EMERGING INFECTIOUS DISEASES®

Vector-borne Infections

February 2013

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

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 Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text.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 in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

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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.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. 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 (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

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 of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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On the Cover

Georges de La Tour (1593–1652)

***La Femme à la puce (The Flea Catcher)* (1638)**

Oil on canvas (90 cm x 120 cm)

Musée Lorrain, Nancy.
Photo P. Mignot

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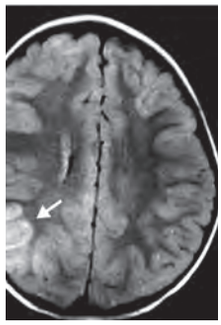

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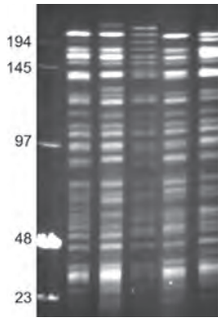

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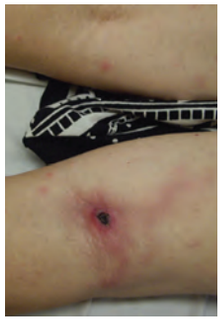

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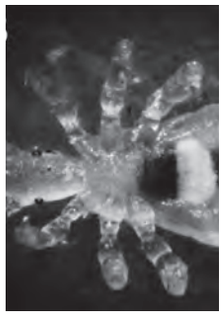
Letters



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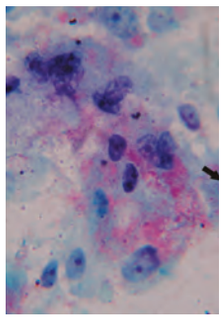

Book Review

About the Cover

Etymologia

Rift Valley Fever and a New Paradigm of Research and Development for Zoonotic Disease Control

Osman Dar, Sabrina McIntyre, Sue Hogarth, and David Heymann

Although Rift Valley fever is a disease that, through its wider societal effects, disproportionately affects vulnerable communities with poor resilience to economic and environmental challenge, Rift Valley fever virus has since its discovery in 1931 been neglected by major global donors and disease control programs. We describe recent outbreaks affecting humans and animals and discuss the serious socioeconomic effects on the communities affected and the slow pace of development of new vaccines. We also discuss the mixed global response, which has largely been fueled by the classification of the virus as a potential bioterrorism agent and its potential to migrate beyond its traditional eastern African boundaries. We argue for a refocus of strategy with increased global collaboration and a greater sense of urgency and investment that focuses on an equity-based approach in which funding and research are prioritized by need, inspired by principles of equity and social justice.

Since Rift Valley fever virus (RVFV) was first identified in 1931, after an investigation of an epizootic among sheep on a farm in the Great Rift Valley of Kenya, the understanding of this zoonotic disease has grown considerably (1). With the rapid progress of molecular biology and genetic techniques in recent years, studies of prevailing circulating variants of RVFV have pointed to a recent common ancestor that existed during 1880–1890. This finding lends weight to the predominant hypothesis on the origins of human outbreaks of Rift Valley fever, which suggests that the development of industrialized agriculture systems and the introduction of highly susceptible European breeds of livestock into East Africa during the colonial era led to amplification of the virus in animal and arthropod vectors and may have been responsible for the establishment of the disease (2).

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Although the disease disproportionately affects vulnerable communities with low resilience to economic and environmental challenges, RVF has remained largely neglected by major global donors and disease control programs. With high numbers of competent vector species present in disease-free regions, the intensification of international trade in live animals, and the uncertain effects of climate change, RVF is now considered a major challenge in global zoonotic disease control (*2*).

Recent Outbreaks

The potential of RVFV to migrate was established after large outbreaks of RVF occurred among animals and humans in Egypt in 1977, in other geographic zones of Africa, and then outside the African continent in Saudi Arabia and Yemen in 2000 (*3*,*4*). The Figure illustrates how the disease has traveled away from its original identified location in humans and animals.

The Table further demonstrates the spread of the disease; 7 of 9 major outbreaks in the past 15 years resulted in human cases outside the Rift Valley region in East Africa. The Table also highlights the difficulty of developing adequate surveillance systems and therefore the difficulty

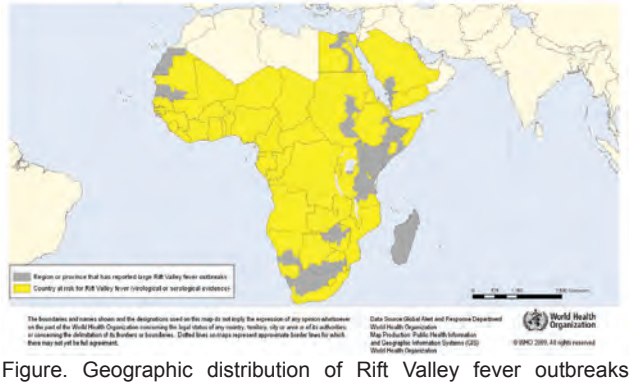

Figure. Geographic distribution of Rift Valley fever outbreaks in animals and humans, 1997–2010 (5). Source: World Health Organization (full map available online, wwwnc.cdc.gov/EID/article/19/2/12-0941-F1.htm).

of accurately estimating morbidity and mortality rates for human populations in resource-poor settings. In the 5 outbreaks for which estimated numbers of human cases have been published, ≈339,000 infections are believed to have occurred. In the 4 outbreaks for which estimated and reported cases are documented, numbers of estimated cases are 78× higher than numbers of reported cases (Table). This difference between estimated and known numbers of cases highlights the inherent complexity of managing outbreaks, monitoring their spread, and mitigating their effects.

Socioeconomic Effects

There is a paucity of studies that have examined the socioeconomic effects of past outbreaks of RVFV, which reflects a lack of research focus on the broader social effects of the disease. One study that did examine the socioeconomic effects of the 2006/2007 RVFV outbreak in Kenya highlighted the concern that the outbreak had tended to disproportionately affect impoverished pastoralist communities, with those in the North Eastern Province of Kenya being hardest hit (*25*,*26*). The lack of understanding of the epidemiology and pathophysiology of RVFV, poor compliance with international health and safety standards by animal exporters, and the limited options for prevention and treatment have periodically led to summary bans of imports of livestock from disease-endemic areas.

The ban of livestock imports to the Middle East from East Africa, instituted after the 1997/1998 RVFV outbreak in Kenya and Somalia, particularly affected the export trade out of Somalia. The ban was variably enforced by several Middle Eastern countries but most notably by Saudi Arabia, which imports large numbers of ruminants for the annual Hajj pilgrimage. In 1997, the year before the onset of the ban, 2.8 million live animals were exported from the Somaliland port of Berbera, making it the single biggest exporting port for ruminants in the world that year. With the livestock trade accounting for 65% of gross domestic product in Somaliland, the export ban had a devastating effect on a region already suffering in the grip of a protracted civil war (*27*). Estimated losses from export sales out of Somaliland alone for the first 16 months of the ban from February 1998 to May 1999 totaled $109 million (*28*). By the time the ban on animal imports was lifted by Saudi Arabia in 2009, this drought- and war-affected region of East Africa had already endured many years of lost income because of prevailing fears concerning RVFV.

Table. Major Rift Valley fever outbreaks and reported cases among humans, 1997–2010*

Outbreak dates	Geographic distribution	Estimated no. cases	No. cases reported	No. deaths confirmed	Precipitation	Control measures
1997 Dec–1998 Jan	Kenya, Somalia, Tanzania	89,000	No documented reports	478	Heavy rainfall and flooding	Active surveillance; safety education; distribution of masks, gloves; slaughterhouse monitoring
1998 Sep–Dec	Mauritania	No documented estimates	300–400	6	Heavy rainfall	Active surveillance; public awareness/education; mosquito control; animal movement control
2000 Aug–2001 Sep	Saudi Arabia, Yemen	20,000†	886	123	Rainfall; virus introduction	Active surveillance; public awareness/education; mosquito control; animal movement control
2006 Nov–2007 Mar	Kenya Somalia Tanzania	75,000 30,000 40,000	700 114 264	158 51 109	Heavy rainfall and flooding	Active surveillance; public awareness/education; mosquito control; ban on livestock slaughtering; closure of livestock market; vaccination Jan 2007
2007 Sep–2008 Jan	Sudan	75,000	747	230	Heavy rainfall and flooding	Active surveillance; public awareness/education; targeted vaccination; ban of livestock imports by Saudi Arabia and Egypt
2008 Jan–Jun	Madagascar	10,000	476	19	Heavy rainfall	Active surveillance; public awareness; mosquito control; animal movement control
2008 Oct–2009 May	Madagascar	No documented estimates	236	7	Heavy rainfall	Active surveillance; public awareness; mosquito control; animal movement control
2010 Feb–2010 May	South Africa	No documented estimates	242	26	Sustained heavy rains	Public awareness/education; mosquito control
2010 Sep–2010 Dec	Mauritania	No documented estimates	63	13	Heavy rainfall	Public awareness; mosquito control; animal movement control

*Sources: (*6*–*24*). FAO, Food and Agriculture Organization of the United Nations.
†Data available for Jizan region only.

Vaccine Development and Production

The slow pace of development of new vaccines (online Technical Appendix; wwwnc.cdc.gov/EID/article/19/2/12-0941-Techapp1.pdf) and diagnostic kits for RVFV and the limited supplies and relatively high cost of those currently available mean that there is a chronic worldwide shortage and lack of availability in areas where they are most needed. To address these problems, a "pull" strategy has been suggested by the Food and Agriculture Organization of the United Nations for the development of new RVFV vaccines. In this model, governments commit themselves to buy, at an agreed-upon price, whichever vaccine meets predefined requirements, thus giving an incentive to pharmaceutical companies to pursue the development of the most promising vaccine candidate. However, this strategy does not seem to be having much effect in the short term; the current financial crisis is limiting the purchasing power of national governments (*29*).

Before modern safety standards were instituted in laboratories, RVFV was regularly transmitted between laboratory staff; 47 cases were documented worldwide (*30*,*31*). International regulations for working with the live virus, and particularly for the production of vaccine and diagnostic test kits, typically require biosafety level 3 (BSL-3) laboratory facilities as a minimum and enhanced BSL-3 Ag/ABSL-3 (with many of the features of a BSL-4 laboratory) for working with live RVFV and loose-housed animals (*30*).

Fortunately, with the advent of recombinant genetic technology and the development of reverse transcription PCR techniques obviating the need to handle and store live virus, new vaccines and diagnostic tests in development can now be produced in laboratories of lower BSL (*29*,*32*). However, for the standard techniques that do involve storage and handling of live virus, because no reported laboratory infections have occurred since modern standard infection control procedures were introduced in the early 1980s (*33*), the case could be made for lowering currently prescribed BSL requirements. If laboratory workers handling live virus in these settings are all vaccinated, the laboratories required could possibly be reduced to BSL-2 with controlled access in disease-endemic countries, and to BSL-2 with controlled access and additional enhancements for working with animals in non–disease-endemic countries. Such a change could lower global production costs of vaccines and diagnostic tests and increase their accessibility by communities most affected by RVFV.

Global Interest, Challenges, and Cooperation

Interest in RVFV and investment in its control were only substantially increased among the global health research and policy community after greater awareness of its potential to migrate beyond its traditional East African boundaries was noted. However, the recognition that much of the industrialized world has animals and arthropod vectors capable of transmitting the virus seems to have focused and accelerated efforts to develop improved tools for outbreak forecasting, monitoring, diagnosis, and prevention.

In more recent years, the classification of the virus as a potential bioterrorism/agroterrorism agent has also helped spur investment and activity, particularly in the area of vaccine development and diagnostics (*34*). Although this theoretical risk has contributed to increased funding over the past few decades, most notably from military sources such as the US Army Medical Research Institute of Infectious Diseases, this concern might also have acted as an impediment to the collaborative aspects of this high-quality work, with research being restricted to fewer, more expensive laboratories (*35*).

Growing restrictions stemming from biosecurity concerns now affect research activity across a range of infectious diseases and have most recently been highlighted by concerns over the publication of research into the production of genetically engineered variants of the influenza A subtype H5N1 virus (*36*). Limiting the dissemination of such research findings could, in any case, curtail technology transfer crucial to studying viruses such as RVFV and could theoretically cause expert technical knowledge and skills to be less accessible. This possibility not only has the potential to delay progress in developing new treatments and vaccines but could also increase their costs by limiting where they could be produced, resulting in decreased production capacity and competition.

Increased sales costs of vaccines have a variety of negative consequences; in particular, this increase could put at risk well-established mechanisms of international cooperation in global infectious disease surveillance. This risk was dramatically highlighted in 2006 and 2007 when Indonesia refused to share samples of influenza subtype H5N1 isolates with the World Health Organization. The event caused a risk to global health and occurred in direct protest to the inequitable sharing of virus samples and vaccine development technology (*37*).

Despite some of these challenges, some positive developments have occurred in global collaborative efforts for controlling zoonotic diseases, including RVFV. These include initiatives like the One Health (*38*) approach of integrating animal and human health challenges and the closer integration of multilateral agencies such as the World Organisation for Animal Health, the Food and Agriculture Organization of the United Nations, and the World Health Organization. These efforts have already resulted in improved outbreak forecasting and surveillance of RVFV in humans and animals, facilitated by the development of initiatives such as the Global Early Warning

System (*39*,*40*). In addition, the rapid increase in socioeconomic interest and investment in RVFV-affected regions of Africa from emerging economies such as the People's Republic of China, and Middle Eastern countries such as Saudi Arabia, provides an opportunity for their increased involvement in, and funding of, RVFV control. Countries benefiting from this socioeconomic interest and investment should develop the necessary information base and negotiating skill to successfully ensure that funds are channeled to such opportunities.

Conclusion

In recent years, the perceived risk of RVFV becoming established in Europe and North America, and the theoretical risk of it being used as a bioterrorism agent, has brought a welcomed increase in investment to combat the disease yet has skewed priority areas of focus for that investment. The ideal that should be adopted is a more equity-based approach in which funding and research are prioritized on a needs-identified basis for the aid of those most disadvantaged in the global community. This approach would concentrate efforts on those interventions that most positively affect these vulnerable communities and, in addition, prevent or minimize the spread of the disease to previously non–disease-endemic high-income countries.

Such an approach would ensure research and policy emphasis on the socioeconomic effects of RVFV outbreaks. Interventions could then address international trade policies and their ramifications on livestock trade and the development of appropriate support systems within exporting countries to mitigate and minimize the risk of bans being instituted. In addition, encouraging farmers to focus their livestock-rearing efforts on breeds more resistant to infection with RVFV and a greater study of the genetic factors that make these breeds resistant should also be promoted as part of this global effort. Developing better surveillance systems is key.

