Annual Scientific Meeting of The Society for Healthcare Epidemiology of America (SHEA) 2011
Dallas, Texas, USA
http://www.shea2011.com

2012
June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Pandemic (H1N1) 2009 Infection in Patients with Hematologic Malignancy

CME Questions

1. A 43-year-old woman who recently began induction chemotherapy for acute myelocytic leukemia presents with a 3-day history of worsening symptoms, and is concerned about possible influenza. Which of the following symptoms would be most indicative of H1N1 influenza infection?

A. Nausea and vomiting
B. Cough and fever
C. Diarrhea
D. Dyspnea

2. You suspect influenza infection, and perform a nasopharyngeal wash for analysis. You consider initiating antiviral therapy. Which of the following statements about antiviral therapy is most accurate?

A. Most patients were treated within 48 hours of the onset of symptoms
B. Many patients received broad-spectrum antibiotics, but no concomitant bacterial infections were found
C. No patients recovered from H1N1 infection without antiviral therapy
D. High-dose oseltamivir was more effective than standard-dose therapy

3. The patient is diagnosed with lower respiratory tract infection (LRTI) and admitted into the hospital. Which of the following statements about patients with LRTIs is most accurate?

A. All patients with LRTIs were hospitalized
B. No patients with LRTIs were admitted to the intensive care unit
C. Only focal consolidations were seen on chest CT scans of LRTI patients
D. Patient age had no effect on the risk for LRTIs

4. Which of the following treatments is appropriate for this patient?

A. Zanamivir treatment for 7 days because of possible resistance to oseltamivir
B. Standard-dose oseltamivir for 7 days to avoid potential adverse events
C. High-dose oseltamivir for 5 days to limit antiviral exposure
D. Weekly serial viral polymerase chain reaction (PCR) testing until there is a negative result

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

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, South Korea

CME Questions

1. Which of the following best describes the pattern of oseltamivir resistance seen with pandemic (H1N1) 2009 virus in South Korea?

A. Resistance was seen synchronous with amantadine resistance
B. Resistance was seen in the community
C. Resistance was seen in hospitalized patients
D. Resistance was seen only in immunosuppressed patients

2. A 45-year-old man from South Korea with pandemic (H1N1) 2009 infection is found to have resistance to oseltamivir. Which of the following is the most likely outcome of this resistance?

A. Increased transmission of virus to staff and family
B. Higher risk for resistance in the community
C. Increased length of hospital stay
D. None of the above

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

Director, Division of Viral Diseases
National Center for Immunization and Respiratory Diseases
Office of Infectious Diseases
Centers for Disease Control and Prevention
Department of Health and Human Services
Opens Friday, October 29, 2010 to Monday, November 29, 2010

The National Center for Immunization and Respiratory Diseases (NCIRD) is seeking an exceptional candidate to serve as the Director of its Division of Viral Diseases in Atlanta, GA. The Division has responsibility for CDC's surveillance, epidemiology, research, domestic vaccine policy, and laboratory activities related to the following viral vaccine–preventable diseases: measles, mumps, poliovirus, rotavirus, rubella, varicella, and herpes zoster. DVD also manages CDC's activities for non–vaccine-preventable viral diseases, including non-polio picornaviruses, such as enteroviruses, parechoviruses, and cardioviruses; the non-rotavirus causes of viral gastroenteritis, such as norovirus, astrovirus, and picobirnavirus; the respiratory viral diseases other than influenza, such as respiratory syncytial virus, the parainfluenza viruses, the coronaviruses, including SARS coronavirus, rhinoviruses, and adenoviruses; the parvoviruses; the molecular virology of henipaviruses; and the herpes viruses, including cytomegalovirus, human herpes viruses 6–8, Epstein-Barr virus, and others (not including human herpes viruses 1 and 2). The Division has just over 200 professional and support staff and a core budget of about $25 million that is supplemented by various initiative and project-specific funds. Division activities include CDC's studies to characterize the clinical and epidemiologic features of infection and burden of disease, studies of vaccine efficacy and impact on disease, outbreak investigations, development and evaluation of novel diagnostics, studies of immunity and disease pathogenesis, and novel pathogen discovery. The Division has extensive collaborative, cooperative, and consultative relationships with other programs at CDC, state and local health departments, academic institutions, other federal agencies, and the World Health Organization and other international agencies. Division laboratories serve as designated national and international reference centers for various viral diseases.

Qualification Requirements:
Applicants must possess an M.D., Ph.D., or other earned doctorate in disease prevention or related health sciences, public health, epidemiology, microbiology, or molecular biology and must demonstrate (1) High degree of public health prominence and expertise, and a distinguished record of accomplishments in the field of public health surveillance and epidemiology, such that the individual can readily command the respect of the national and international public health communities, and stimulate their interest in infectious disease prevention and control strategies at CDC; (2) Strong leadership experience in creating a vision and setting direction in a scientific organization; (3) Leadership experience in directing and managing a public health research organization, including establishing strategic plans and policies, developing and presenting budgets, evaluating program accomplishments, and building and maintaining a diverse staff; (4) Experience in developing and implementing national, state, or local policies, programs and research related to field of public health surveillance and epidemiology in the area of viral infectious disease prevention; (5) Experience that indicates the ability to deal effectively with high-level government officials, the scientific and academic communities, national or international medical and health-related organizations, diverse community and non-governmental groups, media, and the public at large. A supervisory probationary period will be required.

How to Apply: **Title 42 applicants** should submit one of the following: (1) An OF-612 (Optional Application for Federal Employment), or an SF-171 (Application for Federal Employment); (2) Curriculum Vitae or Resume; (3) or any other application form you choose. Applications should be submitted by E-mail to: seniorpositions@cdc.gov. Reference Vacancy Announcement #, **AD10-11-002** at USAJOBS.GOV for additional instructions.

Title 5 applicants should (1) Register at USAJOBS; (2) Respond to the on-line application and assessment question referencing the appropriate Vacancy Announcement #s: **HHS-CDC-D3-2011-0051/HHS-CDC-T3-2011-0078 for Medical Officer or HHS-CDC-D3-2011-0045/ HHS-CDC-T3-2011-0053 for Supervisory Health Scientist**; (3) Fax required documentations (if applicable).

Executive level compensation package is commensurate with qualifications and experience. Moving expenses are authorized. CDC is an Equal Opportunity Employer. CDC offers a smoke-free environment. For further information, prospective applicants are encouraged to contact Robert Pinner, Chair, Division of Viral Diseases Search Committee, at rwp1@cdc.gov or 404 639 7710.
APPLICATIONS MUST BE RECEIVED NO LATER THAN **Monday, November 29, 2010**

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

November 2010

Enteric Infections

National Gallery in Prague, Czech Republic

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 some 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 Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

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, and figure legends. Appendix materials and figures should be in separate files.

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. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

Videos. High-quality video files are accepted in the following formats: AVI, MOV, MPG, MPEG, and WMV. The files should be no longer than 5 minutes in length.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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 abstract, figures, or tables. Include biographical sketch.

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. Include biographical sketch.

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. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

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.

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Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

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. Email to eideditor@cdc.gov.