Fears of RVFV being used as a bioterrorism agent should not sideline the real security effects of the disease in driving impoverished communities to find other, more dangerous means of income. Did the bans on livestock from Somalia, for instance, and the resulting lost economic opportunities afforded by a well-developed functioning ruminant export market, contribute to the drive of persons and communities to seek alternative sources of income, including taking part as combatants in the civil war in or in the piracy trade that has developed in the region? Are the stringent measures being imposed on laboratories that store or work with the virus serving to concentrate technical expertise and industrial know-how in the hands of scientists in a very few industrialized countries, thus contributing to limited scientific inquiry and collaboration, which further escalates costs? Although these questions are yet to be answered conclusively, exploring the case for lowering current BSL requirements of laboratories and production facilities could be 1 method of mitigating these costs.

A greater sense of urgency and investment is required for controlling, better managing, and preventing future large-scale outbreaks of RVFV. Future long-term success lies in building on global collaborative initiatives, the closer integration of multilateral agencies, and a wider participation from livestock-importing countries and emerging economies that are investing in RVFV-endemic countries. A worldwide strategy, both in tune with and inspired by principles of equity and social justice, could ultimately deliver the best outcomes in combating this neglected tropical disease.

Dr Dar is a consultant in communicable disease control at the London School of Hygiene and Tropical Medicine and at the UK Health Protection Agency. He is also an honorary research fellow at the Chatham House Centre on Global Health Security. His research interests focus on global health, especially in low-income and conflict settings.

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Eastern Equine Encephalitis in Children, Massachusetts and New Hampshire, USA, 1970–2010

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Learning Objectives

Upon completion of this activity, participants will be able to

- Assess the clinical presentation of eastern equine encephalitis (EEE),
- Distinguish common ancillary findings in cases of EEE,
- Analyze risk factors for poor outcomes of EEE, and
- Evaluate the most common outcomes of EEE.

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We describe the clinical, laboratory, and radiographic characteristics of 15 cases of eastern equine encephalitis in children during 1970–2010. The most common clinical and laboratory features were fever, headache, seizures, peripheral leukocytosis, and cerebrospinal fluid neutrophilic pleocytosis. Radiographic lesions were found in the basal ganglia, thalami, and cerebral cortex. Clinical outcomes included severe neurologic deficits in 5 (33%) patients, death of 4 (27%), full recovery of 4 (27%), and mild neurologic deficits in 2 (13%). We identify an association between a short prodrome and an increased risk for death or for severe disease.

Eastern equine encephalitis (EEE) is a highly virulent reemerging arboviral encephalitis in humans; the disease is endemic to the eastern United States and the Gulf Coast (*1*,*2*). EEE is characterized by a nonspecific prodrome followed by severe headache, high fevers, lethargy, and seizures (*1*,*3*,*4*). The meningoencephalitis often progresses rapidly to coma and death; mortality rates are 30%–70% (*4*). On average, there are 6 cases per year in the United States, some occurring sporadically and others in epidemics

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(*5*). Over the past decade, EEE virus (EEEV) transmission has resurged in the northeastern region of North America. In the United States, overlapping epidemics among humans occurred in Massachusetts during 2004–2006 and in New Hampshire during 2005 and 2007 (*1*). During consecutive years of EEEV activity from 2009 to 2011, New York State reported its first human cases of EEE in 26 years (*6*). In 2012, a total of 7 human cases of EEE were diagnosed in Massachusetts (*7*)—this is the highest number of annual cases in the state since 1956. Moreover, the first human cases of EEE in Vermont were reported in 2012 (*8*), and public health surveillance indicates that the range of EEEV has extended into the state of Maine (*9*) and into Nova Scotia, Canada (*10*).

The natural history of EEEV infections in children has not been well characterized. This mosquito-borne illness in humans was first described during an outbreak in Massachusetts in 1938 (*11*), and Farber et al. (*12*) described 8 cases of EEE in children during that outbreak. Since then, case series of EEE have been published (*1*,*3*,*4*,*13*), but investigations of EEE in children are lacking. We conducted this study to determine the clinical, laboratory, radiographic, and pathologic features of EEE in children and to assess features associated with the clinical outcome of EEE in children.

Methods

Data Sources

Cases of EEE at Boston Children's Hospital in Boston, Massachusetts, during 1970–2010 were identified by searching hospital records and records of the Massachusetts Department of Public Health. Institutional review board approval was obtained.

Case Definitions and Laboratory Confirmation

Case-patients were defined as children with illness compatible with encephalitis and with laboratory confirmation of EEEV infection (*14*). EEE was confirmed by the William A. Hinton State Laboratory Institute, Department of Public Health, Boston, on the basis of at least 1 of the following: isolation of EEEV or demonstration of specific viral antigen or genomic sequence from cerebrospinal fluid (CSF) or brain tissue; detection of virus-specific IgM in CSF by antibody-capture ELISA; ≥4-fold increase in acute-phase versus convalescent-phase serum antibodies; or detection of EEEV-specific IgM in CSF by ELISA, followed by confirmation of virus-specific neutralizing antibodies by plaque-reduction neutralization assay.

Case-Patient Information

A records search revealed a total of 15 cases; medical records were available for 14. Partial records from the Massachusetts Department of Public Health were available for patient 2. Patient 1 has been described (*15*). We defined the illness prodrome as the time between initial nonspecific symptoms and the first major neurologic symptom, such as headache with photophobia, stiff neck, altered mental status, or seizures. We used the Pediatric Cerebral Performance Category Scale (PCPC) to classify outcomes, which were determined on the basis of the clinical condition of the patient at the time of hospital discharge (*16*). Normal, mild, and moderate disabilities were classified as favorable outcomes (PCPC scores 1–3); severe disability, coma, and death were classified as unfavorable outcomes (PCPC scores 4–6).

Radiograph Interpretation

Radiographs were reviewed by a neuroradiologist who was aware of the EEE diagnosis but blinded to the specifics of the patient's history and previous radiographic and pathologic findings. Lesions seen on radiographs were scored on a scale of 0–3 for the degree of T2 fluid-attenuated inversion recovery enhancement (0, no lesion; 1, 2, and 3, minimal, moderate, and severe enhancement, respectively).

Pathology

Postmortem CNS specimens from 2 patients were analyzed by histopathologic and immunohistochemical examination. Control CNS tissues were postmortem specimens from patients with other forms of encephalitis.

Statistical Methods

The Mann-Whitney rank-sum test was used to compare the length of prodromes for patients with favorable outcomes with those for patients with unfavorable outcomes; $p<0.05$ was considered statistically significant. Pearson's correlation was used to determine relationships between continuous variables.

Results

Demographic and Epidemiologic Characteristics

During 1970–2010, a total of 19 cases of EEE in children in Massachusetts and New Hampshire were reported to the Centers for Disease Control and Prevention; our case series includes 15 of those 19 cases. Thirteen of the patients were from Massachusetts and 2 were from New Hampshire. The median age was 5.3 years (range 0.5–14.7 years). Medical care was sought for all patients during August, September, or October. The clinical features, diagnostic results, treatments, and outcomes for each patient are described in Table 1.

Clinical and Laboratory Characteristics

Of the 15 patients, 4 (27%) died, 5 (33%) had severe neurologic sequelae, 2 (13%) had mild to moderate deficits,

and 4 (27%) fully recovered. The median duration of hospitalization was 16.0 days. The median duration of hospitalization for patients who died was 6.6 days.

Fever was reported for all patients on or near day 1 of hospitalization. Signs and symptoms of meningoencephalitis, such as seizures, headaches, nausea, vomiting, and stiff neck, were observed for all patients (Table 2). Of the 10 patients who had seizures, 5 had complex partial seizures, 3 had generalized tonic–clonic seizures, and 2 had both types of seizures.

The prodrome phase lasted ≥3 days for 7 patients and <3 days for 8 patients (median 2 days, range 0–8 days) (Table 1). Patients with a longer prodrome typically reported a few days of nonspecific symptoms, such as fever, headache, and nausea followed by slower progression of neurologic symptoms, including photophobia, stiff neck, altered mental status, and seizures. In contrast, patients with a shorter prodrome often had rapidly declining mental status and seizures. Of the 15 patients, 13 (87%) became stuporous or comatose during the first 3 days of hospitalization (median 2 days). The remaining 2 patients became delirious but remained responsive.

CSF samples from all patients had neutrophilic pleocytosis, and 12 (86%) had elevated levels of protein (Table 3). Lumbar puncture was performed a median of 2.0 days (range 0–8 days) after symptom onset. Of 14 patients, 13 (93%) had an elevated peripheral leukocyte count on or before day 1 of hospitalization, and 8 (57%) had peripheral neutrophilia.

Electroencephalogram results were available for 14 patients. A pattern of generalized slowing of brain electrical activity was observed in 13 patients; subclinical

Table 1. Clinical features of and diagnostic studies for 15 children with eastern equine encephalitis, Massachusetts and New Hampshire, 1970–2010*

Patient no.	Admitted to hospital	Age, y/sex	Prodome length, d	Blood leukocytes, 10^3 cells/μL	CSF leukocytes, cells/μL	CSF protein, mg/dL	ICP checked	Treatment	Outcome at discharge	Status at follow-up
1	1970 Aug	0.5/M	2	28.0	300	64	No	AEDs, mannitol	Severe disability	No change
2	1974 Aug	1.2/M	2	NA	NA	NA	NA	NA	Death	–
3	1982 Sep	0.8/M	1	17.1	11	66	No	Vidarabine, acyclovir, AEDs, dexamethasone	Severe disability	Improved
4	1982 Sep	14.7/F	2	17.3	1,004	80	Yes	AEDs, mannitol	Severe disability	Improved
5	1983 Aug	11.2/M	3	21.0	720	61	No	Mannitol	Full recovery	No change
6	1984 Aug	6.5/F	0	21.0	1,384	46	Yes	Acyclovir, AEDs, pentobarbitol coma, dexamethasone, mannitol	Severe disability	Improved
7	1995 Aug	0.5/M	4	13.6	451	73	No	Acyclovir, AEDs, IVIG, dexamethasone, cyclophosphamide	Severe disability	NA
8	2000 Sep	4.0/F	5	22.7	258	33	No	Acylovir, AEDs	Full recovery	No change
9	2001 Aug	3.9/F	5	12.0	1,200	54	No	Acylovir, AEDs	Mild to moderate disability	Improved
10	2004 Aug	13.1/M	0	8.4	860	84	No	Acylovir, AEDs dexamethasone, mannitol, 3% saline	Death	–
11	2004 Oct	11.5/F	8	16.5	616	73	No	Acylovir	Full recovery	No change
12	2005 Aug	5.3/F	2	34.8	847	73	No	Acylovir, AEDs, 3% saline	Death	–
13	2006 Aug	9.9/M	2	23.0	1,085	81	Yes	Acylovir, AEDs, pentobarbitol coma, interferon α-2b, dexamethasone, IVIG, 3% saline	Death	–
14	2007 Aug	13.3/M	6	20.4	888	124	No	Acylovir, AEDs, IVIG, 3% saline	Mild to moderate disability	No change
15	2009 Sep	3.5/F	5	17.8	258	39	No	Acylovir, AEDs, dexamethasone	Full recovery	No change

*CSF, cerebrospinal fluid; ICP, intracranial pressure; AEDs, anti-epileptic drugs (phenobarbital, phenytoin, fosphenytoin, carbamazepine, oxcarbazepine, lorazepam, levetiracetam); ND, not done; –, patient died or did not return for follow-up; IVIG, intravenous immunoglobulin.

status epilepticus was noted for 1 of these patients, and epileptiform activity was noted for 5. No correlation could be drawn between electroencephalogram findings and outcome.

Neuroimaging Findings

Six of the 9 initial head computed tomography (CT) scans were abnormal. The initial CT scan was performed at a mean of 1 day (range 0–2 days) after the onset of neurologic symptoms. Lesions were most commonly found in the basal ganglia, cerebral cortex, and thalamus (Table 4 and Figure 1, panel A). These abnormalities were often subtle and initially were underreported, particularly the cortical lesions.

Magnetic resonance imaging (MRI) was performed for 12 patients; all images showed evidence of abnormalities. The initial MRI was performed a mean of 2 days (range 1–4 days) after onset of neurologic symptoms. MRI results most commonly revealed abnormalities in the meninges, the cerebral cortex, basal ganglia, and thalamus (Table 4 and Figure 1, panels B–D). Lesions were most apparent on fluid-attenuated inversion recovery, diffusion-weighted imaging, and T1-weighted postgadolinium sequences, and they were more evident on MRI than CT images. The abnormalities seen on MRI or CT colocalized with areas of inflammation and EEE virus seen in pathology specimens (Table 5), but the location and intensity of the abnormalities seen on images did not correlate with clinical outcomes.

Treatment

All patients received empiric antimicrobial therapy, and most received acyclovir for empiric coverage of herpes simplex encephalitis. Patients also received antiepileptic medications; immunomodulatory agents, including intravenous immunoglobulin, steroids, cyclophosphamide, and interferon-α2b; and treatments for increased intracranial pressure. Intracranial pressure was monitored for 3 patients (Table 1). No patient underwent intraventricular drain placement or a decompressive craniectomy.

Table 2. Clinical signs and symptoms at the time of hospital admission for 14 children with eastern equine encephalitis, Massachusetts and New Hampshire, 1970–2010

Sign or symptom	No. (%) patients
Fever	14 (100)
Seizure	10 (71)
Headache	9 (64)
Neck stiffness	9 (64)
Nausea/vomiting	9 (64)
Myalgia/arthralgia	4 (29)
Plantar reflex	4 (29)
Photophobia	4 (29)
Sore throat	2 (14)
Cranial nerve palsy	1 (7)
Abdominal pain	1 (7)
Diarrhea	0

Characterization of Prognostic Factors

To identify variables associated with clinical outcomes, we compared clinical, laboratory, and imaging findings for patients with favorable outcomes with those for patients with unfavorable outcomes. The length of prodrome was associated with clinical outcome at the time of discharge (Figure 2). The prodrome for patients with a favorable outcome was significantly longer than the prodrome for patients with an unfavorable outcome (median 5 vs. 2 days; $p = 0.002$). The 8 patients for whom the prodrome lasted ≤2 days had a poor outcome; all 4 deaths occurred in this group. For these 8 patients, the relative risk was 6.0 for an unfavorable outcome. Of the 7 patients for whom the prodrome lasted ≥3 days, 6 had a favorable outcome, including 4 patients who fully recovered.

Outcome was not associated with any other variable, including patient age, seizure, headache, photophobia, quantity of cerebrospinal fluid pleocytosis, total protein in the cerebrospinal fluid, serum sodium level, or leukocyte count in the blood. Moreover, no significant association was observed between the patient's clinical outcome and the therapy received.

Patient Follow-up

Follow-up information was available for 10 of the 11 surviving patients. Duration of follow-up ranged from 3 months to 20 years (median 14.5 months). Patient outcomes

Table 3. Laboratory values at the time of hospital admission for 14 children with eastern equine encephalitis, Massachusetts and New Hampshire, 1970–2010

Variable	Median value (range)	Reference range
Blood		
Leukocytes, thousand cells/μL	19.1 (8.4–34.8)	5.7–9.9
Neutrophils, %	84 (40–95)	39–77
Hematocrit, %	36.5 (30–43.9)	31.5–38.0
Platelets, thousand cells/μL	230 (155–416)	198–371
Sodium mmol/L	137 (130–144)	135–148
Cerebrospinal fluid		
Leukocytes, cells/μL	784 (11–1,384)	0–5
Neutrophils, %	83 (61–100)	0–2
Red blood cells, cells/μL	10 (0–721)	0
Glucose, mg/dL	78 (40–135)	60–80
Total protein, mg/dL	70 (33–124)	15–45

Table 4. Results of neuroimaging studies for 9 children with eastern equine encephalitis, Massachusetts and New Hampshire 1970–2010*

Location studied	No. (%) patients with abnormal finding	
	MRI, n = 8	CT, n = 9
Meninges	8 (100)	0
Cortex	7 (88)	5 (56)
Basal ganglia	7 (88)	6 (67)
Thalamus	5 (63)	4 (44)
Brainstem	2 (25)	2 (22)
Subcortical white matter	1 (13)	1 (11)
Periventricular white matter	0	1 (11)
Hydrocephalus	0	2 (22)
Any	8 (100)	6 (67)

*MRI, magnetic resonance imaging; CT, computed tomography.

at follow-up visits were improved (40%) or unchanged (60%) since the time of discharge. Of the 4 patients with severe deficits at hospital discharge, 3 showed improvement at the follow-up visit (Table 1). Patient 3 progressed from being comatose to being able to communicate nonverbally by 30 months after hospital discharge. Patient 4 recovered speech, comprehension, and the ability to self-feed by 18 months after hospital discharge. Patient 6 progressed from being nonvocal and having limited spontaneous movements at discharge to speaking in full sentences by 21 months after hospital discharge. These results demonstrate the opportunity for substantial cognitive and motor rehabilitation in patients with EEE.

Pathologic Findings

Tissue sections from the postmortem brain specimens of patients 10 and 12 demonstrated severe meningoencephalitis. Gross findings were notable for marked, diffuse cerebral edema. There was prominent acute and chronic perivascular inflammation within the cortex, thalamus, basal ganglia, and brainstem (Figure 3, panels A, B).

A multifocal, patchy distribution of EEEV-stained tissue was observed in specimens from patients10 and 12. In the cerebral hemispheres, clusters of infected cells were predominantly in the gray matter. There were distinct, punched-out islands of pallor in the thalamus and the basal ganglia, representing areas of tissue rarefaction and damage (Figure 3, panels C, E). These regions corresponded to areas demonstrating EEEV infection (Figure 3, panels D, F and Table 5). On the basis of cell morphologic appearance and immunohistochemical staining for neuronal cell marker NeuN, neurons appeared to be the predominant cell population infected by EEEV (Figure 4, panels A–C, Appendix, wwwnc.cdc.gov/EID/article/19/02/12-0039-F4.htm).

The anatomic location of EEEV, inflammatory infiltrates, neuronal death, and rarefied tissue corresponded to the location of the abnormalities seen on MRI (Table 5). These findings provide direct evidence that the abnormalities seen on neuroimaging represent virus-induced inflammatory changes.

Discussion

Similar to patients described in the original reports of EEE in the United States (*12*,*17*), the patients in our study sought care for fever and signs of encephalitis during the late summer months in the New England region of the United States; the patients exhibited a peripheral and CNS neutrophilic immune response. Shortly after seeking care, most patients showed a decline in the level of consciousness and often became comatose. In some patients, encephalitis worsened and was associated with increased intracranial pressure and severe neurologic damage or death. Other patients recovered with minimal or no disability.

The correlation between the length of prodrome and clinical outcome is a key finding of our study. A short

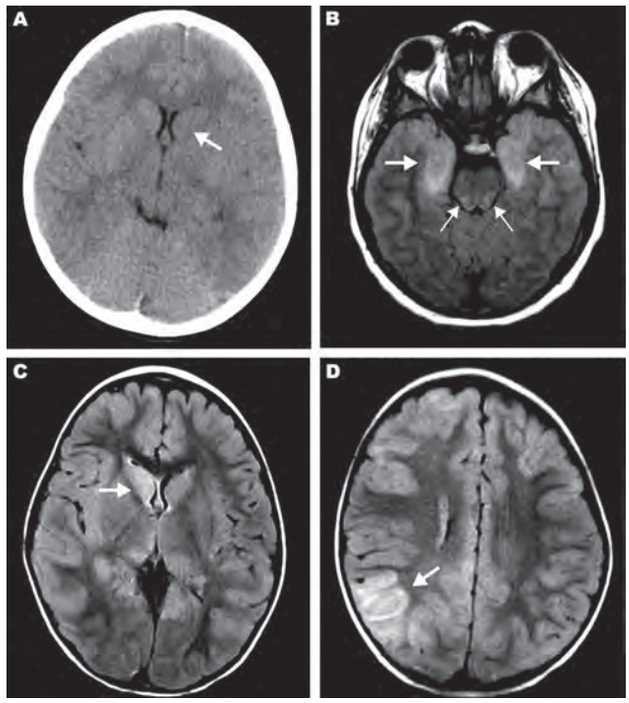

Figure 1. Magnetic resonance images (MRIs) and computed tomography (CT) neuroradiographs showing lesions in brains of 3 children with eastern equine encephalitis. A) Results of noncontrast CT scan of the brain of patient 12 on hospital day 2; the neuroradiograph shows subtle hypoattenuation of the left caudate head (arrow) and diencephalic region. B) Axial fluid attenuated inversion recovery (FLAIR) image from brain MRI scan of patient 14 on hospital day 2; the image shows abnormal T2 hyperintense regions of the bimesial temporal regions (thick arrows) with accompanying abnormal T2 hyperintense regions of the dorsal pontomesencephalic regions (thin arrows). C, D) FLAIR images from brain MRI scan of patient 15 on hospital day 3. C) Abnormal T2 hyperintense caudate and thalamic nuclei, most prominent on the right (arrow). D) Abnormal T2 hyperintense regions are most prominent in the right parietotemporal gray matter (arrow) and subcortical white matter but are also seen scattered throughout.

Table 5. Colocalization of lesions, virus, and brain inflammation in 2 children in a study of EEE, Massachusetts and New Hampshire, 1970–2010*

Patient, anatomical location	Inflammation†	EEE virus†	MRI showing lesion(s)‡
Patient 10			
Frontal cortex	+++	+++	+++
Parietal cortex	++	++	++
Temporal cortex	++	+++	++
Occipital cortex	+	–	–
Thalamus	+++	+++	++
Basal ganglia	++	+++	+++
Cerebellar cortex	+	+	–
Patient 12			
Frontal cortex	++	++	++
Parietal cortex	+	+	–
Temporal cortex	++	++	–
Occipital cortex	+	++	–
Thalamus	++	++	+++
Basal ganglia	++	++	+++
Cerebellar cortex	+	++	–
Parameter comparison		Correlation coefficient§	
Inflammation vs. EEE virus		0.82	
Inflammation vs. lesion on MRI		0.68	
EEE virus vs. lesion on MRI		0.52	

*Lesions were observed by use of MRI (magnetic resonance imaging). EEE, eastern equine encephalitis; –, absent or none found.
†The degree of cerebral inflammation and amount of EEE virus antigen were scored 0–3 on the basis of histopathologic and immunohistopathologic findings.
‡Lesions seen on MRIs were scored 0–3 on the basis of the degree of T2 fluid-attenuated inversion recovery enhancement.
§Determined by using the Pearson correlation.

prodrome was associated with death or severe neurologic disability; a long prodrome was associated with mild to moderate disability or full recovery. The association between prodrome length and outcome may help identify a subgroup of patients at higher risk for severe disease and for whom more aggressive medical and surgical management may be warranted. In our series, multiple patients showed clinical improvement after medical management of increased intracranial pressure.

Only 3 (20%) patients in our study received intracranial pressure monitoring and none underwent decompressive craniectomy. The treatment choice reflects, in part, the primary signs and symptoms observed at the bedside (intractable seizures vs. signs of uncontrolled cerebral swelling) and changes in medical practice over the past 4 decades. Neuroimaging conducted early in the course of the disease disclosed scattered, focal lesions without gross swelling or herniation; however, severe swelling was clearly present at autopsy. Barnett and colleagues (*18*) observed severe cerebral swelling, peaking around day 12, in 60% of adults with encephalitis. Furthermore, in that early series, all patients with low intracranial pressure (<20 mm Hg) survived, and nearly all those with increased intracranial pressure died. More recently, routine intracranial pressure monitoring and cerebral perfusion pressure–directed therapies have been recommended for patients with worsening encephalitis (*19*). Decompressive craniectomy has been suggested for patients with conditions refractory to medical management (*20*,*21*).

We did not observe some previously reported associations between poor outcome and the degree of CSF pleocytosis (*3*), serum hyponatremia (*3*), age (*4*), or electroencephalogram abnormalities (*4*). The small sample size in our study may explain the difference between findings in our study and others; power calculations indicate that our case series would not be expected to show these associations.

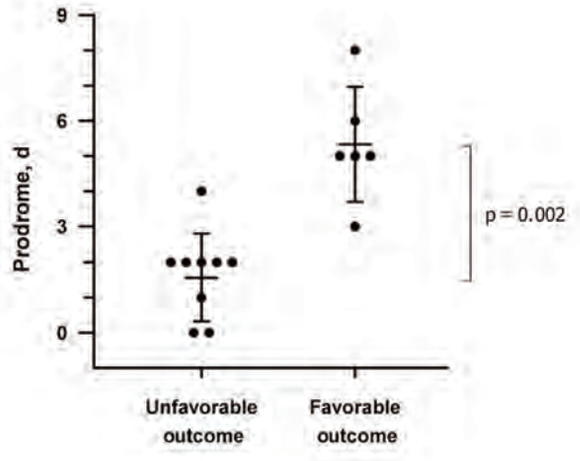

Figure 2. Association of length of prodrome with clinical outcome in children with eastern equine encephalitis. Clinical outcome at the time of hospital discharge was defined by using a modified Pediatric Cerebral Performance Category scale (PCPC) (*27*). Error bars represent standard deviation; the central lines are the mean. The Mann-Whitney rank-sum test was used to compare the prodrome lengths for patients with favorable outcomes with those for patients with unfavorable outcomes. Prodrome is defined as the time from initial illness symptom to first neurologic symptom. Unfavorable outcome is defined as a PCPC score of 4–6, which includes death and severe neurologic disabilities. Favorable outcome is defined as a PCPC score of 1–3, which includes complete recovery and mild to moderate neurologic disability.

A limitation of our study is the potential for recall bias with regard to timing of the prodrome. The timing and symptoms of the prodrome in this study depended on the recall of the patient (or parent) and documentation by the medical provider. However, these are the same data that clinicians use to make treatment decisions, so this association, despite recall bias, is probably clinically relevant. Prospective study of EEE with larger sample sizes would overcome some of the limitations of this study and would be a means of assessing the utility of prodrome length as a clinically relevant tool for predicting outcome. However, the rarity of the disease makes a prospective study difficult to perform.

Our finding of the predilection for focal basal ganglia lesions and thalamic lesions in patients with EEEV is consistent with findings in prior studies (*3*,*22*–*24*). In addition to the characteristic basal ganglia and thalamic lesions, we observed a pattern of focal cortical lesions in almost all patients. This finding extends the neuroimaging findings of EEE to include focal cortical lesions in children. The prominence of cortical lesions in EEE in children may reflect pathophysiologic differences between children and adult patients. It remains unclear why EEE in children causes a characteristic pattern of multifocal lesions with a predilection for the basal ganglia, thalamus, and cortex. Other viral encephalitides also exhibit a tropism for certain neuroanatomic locations. For example, Japanese encephalitis often affects the thalamus and basal ganglia (*25*), and herpes simplex virus has a predilection for the temporal lobes. Recognition that focal cortical CNS lesions are characteristic features of EEE in children may assist in the diagnosis of EEE.

In addition, in our study, the rate of seizures (>70%), specifically, complex partial seizures, was higher than that found by Deresiewicz et al. (25%) (*3*). Because complex partial seizures are often secondary to focal cortical lesions, it is tempting to speculate that these focal cortical lesions caused more complex partial seizures.

The pattern of histologic findings in our study, including inflammation of the leptomeninges; chronic and acute perivascular inflammation; evidence of neuronal injury and cell death (*11*,*26*,*27*); and presence of EEEV antigen specifically in neurons, is similar to the pattern of findings in previous reports (*27*). We extend these observations by demonstrating that lesions observed on radiographs correspond to pathologic regions of EEEV infection and CNS inflammation.

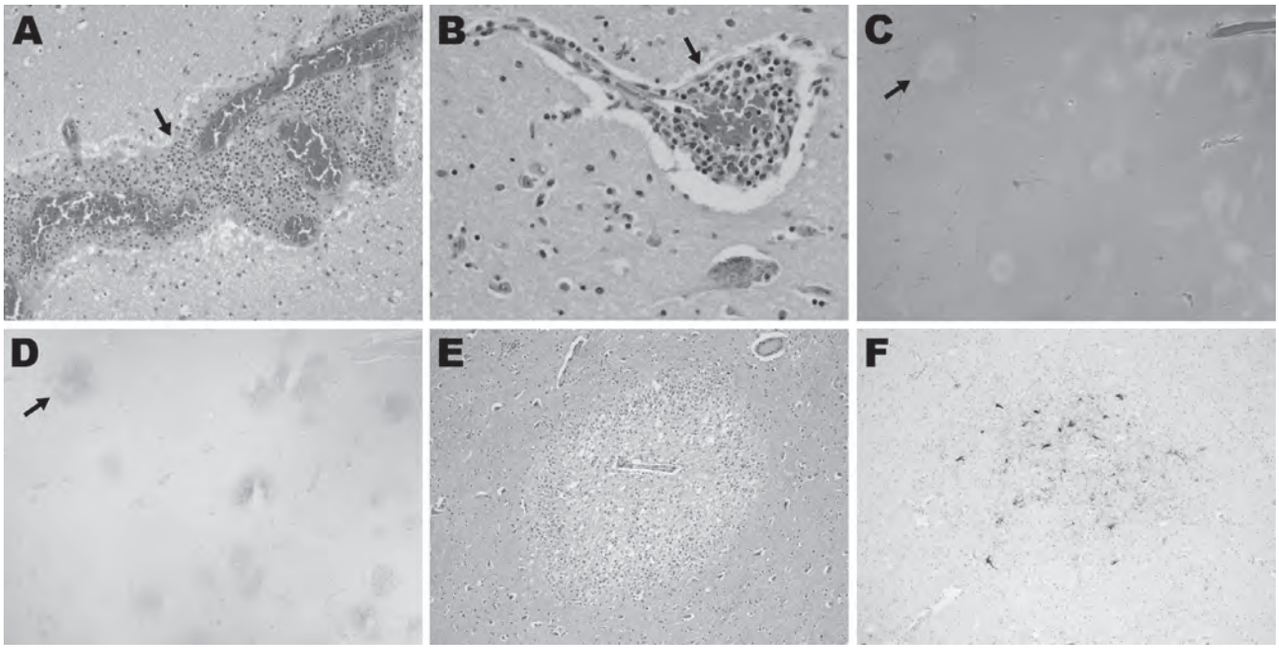

Figure 3. Histopathologic features for patient 12 in a study of children with eastern equine encephalitis (EEE), Massachusetts and New Hampshire, 1970–2010. The postmortem samples of central nervous system tissue were obtained 10 days after the onset of symptoms. A) Hematoxylin and eosin (H&E)–stained section of temporal lobe, showing meningeal inflammation (arrow) (magnification ×200). B) H&E-stained section of midbrain, showing perivascular inflammation (arrow) (magnification ×400). C–F) EEE virus (EEEV) colocalizes with areas of tissue injury in the brain. C) H&E-stained section of the basal ganglia, demonstrating foci of marked tissue rarefaction (arrow) (magnification ×12.5). D) Immunohistochemistry of section adjacent to that shown in panel C; staining of the basal ganglia with EEE immune ascites demonstrates foci of EEEV (arrow) that correspond with areas of tissue rarefaction in panel C (magnification ×12.5). E) H&E stained section of thalamus (magnification ×100). F) Immunohistochemistry with EEE immune ascites demonstrates EEE viral antigens in the thalamus. Specificity for EEEV immunoreactivity of this ascites fluid was confirmed by the lack of staining on control brain specimens (data not shown).

Conclusions

The rates of illness and death are high among children with EEE. Key features of this disease include signs of encephalitis during late summer and early fall, neutrophilic cerebrospinal fluid pleocytosis, and abnormal neuroimaging results, including the finding of lesions in the basal ganglia and cerebral cortex. A short prodrome is associated with unfavorable outcomes and, when present, warrants close monitoring and management of intracranial pressure.

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Lessons and Challenges for Measles Control from Unexpected Large Outbreak, Malawi

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Despite high reported coverage for routine and supplementary immunization, in 2010 in Malawi, a large measles outbreak occurred that comprised 134,000 cases and 304 deaths. Although the highest attack rates were for young children (2.3%, 7.6%, and 4.5% for children <6, 6–8, and 9–11 months, respectively), persons >15 years of age were highly affected (1.0% and 0.4% for persons 15–19 and >19 years, respectively; 28% of all cases). A survey in 8 districts showed routine coverage of 95.0% for children 12–23 months; 57.9% for children 9–11 months; and 60.7% for children covered during the last supplementary immunization activities in 2008. Vaccine effectiveness was 83.9% for 1 dose and 90.5% for 2 doses. A continuous accumulation of susceptible persons during the past decade probably accounts for this outbreak. Countries en route to measles elimination, such as Malawi, should improve outbreak preparedness. Timeliness and the population chosen are crucial elements for reactive campaigns.

During the prevaccine era, 130 million measles cases occurred annually worldwide, and measles was a leading cause of childhood death (*1*). Measles vaccines have dramatically reduced cases and deaths during recent decades. The Measles Initiative developed a joint strategic plan to reduce measles-related deaths by strengthening routine immunization, supplementary immunization activities (SIAs) in the form of mass vaccination campaigns, reinforced surveillance, and adequate case management (*2*,*3*). In 2000, the World Health Organization (WHO) Regional Office for Africa adopted a plan to reduce measles-related deaths by 50% by the end of 2005 (*3*,*4*), and measles-related deaths decreased from 535,300 in 2000 to 139,300 in 2010 (*5*). A recent WHO resolution called for measles elimination in the African Region by 2020 (*6*).

Malawi's Expanded Program on Immunization (EPI), established in 1979, recommends 1 dose of measles-containing vaccine (MCV) for infants 9–11 months of age (*7*). After implementation of EPI, cases declined from >162,000 in 1980 to an annual average of 8,000 cases throughout the 1990s. Additional initiatives toward measles control (*8*) comprised a catch-up campaign in 1998 directed toward children 9 months–14 years of age and follow-up campaigns in 2002, 2005, and 2008 for children 9–59 months (*9*); administrative reported vaccine coverage was close to 100% (*10*). Before 2010, the last large epidemic in Malawi occurred in 1996 and 1997, when ≈10,000 cases were reported nationwide each year.

Despite Malawi's measles control successes during the past 2 decades, a large outbreak occurred in 2010, with as many cases as in the 1980s. Médecins Sans Frontières (MSF) with the Ministry of Health (MoH) reinforced surveillance, provided case management, and vaccinated 3,343,112 children through outbreak response immunization (MoH/MSF) during epidemiologic weeks 18–26 (May–June) (Figure 1, panel A). The MoH implemented additional reactive vaccination campaigns for 1) children 9–59 months of age in some districts in epidemiologic weeks 10–14 (March–April) and 2) children 9 months–15 years of age nationwide in epidemiologic weeks 33–34 (August).

To describe this epidemic and outcomes from outbreak response vaccination, we analyzed national surveillance data. We also addressed factors that might explain the

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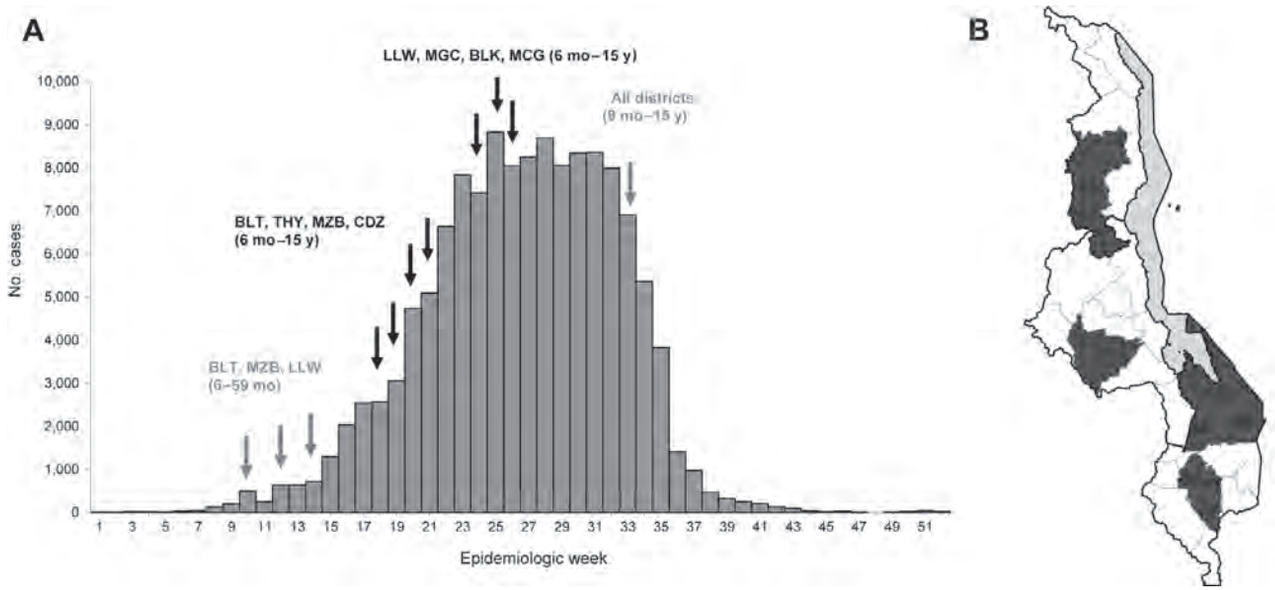

Figure 1. Weekly distribution of measles cases and time when the reactive vaccination campaigns were implemented (A) and districts where outbreak-response immunizations were conducted by the MoH and MSF (B), Malawi, 2010. MoH, Ministry of Health; MSF, Médecins Sans Frontières; BLT, Blantyre; MZB, Mzimba, LLW, Lilongwe; THY, Thyolo; CDZ, Chiradzulu; MGC, Mangochi; BLK, Balaka; MCG, Machinga. Black arrows indicate compaigns implemented by MoH/MSF; gray arrows indicate campaigns implemented by MoH only.

outbreak, including results from coverage and vaccine effectiveness surveys.

Field Studies

Measles Surveillance

National surveillance in Malawi is based on the Integrated Disease Surveillance and Response strategy. Each month, health officers from public health facilities and selected private centers report cases and deaths to the district level. Data are electronically compiled and transmitted monthly to the national level. A standardized individual collection form is used to collect information about age, sex, health facility, date of consultation, date of onset, whether specimen was sent to the laboratory, treatment, and outcome (alive or dead).

Health care providers used WHO case definitions to diagnose measles cases and to report case-patients in the surveillance system. Suspected measles was defined as generalized maculopapular rash and fever (≥38°C) and at least 1 of the following: cough, runny nose, or conjunctivitis; or as suspected measles reported by a health professional. A measles-related death was defined as the death of a person with measles within 30 days after rash onset, unless the death was unrelated to the disease. Samples from suspected case-patients were sent for laboratory confirmation to detect measles IgM. Three confirmed cases at the district level constituted an outbreak. Once an outbreak was confirmed, additional cases were confirmed by epidemiologic link if they met the clinical case definition (*1*).

During the 2010 outbreak, the surveillance system was strengthened by reinforcing health officer training (case definition and data collection), retrospective review of health registers, weekly communication to the district level for data sharing, monitoring of data completeness, and electronic compilation and cleaning of the line list. Data collection strengthening was interrupted in epidemiologic week 35 when MSF involvement in measles case management ended.

Data were entered in Excel (Microsoft, Redmond, WA, USA). Attack rates (ARs) were defined as the number of measles cases divided by the population at risk and case-fatality rates (CFRs) as the number of measles-related deaths divided by the number of measles cases. Population projections for 2010 were calculated by applying a growth rate of 3.28% to the 2008 Malawi Population and Housing Census figures.

Vaccine Coverage and Effectiveness

We conducted age-stratified surveys (epidemiologic weeks 27–31 [July–August 2010]) to assess coverage in the districts where MoH/MSF conducted vaccination campaigns (Figure 1, panel B). Children 6 months–15 years of age were eligible for inclusion in the surveys. For each district, we required a sample of 983 on the basis of the following assumptions: 80% vaccine coverage, an α error of 0.05, an absolute precision of 0.05, and a design effect of 4.

We used cluster sampling (40 villages of 25 children in each district); clusters were allocated proportionately to the

population of each village or health center catchment area where village populations were not available. In the urban areas of Blantyre and Lilongwe, spatial-based sampling was used to select randomly the first household of clusters (*11*). In rural areas, the households in the villages were numbered, and the first household was randomly selected. Subsequent households were selected by proximity. Households were defined as persons living and eating together under the same roof. If a head of household or adult was absent, the survey team returned later in the day; if a head of household was absent after 2 attempts, the household was skipped and replaced.

A standardized questionnaire was used to collect demographic data, vaccination status, and vaccination history (place, date of vaccination, and injection site on the body) for routine vaccination, last SIAs (October 2008) and MoH/MSF campaigns (May–July 2010), reasons for nonvaccination for routine vaccination and MoH/MSF outbreak response immunization, and previous measles episodes. Proof of vaccination status was verified in the health passport; otherwise we relied on verbal recall. We also asked for respondents' age and degree of literacy.

To estimate vaccine effectiveness, we identified all children born after October 2003 (oldest age group toward which the 2008 follow-up SIAs were directed) with known vaccination status and no previous measles episode before 2010 among those recruited for the vaccine coverage survey. The main exposure of interest was vaccination status. Children were classified as not vaccinated, vaccinated with the routine dose only (routine vaccination ascertained by health passport), or vaccinated with 2 doses (routine vaccination ascertained by health passport plus the 2008 SIAs). Case-patients were defined as any child 9 months–15 years of age whose illness met the WHO measles case definition from January 2010 to the date of MoH/MSF outbreak response immunization in the different districts. Additional variables considered as possible confounders were age and literacy of the main caregiver and district of residence.

Vaccination coverage estimates were obtained by taking into account the survey design; sampling weights and design effect (deff) were applied to obtain the estimates (*12*). Associations between vaccination status and outcome were assessed through binomial regression (log link). The exponential of the coefficient for the vaccination variable was computed to estimate the adjusted risk ratio, and p values <0.05 were regarded as significant. The adjusted level of vaccine protection was computed as follows: (1 – adjusted risk ratio) × 100. Confidence intervals were calculated by taking into account the deff.

Data were entered by using EpiData 3.1 (EpiData Association, Odense, Denmark). Data analysis was performed by using Stata 10.0 (Stata Corp., College Station, TX, USA).

Ethical Considerations

The study was implemented in collaboration with the MoH after obtaining authorization. Privacy, confidentiality, and rights of patients were ensured during and after the study. Verbal informed consent was obtained from each head of household visited after detailed explanation about the existence of the outbreak, study objective, and planned use of the data collected. The survey data were entered and analyzed anonymously.

Main Findings

Description of the Outbreak

In January 2010, sporadic suspected measles cases were reported in Blantyre and Zomba districts (Southern Region) and in Mzimba and Nkhata Bay districts (Northern Region). The first samples were confirmed measles IgM positive on week 3. For the first few weeks, most reported cases were from the urban area of Blantyre, but by the end of March, all 3 regions were affected; the bulk of cases came from a few districts: Blantyre, Chiradzulu, Mangochi, Machinga, Zomba, and Thyolo (Southern Region); Mzimba (Northern Region); and Lilongwe (Central Region). By mid-July, all districts reported measles cases.

During weeks 1–52, a total of 134,039 measles cases and 304 deaths were reported. At the national level, the epidemic plateaued on week 23, and reported cases started to decrease in week 32 (Figure 1). The overall cumulative AR was 0.96%. The CFR was 0.23%.

The most affected areas were the Central Region and the Southern Region, where cumulative ARs were 1.09% and 1.03%, respectively; in the Northern Region, the AR was 0.28%. In the Southern Region, cases peaked in week 23, and in the Central Region, in week 32 (Figure 2).

Measles cases were equally distributed between male and female patients (M:F ratio 1.03:1) (Table 1). Median age of case-patients was 7 years (interquartile range 1–16). A total of 54,138 case-patients were <5 years of age, constituting 42% of all cases; 30% of case-patients were 5–14 years old, and 28% were adults (≥15 years). The most affected age groups were infants <1 year of age; AR was highest among children 6–8 months of age, followed by those 9–11 months (Figure 3).

Vaccine Coverage and Effectiveness

A total of 9,381 households were visited, and 21,993 children participated in the surveys. The median age of children was 6.8 years (interquartile range 3–10 years).

In the 8 districts surveyed, 95.0% (95% CI 93.7%–96.0%, deff 1.5) of children 12–23 months of age were vaccinated through EPI (Figure 4). A similar percentage (96.9% [95% CI 96.4%–97.3%, deff 4.0]), was observed overall. Reason for nonvaccination was obtained for 661

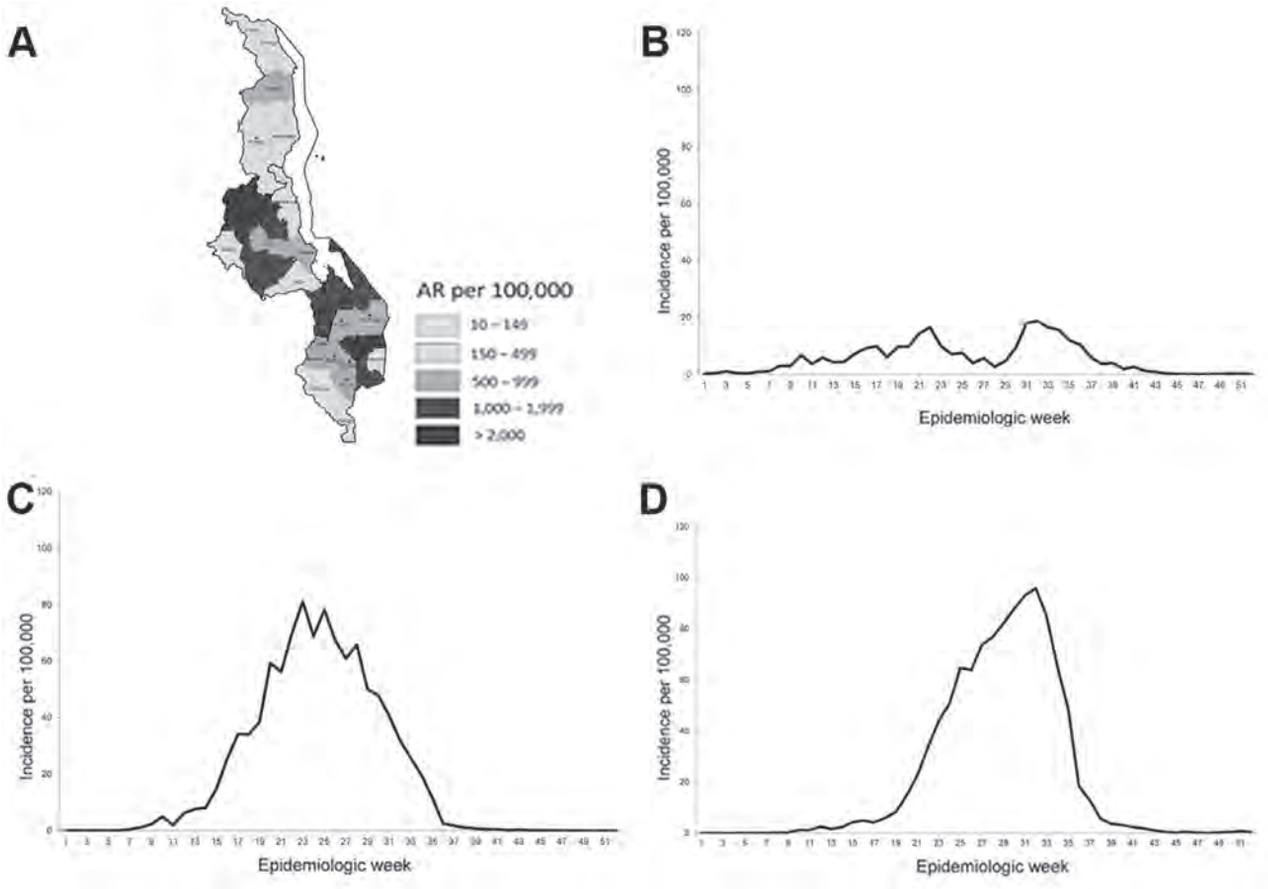

Figure 2. Measles cumulative attack rates (ARs) by district and weekly incidence, in Malawi, 2010. A) Malawi. The white area is Malawi Lake. Asterisks indicate districts in which children were vaccinated. B) Northern Region. C) Southern Region. D) Central Region.

(88.6%) children not vaccinated through EPI. Caregivers decided not to vaccinate the child in 61.8% of instances; the rest were unable to vaccinate the child despite their willingness because of reasons such as distance and cost. The main reason for nonvaccination was the caregiver's belief that the child was too young (36.0%); few (4.4%) declared religious objection to vaccination.

As reported by the caregiver, 60.8% (95% CI 58.3%–63.1%, deff 5.7) of children 9 months–5 years of age at the time of the SIA in 2008 were vaccinated against measles during this SIA; 0.7% of these children received the first dose, 60.1% received the second dose, and 39.2% were not vaccinated. Children not included in the age group toward which immunization was directed were vaccinated during the SIA (11% of children 0–9 months and 19% of children 5–10 years). Among the 159 (1.7%) children 9 months–5 years of age not vaccinated through EPI by October 2008, 54 (34.8%) were vaccinated in the SIA.

Coverage of the MoH/MSF outbreak response immunization was 95.5% (95% CI 94.9%–96.1%, deff 4.7) and lowest in children 6 months–1 year of age (Figure 4). Results were similar across the 8 districts surveyed (Table 2).

In total, 175 (3.4%) children among the 5,082 in the effectiveness survey reported a measles episode in 2010 before the MoH/MSF outbreak response immunization was implemented. Most (90.8%) children consulted in a health facility, and 21.8% were hospitalized. The vaccine effectiveness adjusted by child's age, sex, and location and the main caregiver's literacy was 83.9% (95% CI 70.8%–90.8%) for 1 EPI dose and 90.5% (95% CI 79.7%–95.5%) for 2 doses (EPI and SIAs) (Table 3). Vaccine effectiveness was highest among children 1–2 years, but effectiveness did not significantly differ by age group (data not shown).

Lessons Learned

In November 2009, WHO and the Global Alliance on Vaccination and Immunization rewarded the Malawi MoH for its outstanding performance in improving child health and immunization. The measles control program in Malawi is, to a large extent, a success story. However, despite high reported coverage for both EPI and SIAs, implemented in

Table 1. Characteristics of persons reported to have measles, Malawi, 2010

Characteristic	No. (%) cases reported	Attack rate*	No. (%) deaths reported	Case-fatality rate†
Total Malawi	134,039 (100)	0.96	304 (100)	0.23
Northern Region	5,054 (4)	0.28	24 (8)	0.47
Central Region	64,688 (48)	1.09	152 (50)	0.23
Southern Region	64,297 (48)	1.03	128 (42)	0.20
Sex	133,834 (100)		304 (100)	
M	67,949 (51)	0.97	126 (41)	0.19
F	65,885 (49)	0.94	178 (59)	0.27
Age group	131,725 (100)		292 (100)	
0–5 mo	7,243 (6)	2.26	10 (3)	0.14
6–8 mo	10,615 (8)	7.61	27 (9)	0.25
9–11 mo	7,543 (6)	4.5	21 (7)	0.28
12–59 mo	28,737 (22)	1.38	81 (28)	0.28
5–14 y	39,979 (30)	1.02	69 (24)	0.17
15–19 y	13,641 (10)	1.0	14 (5)	0.10
≥20 y	23,967 (18)	0.4	70 (24)	0.29

*Per 100 persons.
†Per 100 measles cases.

recent years in a timely manner, in 2010 Malawi faced its largest measles outbreak in >2 decades, with a number of reported cases comparable to the prevaccine era.

Malawi reported fewer deaths from measles than did other African countries where measles outbreaks recently occurred (*13*–*15*). Although measles-related deaths are known to be underreported, perhaps the best explanation for low numbers of reported deaths is the high measles vaccine coverage combined with the lower CFR among vaccinated case-patients (*16*,*17*). In addition, a high proportion of cases occurred in older children and young adults, who are at lower risk for death (*18*–*20*). The wide age distribution documented in this outbreak corresponds to a setting with good coverage and adequate vaccine effectiveness but insufficient to achieve elimination (*18*).

The EPI measles coverage (first dose of MCV) was high according to administrative estimates and from the results of vaccine coverage surveys in selected districts. Nonetheless, children 9–11 months of age were one of the most affected age groups during the 2010 outbreak. The routine vaccine coverage was low for this group, suggesting that children are vaccinated toward the end of or after the recommended period for EPI. The main reason for nonvaccination was the belief that the child was too young to be vaccinated. This issue highlights the need to reinforce routine vaccination, even in high-performing programs such as that in Malawi, and to include clear advice on the age for vaccination (*19*). In Malawi, the first measles dose is recommended at 9–11 months; however, older children also should be vaccinated through the routine program if they have not been vaccinated at the recommended age.

ARs during the 2010 outbreak were highest for age groups not yet vaccinated or for children who had received only 1 dose of MCV (i.e., children born after the last SIAs in October 2008). However, ARs also were relatively high for age groups eligible for SIAs. The reported administrative coverage of the last SIA in 2008 was 98% (*10*), but survey results indicate that only 61% of children eligible for the SIA in 2008 were vaccinated. The discrepancy between survey figures and the administrative coverage might

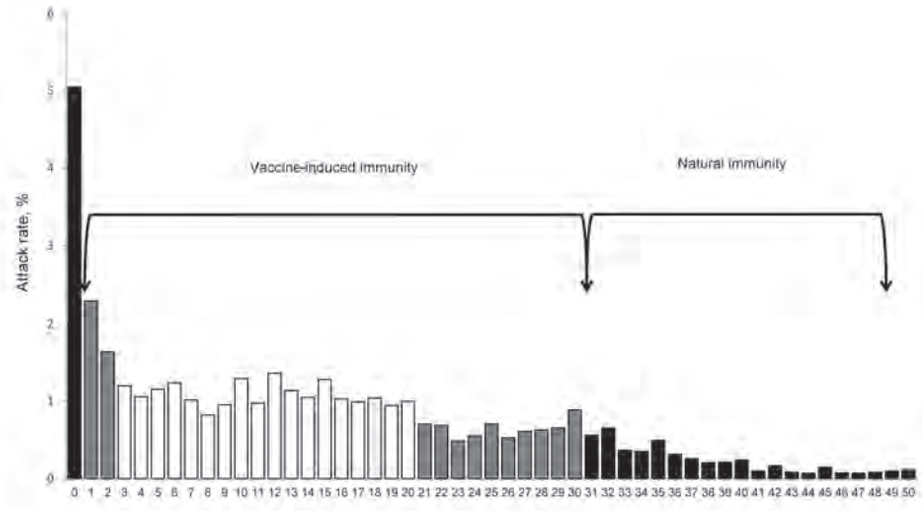

Figure 3. Age distribution of children with measles reported in Malawi, 2010. Vaccine-induced immunity of children in age groups vaccinated mostly through a 2-dose vaccination strategy, whether they have been offered 1 dose of vaccine (2 years, 21–30 years of age) or 2 doses of vaccine (3–20 years of age) and natural immunity of children in age groups mostly immunized through natural infection (>31 years of age). Black bars, no vaccine offered; gray bars, 1 vaccine dose offered; white bars, 2 vaccine doses offered.

be explained by denominator problems (administrative coverage), by possible recall bias (survey coverage), and by the fact that children out of the age groups toward which routine vaccination is directed are vaccinated during the SIA as documented in our survey. Most children vaccinated during the SIA already had been vaccinated through EPI; the SIA captured only one third of those not yet vaccinated.

We estimated the vaccine effectiveness for 1 dose to be 84%, meaning that each year 16% of children vaccinated through the routine program in Malawi are not protected. This percentage is expected for a vaccination that recommends giving a first dose to children at 9 months of age (the seroconversion rate at this age is 85%–90%) (*20*) but is insufficient to prevent measles outbreaks, as was shown in Malawi. One option for increasing vaccine effectiveness is to provide the first measles dose at 12 months, when seroconversion is more likely (*21*). WHO recommends this strategy in the absence of large outbreaks and documented good coverage. Nonetheless, when this schedule is followed, the children remain susceptible to measles infection for an additional 3 months, and in countries with a high birth rate (such as Malawi), this schedule may result in the buildup of a large cohort of susceptible children. Because the youngest children are at higher risk for measles-related death (*17*), careful consideration must be given to any increase in the age at first measles vaccination, especially if the risk for an outbreak remains high (*22*).

Reaching all children with 2 doses of MCV should be the standard for all national immunization programs—especially in countries with an elimination goal—to increase the seroconversion rates (*20*). However, in our study the estimated effectiveness of the second dose was lower than expected; for children receiving the second measles vaccine dose through the 2008 SIA, vaccine effectiveness was 91%. Factors that can reduce vaccine protection include failure in the cold chain, interaction with maternal antibodies, waning immunity, and the HIV pandemic (*23*). The vaccine is less effective in HIV-infected children because of quick loss of protective antibodies after immunization (*24*). In Malawi, ≈1%–2% of children <15 years of age are HIV infected (*25*). WHO recommends vaccination at 6 months in addition to the normal schedule in areas with high HIV prevalence and measles transmission (*20*), and incorporating this guidance into the routine program remains a priority.

Despite several reactive vaccination campaigns, the epidemic spread throughout Malawi. WHO guidelines on measles outbreak response suggest that reactive vaccination should be implemented as quickly as possible; at-risk groups and affected areas also should be considered as areas that are not yet affected but have high epidemic risk (*1*). The MoH campaign conducted early during the outbreak (March–April) was not wide enough in terms of focus population and geographic extension to contain the epidemic. The MoH/MSF nonselective campaigns were implemented in several districts for children 6 months–15 years of age, but more than one third of cases were reported among older persons. Despite overall high vaccine coverage obtained without major differences across districts, most of these campaigns were implemented late in the epidemic (during or after the peak) (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article12-0301-Techapp1.pdf). In addition, campaigns were conducted in some of the most affected districts but not in neighboring districts with high transmission. The national mass vaccination campaign conducted by the MoH in August (reported administrative vaccine coverage of 107%) also was implemented late in the course of the epidemic and also focused on persons <15 years of age. The timeliness of the intervention is probably the major factor determining the effect of the outbreak response; the delay in Malawi probably decreased the effect of the immunization campaigns. Our experience in 2010 shows that better preparedness plans for outbreak response are needed to improve the timeliness of

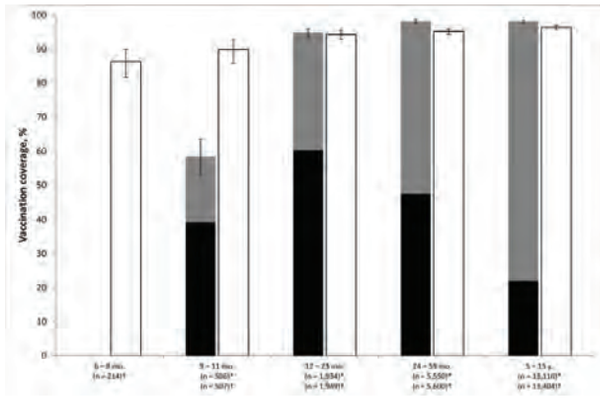

Figure 4. Measles vaccine coverage for the Expanded Program on Immunization and outbreak-response immunization conducted by the Ministry of Health and Médecins Sans Frontières in 8 districts (Blantyre, Mzimba, Lilongwe, Thyolo, Chiradzulu, Mangochi, Balaka, and Machinga), Malawi, 2010. *EPI; †ORI; black bars, vaccination ascertained by health passport; gray bars, EPI vaccination ascertianed by oral reporting; white bars, ORI vaccination ascertained by oral reporting.

Table 2. Measles vaccination coverage estimates in 8 districts, Malawi, 2010*

Vaccination coverage, age group	Estimates, % (95% CI)						
	Blantyre	Chiradzulu	Lilongwe	Mzimba	Thyolo	Mangochi	Bal-Mach
Routine EPI vaccination, 9 mo–15 y	98 (96–98)	99 (98–99)	95 (92–97)	95 (93–97)	97 (96–98)	96 (95–97)	98 (97–99)
MoH/MSF reactive campaign, 6 mo–15 y	93 (91–94)	98 (97–98)	96 (94–97)	92 (90–94)	97 (95–97)	96 (95–98)	96 (94–97)
SIA October 2008, 9 mo–5 y	47 (40–54)	57 (52–63)	60 (55–65)	47 (41–53)	69 (64–74)	63 (57–68)	6 (63–74)

*Bal-Mach, Balaka-Machinga; EPI, Expanded Program on Immunization; MoH/MSF, Ministry of Health/ Médecins Sans Frontières; SIA, supplementary immunization activity.

Table 3. Results from the vaccine effectiveness study in 8 districts, Malawi, 2010*

Vaccination status	Total	No. cases	Attack rate, %†	Vaccine effectiveness	
				Crude, % (95% CI)	Adjusted, % (95% CI)‡
Unvaccinated	793	91	13.3	Referent	Referent
Vaccinated with MCV					
1 dose§	2,512	62	2.4	82.0 (72.9–88.1)	83.9 (70.8–90.8)
2 doses¶	1,777	22	1.1	91.6 (84.7–95.4)	90.5 (79.7–95.5)

*Districts are Blantyre, Mzimba, Lilongwe, Thyolo, Chiradzulu, Mangochi, Balaka, and Machinga. MCV, measles-containing vaccine.
†Weighted and adjusted by the study design.
‡Adjusted by age, sex, and literacy of the caregiver.
§Routine immunization.
¶Routine immunization and 2008 supplementary immunization activities.

such interventions. Prior evidence favored conducting timely reactive campaigns directed toward children 6 months–15 years to maximize cases averted (*26*). However, in areas such as Malawi, where older age groups are highly affected, efforts directed toward persons >15 years of age would have had a greater effect on the transmission dynamics.

Our field studies have some key limitations. First, despite the effort to detect and report all suspected measles cases and related deaths to the national surveillance system, only case-patients seeking treatment at health facilities were recorded. Thus, the actual number of measles cases and measles-related deaths remain unknown. In addition, completeness of case reporting and sensitivity of the surveillance system were not assessed. However, health services are accessible and functional in Malawi, and free treatment for measles was provided during the epidemic; moreover the survey results showed that most of the children were vaccinated at a health facility. Despite the effort to standardize procedures for data collection in all districts, active surveillance was reinforced more in districts where case management was implemented by MSF. Thus, differences in number of cases reported might partially reflect the difference in performance of the surveillance system.

Second, we conducted surveys only in districts where reactive vaccination campaigns were implemented, which makes extrapolation to the country difficult. In addition, because not all children had health passports, vaccination coverage was assessed by parental recall, leading to possible overestimation or underestimation of coverage. To minimize misclassification of verbally reported vaccination status, we asked parents to name the vaccination place and the part of the body where the vaccine was delivered (e.g., shoulder, leg, other) to determine whether the caregiver correctly remembered a vaccine consistent with measles vaccine delivery. Previous studies in areas of high measles incidence have shown parental recall to be reliable (*27*). We suspect underreporting for the 2008 SIA given that all the information for this estimate was collected from verbal reporting because documentation was not available and the recall period was long.

Third, misclassification of SIA vaccination status might have decreased the vaccine effectiveness estimate for the second dose (*28*) and should be considered as an additional reason to explain the lower-than-expected vaccine effectiveness for 2 doses. Regarding the retrospective ascertainment of measles cases, we used the local term for measles to increase the sensitivity and specificity of the case definition (*29*). However, if misclassification is present, it probably does not differ for vaccinated and nonvaccinated children. Finally, we did not collect information about measles-related deaths, which might slightly downward bias our estimate of vaccine efficacy.

Measles and measles-related deaths have decreased dramatically during recent decades in Malawi thanks to a comprehensive measles control strategy. However, our results highlight the difficulties in avoiding large outbreaks, even with successful routine programs. Control programs need to be adapted to the epidemiologic context, including age range for routine vaccination. SIAs are crucial for reducing the number of susceptible children. This SIA strategy has been successfully implemented in the Americas, which achieved measles elimination in 2003, but reality shows the complexity of obtaining similar outcomes in other locations. SIAs could focus on a wider age range if older nonvaccinated persons are expected. We also highlight the need for the timeliness and choice of the population outbreak response immunizations. Age groups toward which vaccination efforts are directed should be determined according to local measles epidemiology. To provide timely and adequate responses in similar contexts, better preparedness plans for possible outbreaks based on proper risk assessments, including good estimates of vaccine coverage, are urgently needed.

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analyzed by A.M., H.H., H.C., D.N., S.N., and F.J.L. The first draft of the manuscript was written by A.M. and F.J.L. All authors contributed to the writing of the manuscript and agree with the results and conclusions.

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Nipah Virus Infection Outbreak with Nosocomial and Corpse-to-Human Transmission, Bangladesh

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Active Nipah virus encephalitis surveillance identified an encephalitis cluster and sporadic cases in Faridpur, Bangladesh, in January 2010. We identified 16 case-patients; 14 of these patients died. For 1 case-patient, the only known exposure was hugging a deceased patient with a probable case, while another case-patient's exposure involved preparing the same corpse for burial by removing oral secretions and anogenital excreta with a cloth and bare hands. Among 7 persons with confirmed sporadic cases, 6 died, including a physician who had physically examined encephalitis patients without gloves or a mask. Nipah virus–infected patients were more likely than community-based controls to report drinking raw date palm sap and to have had physical contact with an encephalitis patient (29% vs. 4%, matched odds ratio undefined). Efforts to prevent transmission should focus on reducing caregivers' exposure to infected patients' bodily secretions during care and traditional burial practices.

In Bangladesh, 135 probable or confirmed cases of Nipah virus (NiV) infection in humans were identified from 2001 through 2008; 98 (73%) were fatal (*1*). Drinking raw date palm sap, contaminated by NiV from urine or saliva of *Pteropus* spp. fruit bats, has been identified as a vehicle for transmission of NiV to humans in Bangladesh (*2*,*3*). NiV, an RNA paramyxovirus (*4*), was isolated from human respiratory secretions, saliva, and urine during the outbreaks (*5*,*6*). Outbreak investigations in Bangladesh and India have repeatedly implicated person-to-person transmission of NiV, including health care–associated transmission in the Siliguri, India, outbreak in 2004 (*7*–*10*). However, to our knowledge, no evidence of NiV transmission to health care workers had been confirmed in Bangladesh (*11*).

In the area where NiV outbreaks have been repeatedly identified (Figure 1), the Institute for Epidemiology, Disease Control and Research (IEDCR) of the Government of Bangladesh, in collaboration with icddr,b (formerly the International Centre for Diarrhoeal Disease Research in Bangladesh) is conducting hospital-based encephalitis surveillance. To detect outbreaks of NiV infection, the surveillance system identifies sporadic NiV cases during January–March and clusters of encephalitis patients throughout the year. On January 14, 2010, two cousins living in the Faridpur District in Bangladesh (Figure 1) were admitted to Faridpur Medical College Hospital (FMCH) with fever and altered mental status. A team from IEDCR and icddr,b initiated an investigation on January 15, 2010. The objectives of the investigation were to identify the cause of the outbreak and to detect sporadic cases of NiV infection.

Methods

Case Identification and Specimen Collection

Hospital Surveillance

Surveillance physicians maintained a registry of patients who sought treatment with fever or with history of fever with axillary temperature >38.5°C (101.3°F), altered mental status, new onset of seizures, or a new neurologic deficit, either diffuse or localized to the brain (*12*). The

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physicians collected whole blood, cerebrospinal fluid, a throat swab specimen, and urine samples from patients admitted during January–March 2010 in 3 of the 6 active surveillance hospitals (Faridpur, Rajshahi, and Rangpur Medical College Hospitals) and during January–April 2010 at Faridpur Medical College Hospital. These samples were stored in liquid nitrogen in local hospitals and transported biweekly to the IEDCR laboratory.

Outbreak Area

We defined possible case-patients as persons with acute onset of fever living in the outbreak area with onset of illness from January 7 through January 28, 2010. We identified possible case-patients by conducting door-to-door visits of all homes and contacting local physicians in the affected villages. We defined probable case-patients as persons who met the possible case definition and who had new onset of altered mental status or new onset of breathing difficulty. We defined case-patients with laboratory-confirmed NiV infection as those with detectable serum IgM against NiV. To assess asymptomatic NiV infection in the outbreak community, we asked community members who had close physical contact or had shared date palm sap from the same pot with probable or confirmed case-patients within the preceding month, to provide a blood specimen for serologic testing.

We obtained a clinical history and information about exposures during the month before illness from each probable and confirmed case-patient. Friends, relatives, and neighbors of deceased or unconscious case-patients served as proxy informants for interviews.

The investigation team collected acute-phase and subsequent whole blood specimens from possible case-patients. Samples were centrifuged in the local government community clinic, and the separated serum was stored and transported to the IEDCR laboratory in liquid nitrogen. Samples were stored at –70°C.

Laboratory Testing

We tested serum samples at IEDCR with an IgM capture enzyme immunoassay that detects NiV IgM (*13*). We shipped an aliquot of serum, cerebrospinal fluid, throat swab specimens, and urine from patients with probable and confirmed cases of NiV infection and from those with IgM against NiV to the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, for confirmatory testing.

In-Depth Interviews

We conducted in-depth interviews with families of case-patients to explore history of illness and exposures of case-patients. We also had informal discussions with neighbors of case-patients to explore possible modes of transmission.

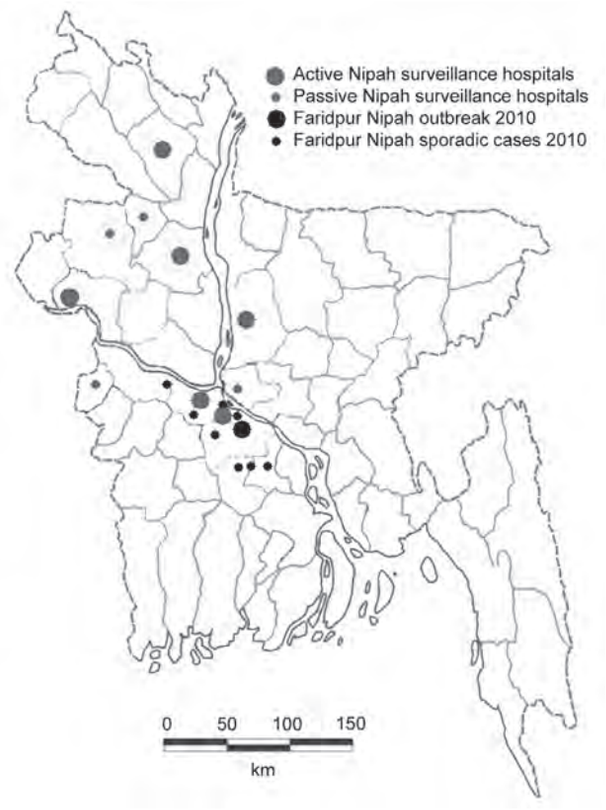

Figure 1. Surveillance hospitals and locations of outbreak clusters and sporadic cases of Nipah virus infection, Bangladesh, 2010.

Case–Control Study

We conducted a matched case–control study to identify risk factors for transmission of NiV. Persons with probable and confirmed cases from the outbreak and with sporadic cases identified from surveillance were considered to be case-patients. The field team selected 4 neighborhood controls for each case-patient, starting from the fourth closest courtyard to the case-patient's residence. The courtyards where other case-patients resided were excluded. In each courtyard, only the household closest to the main entry was selected. The age of every person in the selected household was recorded. Then a control (only 1 from each courtyard), whose age was closest to the case-patient's age, was selected. If that control was absent during the first visit, the team tried 3 times to reach the control. If the team was unsuccessful, no control was selected from that household. This process was repeated at the next closest courtyard household until we had selected the required number of controls.

All case-patients, except 1, were either too sick or too confused to respond or had died, so the field team selected multiple appropriate proxy respondents for interview. We used standardized, structured questionnaires in the Bengali language.

Statistical Analyses

To estimate the association between each exposure and NiV infection, we calculated the matched odds ratio (mOR). We used conditional logistic regression and considered any association to be statistically significant if the p value was <0.05. We analyzed data in STATA 10 (Stata Corp., College Station, TX, USA).

Ethics Approval

Legal guardians of study participants and healthy adult participants provided informed verbal consent for participation in this investigation. The Ethical Review Committee of icddr,b reviewed and approved the protocol for NiV surveillance and outbreak investigation.

Results

Cases

Hospital Surveillance

During January–April 2010, surveillance physicians from the 6 hospitals reported 328 meningoencephalitis case-patients. Of these, 106 (32%) were reported by surveillance physicians in Faridpur, including 4 outbreak case-patients. Ninety-seven (92%) serum samples were collected at the Faridpur surveillance site. Of these, 12 had IgM against NiV (12%), including 4 from the outbreak area.

Outbreak

The outbreak investigation team identified 100 possible NiV-infected persons with febrile illness in the outbreak area during January 5–28, 2010. Of these, 68 persons gave a blood sample for NiV laboratory diagnosis. One person's sample was positive for IgM against NiV and that person was classified as a confirmed NiV-infected case-patient. We collected 4 blood samples positive for IgM against NiV from 7 probable case-patients. The remaining 3 case-patients died before blood samples were collected. We defined these persons as probable NiV-infected case-patients. All but 1 case-patient had altered mental status, and two thirds of case-patients had breathing difficulty (Table 1). Seven (88%) of 8 persons who met the case definition for probable or confirmed NiV infection died. The onset of illness for all of case-patients occurred within 16 days of exposure. Four cases constituted an initial peak, and a second-generation outbreak of 4 cases appeared after contact with the initial case-patients (Figure 2).

Qualitative Findings on Exposure Histories

Sporadic Case-patients

Among 7 patients with sporadic cases of NiV infection, 6 of whom died, 5 had a history of drinking raw date palm sap in the month preceding illness onset, and 2 of these 5 were harvesters of date palm sap. One case-patient attended to a family member who was hospitalized at FMCH with an illness unrelated to encephalitis for 3 days, 4 days before the onset of illness. While staying in the hospital, he spent the days talking with and caring for other patients and slept alongside them.

Another case-patient was a physician in training at FMCH. From January 1 through February 28, 2010, he worked in the adult medicine and pediatric wards and clinically managed cases of meningoencephalitis in 14 patients, including 3 with confirmed NiV infection and other severely ill febrile patients who had not been enrolled in NiV surveillance. He performed physical examination, intravenous canalization, and nasogastric intubation for 1 confirmed case-patient in the adult medicine unit >1 month before the onset of his illness. He also similarly treated the condition of 2 confirmed NiV case-patients in the pediatric unit 4 days before his onset of illness. A handwashing station for physicians did not exist in either the adult medicine or pediatric wards, and his colleagues reported that he did not use gloves or a mask during patient care or wash his hands after patient care. His illness began on February 28, 2010, with fever and myalgia. Subsequently, he experienced coughing, respiratory distress, convulsions, altered mental status, and loss of consciousness. He died on March 6, 2010. The duration between onset of illness of the physician and his contact with 2 known NiV-infected case-patients in the adult medicine ward was 32 days, and the time from his contact with the 2 children with NiV infection to illness was 2–4 days. Two NiV genomic sequences, obtained from 2 of 14 meningoencephalitis patients that the physician had been in contact with, did not match the NiV genomic sequence of the physician's isolates (*14*).

Outbreak Case-patients

Case-patient A, a 45-year-old man with a probable case of NiV infection, lived under a *Pteropus* bat roost. He drank raw date palm sap from his own date palm tree during the last week of December 2009, 1 week before the onset of illness on January 7, 2010. He had fever, headache, and myalgia, which progressed over 6 days to drowsiness, convulsions, confusion, unconsciousness, and death.

On January 7, three neighbors of case-patient A, from a single family (case-patients B, C, and D), bought and drank raw date palm sap from the village date palm sap harvester. All became ill on January 9, within hours of each other. None had any history of contact with any person who had symptoms similar to encephalitis. Probable case-patients A and B died on January 13; confirmed case-patients C and D died on January 16 and 17 (Figure 2).

Among those with second-generation cases, case-patient E (confirmed case) was the wife of case-patient A

Table 1. Demographic and clinical features of outbreak and sporadic case-patients with encephalitis caused by Nipah virus infection, Faridpur, Bangladesh, 2010*

Feature	First-generation outbreak, n = 4	Second-generation outbreak, n = 4	Sporadic, n = 8	All, n = 16
Median age, y (range)	28 (10–45)	55 (32–60)	23 (4–45)	35 (4–60)
Male sex	1 (25)	3 (75)	5 (63)	9 (56)
Clinical features				
Fever	4 (100)	4 (100)	8 (100)	16 (100)
Altered mental status	4 (100)	3 (75)	8 (100)	15 (94)
Unconscious	4 (100)	2 (50)	8 (100)	14 (88)
Difficulty breathing	2 (50)	3 (75)	7 (88)	12 (75)
Headache	4 (100)	2 (50)	4 (50)	10 (63)
Vomiting	4 (100)	1 (25)	3 (38)	8 (50)
Convulsion	3 (75)	1 (25)	3 (38)	7 (44)
Case-fatality rate	4 (100)	3 (75)	7 (88)	14 (88)
Median days (range) from onset of illness to death	7 (4–8)	6 (3–7)	4 (4–17)	5 (3–17)†

*Values are no. (%) case-patients except as indicated.
†n = 14, all of whom died.

and was involved in feeding, comforting, and transporting her husband to FMCH and in bringing his dead body back home on January 13. She cleaned saliva from her husband using her bare hands and did not wash her hands afterwards. She also drank raw date palm sap with case-patient A in last week of December and did not get ill. On January 15, 2010, a fever developed, but she did not have altered mental status. She recovered on the fourth day of illness and was a confirmed case-patient who survived. Case-patient F (confirmed case) was a friend of case-patient A, who frequently visited case-patient A during case-patient A's illness. When case-patient A's condition deteriorated, case-patients E and F took him to the hospital. Case-patient A had to be supported by case-patient F, and the distance between the faces of case-patients A and F was <1 foot while case-patient A was coughing, salivating, and having difficulty breathing. While bringing the dead body back from hospital by bus, case-patient A's head rested on the thigh of case-patient F. Case-patient G (confirmed case) was the uncle of case-patient A who lived in the same village, and he never visited case-patient A during his illness. He had no contact with any other encephalitis case-patient. He arrived only after case-patient A died and had caressed the head of the corpse before the ritual corpse bathing. Case-patient H (probable case), was a neighbor of case-patient A, who did not come into contact with case-patient A during his illness. He carried out the Muslim practice of ritual purification by cleaning the body and washing it. He used pieces of cloth to clean the body orifices (anus, urethra, oral and nasal secretions) with an ungloved hand. He then washed the entire corpse with water. He took a bath 1 hour after the ritual cleansing. Case-patient G came in contact with the corpse of case-patient A 3 hours after case-patient A's death, and case-patient H came in contact with the corpse of case-patient A 6 hours after case-patient A's death. None of these 3 case-patients (F, G, and H), who cared for case-patient A before or after his death, had drunk raw date palm sap in the preceding month. Their illnesses began 8–12 days after contact with case-patient A, they had symptoms similar to those of case-patient A, and they died on January 27 or January 28, 2010, after 4–8 days of illness.

Case–Control Study

The field team enrolled 15 case-patients and 58 controls (Table 2). The mean age of the case-patients and controls was similar (mean age [±SD] 31 ± 5 years for case-patients vs. 30 ± 2 years for controls, $t = -0.24$, $p = 0.81$). Among case-patients with NiV infection and neighborhood controls, NiV case-patients were more likely than controls to have consumed raw date palm sap during the month before the case-patient's illness (69% in case-patients vs. 30% in controls, matched odds ratio [mOR] 7.9, $p = 0.01$) and were more likely than controls to have been in the same room as case-patients (29% vs. 4%, mOR undefined, $p<0.01$) or to have touched (29% vs. 4%, mOR undefined, p value undefined) case-patients.

Discussion

During 2010, we identified an outbreak and several sporadic cases of encephalitis caused by NiV infection. Two case-patients from the outbreak and 3 patients with sporadic cases had IgM against NiV in serum and NiV RNA in oropharyngeal swab samples by conventional and real-time reverse transcription PCR (*14*). We could not collect biological specimens from 3 probable outbreak case-patients, including the source case-patient; however, the onsets of illness of patients with confirmed and probable cases were within 3 weeks of each other in an area where NiV outbreaks have been repeatedly confirmed over the past decade (*2*,*3*,*7*–*10*,*15*,*16*). Clinical features of fever, evidence of brain involvement, and rapid progression to death were also consistent with previous NiV outbreaks (*2*,*3*,*8*–*10*,*12*).

The first 4 case-patients of the initial phase of the outbreak, and 6 of 8 of the patients with sporadic cases, apparently contracted NiV infection by drinking raw date

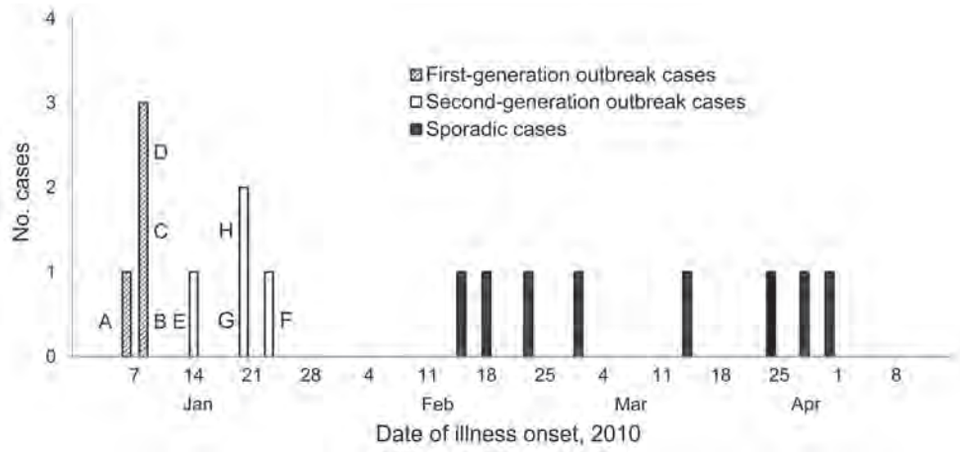

Figure 2. Nipah virus infection cases, Faridpur, Bangladesh, 2010. A–H indicate specific case-patients.

palm sap contaminated with NiV by *Pteropus* bats, an exposure that has been linked to NiV infection in previous outbreaks (*3*,*17*).

The remaining 4 case-patients from the outbreak probably acquired NiV infection from physical contact with the source case-patient. Such person-to-person transmission has been observed in prior NiV outbreaks in Bangladesh (*7*–*10*). The 2 generations of transmission are reflected in the 2 peaks in the epidemiologic curve (Figure 2). Among the second-generation cases, a novel finding was the transmission of NiV from the corpse of the source case-patient to 2 persons who had contact with the corpse before burial. This is the most plausible transmission pathway, because they did not have known exposures to living persons with encephalitis and had no history of drinking raw date palm sap. Because NiV is found in the respiratory secretions of NiV case-patients (*14*), case-patient G may have had intimate hand and facial contact with the corpse's respiratory secretions while performing ritual purification. Consistent with the culturally prescribed method of ritual bathing of a corpse, case-patient H did not wear a mask or gloves during cleansing of the corpse's orifices. He only used 3 pieces of cloth and his bare hands, which then were almost certainly contaminated with NiV. Case-patient H also likely touched his face or nose during or after the ritual purification. Persons commonly touch their own faces subconsciously, and 1 videotaped observational study found that persons touched their own eyes, nostrils, and lips 16 times per hour during normal activities (*18*). During Muslim ritual bathing, water is poured on the body (*19*). Thus, the water may have become contaminated with NiV and came in contact with case-patient H's clothes and body. Similar to other infectious diseases, including severe acute respiratory syndrome and measles, the transmission efficiency of individual NiV case-patients varies (*17*,*20*,*21*). Case-patient A was an unusually efficient spreader of NiV, perhaps because of an unusually high concentration of NiV in his oral secretions.

The dead bodies of all NiV-infected patients who are Muslim in Bangladesh have undergone the same process of ritual bathing, but to our knowledge, corpse-to-human transmission has not been previously recognized. In other NiV outbreaks when NiV infection developed in family members, many persons had contact with the source case-patient during illness and when preparing the corpse, so we were unable to separately assess corpse-to-person transmission. This investigation suggests that occasional NiV transmission could occur during the Muslim ritual purification of a corpse before burial.

This study also documents the death of a physician in Bangladesh from NiV encephalitis after he cared for NiV-infected patients with encephalitis in the surveillance hospital. The physician's colleagues and roommates did not report any history of his drinking raw date palm sap during the month preceding onset of illness. Although the physician had contact with oral secretions of several meningoencephalitis patients during the outbreak, the genetic sequence of NiV found in the physician was distinct from those of 2 hospitalized NiV-infected case-patients who were positive for NiV by reverse transcription PCR (*14*). Indeed, none of the 3 hospitalized patients with confirmed NiV infection was likely to have been the source of the physician's infection. The duration between onset of illness of the physician and his contact with confirmed NiV case-patients was beyond the range of the 6- to 11-day incubation period for NiV (*12*,*17*). During the assumed time of exposure to NiV, he cared for patients in the adult medicine ward; some of them may have had NiV infections that were missed by hospital surveillance. However, we did not identify any patient who met the case definition for meningoencephalitis in that ward 6–11 days before onset of the physician's illness. The clinical spectrum of human NiV infection in Bangladesh also includes patients who sought treatment with respiratory disease as the primary manifestation (*12*), and surveillance may have missed any NiV-infected persons on the ward with this clinical

Table 2. Bivariate analysis of risk factors for Nipah virus infection, Faridpur, Bangladesh 2010*

Risk factor	No. (%) case-patients with risk factors, n = 15	No. (%) controls with risk factors, n = 58	mOR (95% CI)	p value
Male sex	8 (53)	26 (46)	1.3 (0.4–4)	0.62
Climbed tree	5 (33)	16 (29)	1.6 (0.7–3.7)	0.24
Physical contact with living animal				
Cow	11 (73)	32 (57)	2.5 (0.6–9.9)	0.2
Goat	5 (33)	26 (46)	0.4 (0.1–2.2)	0.3
Pig	0	1 (2)	Undefined	Undefined
Chicken	10 (67)	39 (70)	0.9 (0.3–2.9)	0.83
Duck	8 (53)	20 (36)	1.9 (0.7–5.8)	0.23
Dog	1 (7)	4 (7)	0.9 (0.1–7.9)	0.91
Cat	1 (6)	7 (13)	0.4 (0.1–3.9)	0.45
Fruit bat	0	0	Undefined	Undefined
Physical contact with sick animal				
Cow	0	4 (7)	Undefined	Undefined
Goat	1 (7)	1 (2)	0.9 (0.4–2)	0.76
Chicken	0	9 (16)	Undefined	Undefined
Duck	1 (7)	3 (5)	1.2 (0.1–15)	0.89
Ate any animal that had been sick	1 (7)	3 (5)	1.2 (0.1–15)	0.89
Had seen bats in or around residence at night	12 (80)	39 (70)	1.2 (0.1–15)	0.89
Drank raw DPS	9 (69)	17 (30)	7.9 (1.6–40)	0.012
Drank DPS before 9:00 AM	8 (53)	16 (100)	Undefined	Undefined
DPS was				
Purchased	2 (20)	5 (33)	0.4 (0.04–4.8)	0.49
Given	2 (20)	4 (27)	Undefined	Undefined
Collected by a family member	6 (67)	5 (33)	Undefined	Undefined
Consumption of raw DPS during month before onset of illness, per day				
0	5 (33)	40 (71)	1	
<1 glass	4 (27)	4 (7)	32 (2.1–474)	0.01
1 glass	3 (20)	9 (16)	4.3 (0.6–29)	0.13
>1 glass	3 (20)	3 (5)	22 (1.2–404)	0.04
Is DPS harvester by profession	4 (27)	4 (7)	8.7 (0.9–83)	0.06
Household member harvests DPS by profession	4 (27)	4 (7)	8.7 (0.9–83)	0.06
Household distributes or sells DPS	4 (27)	5 (9)	10 (1.1–100)	0.04
Ate fruit				
Banana	11 (73)	33 (60)	1.9 (0.5–6.5)	0.32
Boroy/plum	7 (47)	29 (53)	0.8 (0.2–3.1)	0.75
Papaya	8 (53)	29 (52)	1.1 (0.3–3.2)	0.95
Sofeda	3 (20)	14 (25)	0.7 (0.1–3.7)	0.67
Kamranga	0	6 (11)	Undefined	Undefined
Guava	4 (29)	14 (25)	1.4 (0.2–8.4)	0.71
Tamarind	1 (7)	8 (14)	0.4 (0.4–3.7)	0.42
Custard apple	1 (7)	1 (2)	Undefined	Undefined
Visited another subdistrict	5 (33)	14 (25)	1.6 (0.4–5.7)	0.47
Touched someone with fever and altered mental status who died later	4 (29)	2 (4)	Undefined	Undefined
Was in same room as someone with fever and altered mental status who died later	4 (29)	2 (4)	Undefined	Undefined

*mOR, matched odds ratio; DPS, date palm sap.

manifestation. Another line of evidence suggests that an unidentified NiV-infected patient was hospitalized on that adult medicine ward at that time. One patient with a sporadic case, who visited FMCH as a family caregiver, also provided care for several patients in the men's medicine ward during the same days that the physician attended to patients on that ward. This case-patient may have come in close physical contact with the same unidentified NiV-infected case-patient as the physician.

During 2001, health care workers were infected by NiV in Siliguri, India. Among 66 infected persons, 45 case-patients were hospital staff or family caregivers attending to the patients, and 11 patients were infected from an unidentified, hospitalized index case-patient (*22*). However, during an NiV outbreak in Bangladesh in 2004, health care providers (using minimal personal protective equipment [PPE] and with substantial exposure to NiV case-patients) had no evidence of having acquired NiV infection (*11*). During 2010 in Faridpur, NiV was transmitted from person to person in community and hospital settings. The observed differences in risk for person-to-person transmission between outbreaks suggest that NiV strains may differ in their proclivity for person-to-person transmission.

Because NiV infection is not the major cause of acute meningoencephalitis in Bangladesh, and because

most persons who contract NiV infection have died by the time a diagnosis is made, it is difficult to identify a strategy to prevent person-to-person transmission that could be consistently applied to NiV-infected case-patients. Strategies to reduce care providers' exposure to respiratory secretions could prevent a broad array of saliva-transmitted infections, including NiV encephalitis. Prevention approaches to reduce corpse-to-person transmission of NiV and other potentially fatal respiratory secretion-transmitted viruses should focus on minimizing exposure to saliva and other bodily fluids from the body of a person who died of severe febrile illness. Wearing gloves and a mask during the handling and washing of a dead body before burial would not be feasible in low-income communities, where the annual total per capita spending on health is US $12 per person per year (*23*). Research to identify culturally acceptable cost-effective approaches that can be consistently implemented in low-income settings, for example, washing hands thoroughly with soap and water immediately after corpse contact, could save lives.

This report of nosocomial transmission of NiV to a health care worker in Bangladesh after caring for NiV-infected patients highlights the risk of working without PPE. Barriers to developing an appropriate prevention strategy for nosocomial transmission of NiV in hospitals in Bangladesh include the following: inadequate supplies of PPE for hospital staff, absence of isolation wards, absence of handwashing facilities in hospital wards and physicians' rooms, and inadequate training and monitoring for infection control (*24*). Because saliva is the most likely vehicle for transmission of NiV among care providers, implementation of standard and contact precautions (*25*) that have been culturally and economically customized to fit this setting could reduce NiV transmission. As a first step, we recommend that handwashing stations be established and consistently supplied with soap and water in every ward of the hospital for health care workers and patient attendants. Second, because laboratory diagnosis for NiV infection is not available during the initial evaluation of patients with meningoencephalitis syndrome, during NiV season all hospitals in NiV infection–prone areas should admit patients with meningoencephalitis syndrome into an isolation room or ward and routinely provide gloves and masks for health care workers when they are caring for meningoencephalitis patients. Patient attendants could reduce their exposure to patient saliva and respiratory secretions by frequent handwashing and by avoiding sharing food and beds with patients.

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Laboratory-based Surveillance for Hepatitis E Virus Infection, United States, 2005–2012

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Learning Objectives

Upon completion of this activity, participants will be able to

- Describe the percentage of hepatitis E cases among US patients with hepatitis who were seronegative for acute hepatitis A and B, including those who had and those who had not traveled abroad,
- Compare characteristics of nontravelers vs travelers with hepatitis E, and
- Describe HEV genotypes among nontravelers vs travelers with hepatitis E.

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To investigate characteristics of hepatitis E cases in the United States, we tested samples from persons seronegative for acute hepatitis A and B whose clinical specimens were referred to the Centers for Disease Control and Prevention during June 2005–March 2012 for hepatitis E virus (HEV) testing. We found that 26 (17%) of 154 persons tested had hepatitis E. Of these, 15 had not recently traveled abroad (nontravelers), and 11 had (travelers). Compared with travelers, nontravelers were older (median 61 vs. 32 years of age) and more likely to be anicteric (53% vs. 8%); the nontraveler group also had fewer persons of South Asian ethnicity (7% vs. 73%) and more solid-organ transplant recipients (47% vs. 0). HEV genotype 3 was characterized from 8 nontravelers and genotype 1 or 4 from 4 travelers. Clinicians should consider HEV infection in the differential diagnosis of hepatitis, regardless of patient travel history.

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Hepatitis E in Africa, southern and central Asia, and Central America causes occasional outbreaks of jaundice and, between outbreaks, occurrences of sporadic jaundice. Primarily spread by waterborne transmission, the disease tends to resolve spontaneously, although fulminant hepatic failure can ensue (*1*). In eastern Asia and Europe, sporadic hepatitis E, whether imported after return from international travel or acquired indigenously, has been observed; the indigenous form is thought to be foodborne (*2*). Although the disease is largely self-limiting, in Europe, chronic hepatitis E, which may lead to cirrhosis, is increasingly recognized among solid-organ transplant recipients (SOTRs) (*3*).

The causative agent of hepatitis E is hepatitis E virus (HEV), of which 4 genotypes are found in humans. Genotypes 1 and 2 circulate in regions where waterborne transmission is common; genotype 3 is prevalent in eastern Asia and the West and genotype 4 in eastern Asia. Genotypes 1 and 2 infect humans, but genotypes 3 and 4 infect humans and animals, predominantly pigs (*4*).

In the United States, HEV imported into the country after travel to regions to which waterborne HEV transmission is endemic is well recognized (*5–7*). Recently, 21% of participants of the US-based Third National Health and Nutrition Examination Survey were found seropositive for IgG anti-HEV (*8*). This unexpectedly high prevalence rate would not be ascribable to imported HEV infection alone. Indeed, cases of hepatitis E unassociated with travel abroad have been observed in the United States, implying infection by indigenous HEV strains (*9–14*). Moreover, the increasing number of reports from Europe of hepatitis E among SOTRs (*3*,*15*) suggests that SOTR in the United States might be similarly susceptible to the disease.

We report a study of demographic, clinical, travel-related, and virologic characteristics of persons with hepatitis E derived from a diverse patient base. Critical to this investigation was the application of a validated serologic assay for detecting IgM anti-HEV (*16*), the marker of recent HEV infection, as well as a real-time reverse transcription PCR (RT-PCR) that had been validated to detect, to high sensitivity, HEV RNA (*17*), which is an indicator of active HEV shedding. Together, these 2 assays enabled us to identify patients with incident hepatitis E.

Methods

Samples and Patients

The Centers for Disease Control and Prevention (CDC) conducts HEV testing of serum and stool samples referred by health care providers, public health departments, and diagnostic laboratories in the United States (*18*). Referrers are requested to fill out a standardized questionnaire of patients' demographics, clinical and laboratory test features, and risks for HEV infection, including recent international travel and destinations visited; the completed questionnaire is submitted along with the test specimens (*18*). Persons whose specimens were received during June 2005–March 2012 and reported as being negative for IgM against hepatitis A virus and hepatitis B core antigen, regardless of positivity for IgG against hepatitis C virus, were considered for inclusion into the study.

Assays

An earlier, pangenotypic evaluation by CDC of 6 serologic assays for IgM anti-HEV identified the assay manufactured by Diagnostic Systems (Saronno, Italy) as having the best performance characteristics (*16*). Its diagnostic sensitivity and specificity were 98% and 95.2%, respectively, and its analytic sensitivity was 9 Walter Reed Units/mL. For this study, the assay was used to detect IgM anti-HEV in test samples. IgG anti-HEV was tested by applying an assay from the same manufacturer. Serum and stool samples were tested for HEV RNA by a real-time RT-PCR, capable of detecting HEV genotypes 1–4 to a sensitivity of 4 HEV genome-equivalents/mL, to amplify a 69-bp fragment in open reading frame (ORF) 3 of the HEV genome (*19*). Application of that assay enabled our laboratory to attain perfect detection scores in a recent international evaluation of 20 laboratories conducting HEV RNA testing (*17*). Samples found to be positive for HEV RNA were subjected to another RT-PCR to generate amplicons from a 258-bp segment from ORF1, which were then processed for nucleotide sequencing and phylogenetic analyses (*20*).

Statistics

Distributions of variables were assessed by using the Kruskal-Wallis test and the χ^2 test with the Yates correction or the Fisher exact test, as appropriate. Univariate and bivariate data analyses were conducted by using Epi Info (wwwn.cdc.gov/EpiInfo/html/prevVersion.htm).

Case Definition

A case of hepatitis E was defined as illness in a person in whom IgM and IgG anti-HEV in serum or HEV RNA in serum or stool samples were detected. A person in whom IgM but not IgG anti-HEV was detected in serum was excluded unless HEV RNA was found or IgG anti-HEV was detected in follow-up serum samples. A person in whom IgG but not IgM anti-HEV was detected in serum samples was included if HEV RNA was found in serum or stool samples.

Results

Of 154 persons whose specimens fulfilled the inclusion criteria, 26 (17%) met the case definition for hepatitis

E. Case-patients were between 14–67 years of age (median 43 years); 19 (73%) were male. Fifteen (58%) were white, 9 (36%) South Asian, and 2 (8%) Hispanic. None were seropositive for IgG against HCV. Eighteen (69%) case-patients were jaundiced, and 7 (27%) were SOTRs, the allografts received being kidney (3), liver (2), kidney and pancreas (1), and heart and lungs (1). Fifteen case-patients (58%) who reported not having traveled outside the United States in the previous 2 months were classified as nontravelers; the remaining 11, who had traveled abroad, were classified as travelers.

The Table summarizes the demographic, clinical, and virologic data for individual case-patients. Compared with travelers, nontravelers were older (median age 61 vs. 32 years of age; $p<0.05$) and more likely to be anicteric (not jaundiced; 8/15 [53%] vs. 1/11 [8%]; $p = 0.02$). The nontraveler group also included fewer South Asians (1/15 [7%] vs. 8/11 [73%]; $p<0.001$) and more SOTRs (7/15 [47%] vs. 0; $p = 0.02$). Differences in sex distribution were not significant.

Three case-patients (NT6, NT7, and T9; Table) were documented to have fulminant hepatic failure. Two of them required liver transplantation; 1 died and 1 survived.

HEV RNA was amplified from 12 case-patients (46%). The rate of HEV RNA detection among SOTRs (5/7; 71%) was higher than that among non-SOTRs (7/19; 37%), but this difference was not significant. HEV genotype 1 was characterized from 3 travelers, genotype 3 from 8 nontravelers (including the 5 SOTRs), and genotype 4 from 1 traveler. The Figure displays the genetic diversity of HEV carried.

Discussion

We identified 26 case-patients with hepatitis E in the United States. No distinction was made between acute and chronic hepatitis E. Whereas acute hepatitis among non-

Table. Demographic, clinical, travel-related, and virologic characteristics for patients with hepatitis E, United States, 2005–2012*

Travel history and case-patient no.	Age, y/sex	Race/ ethnicity	State of residence	Transplant (organ)	Jaundice	Countries visited	Anti-HEV SCR IgM	Anti-HEV SCR IgG	HEV genotype	HEV RNA viral load†
No recent international travel‡										
NT1	61/M	White	FL	No	Yes	NA	7.5	5.7	3	NA
NT2	45/M	White	CA	No	Yes	NA	3.7	4	–	–
NT3	63/M	White	SD	Yes (kidney)	No	NA	7.2	5.4	3	NA
NT4	61/M	South Asian	IL	Yes (liver)	No	NA	1.9	5.9	3	NA
NT5	67/M	White	FL	No	Yes	NA	6.3	1.3	–	–
NT6	44/F	Hispanic	TX	No	Yes§	NA	3.1	3.7	3	NA
NT7	21/F	Hispanic	TX	No	Yes¶	NA	2.2	1.6	–	–
NT8	67/M	White	IL	Yes (heart and lungs)	Yes	NA	3	3.3	–	–
NT9	42/M	White	WI	No	Yes	NA	6	6.6	–	–
NT10	62/F	White	IL	Yes (kidney)	No	NA	2.9	8.9	–	–
NT11	26/M	White	PA	Yes (kidney)	No	NA	5.3	8.3	3	7.8×10^2
NT12	40/F	White	NY	Yes (kidney and pancreas)	No#	NA	7.7	12.9	3	1.4×10^3
NT13	64/M	White	CT	Yes (liver)	Yes	NA	9.2	1.3	3	1.4×10^4
NT14	29/F	White	MI	No	No**	NA	6.6	9.8	–	–
NT15	62/M	White	NY	No	No	NA	Neg	9.6	3	1.5×10^3
Recent international travel‡										
T1	35/M	South Asian	DE	No	Yes	India	2.3	4.5	1	1.8×10^2
T2	14/F	South Asian	TX	No	Yes	India	7.3	5.8	–	–
T3	32/F	South Asian	TX	No	Yes	India	3.7	5.8	–	–
T4	24/M	South Asian	TX	No	Yes	India	2.3	2	–	–
T5	35/M	White	IL	No	No	India and Indonesia	2.9	8.9	–	–
T6	24/M	White	MD	No	Yes	Afghanistan and Dubai	6.9	9.4	–	–
T7	63/M	White	AL	No	Yes	China	7.9	Neg	4	2.4×10^2
T8	23/M	South Asian	ME	No	Yes	Bangladesh	7.6	10.8	–	–
T9	53/M	South Asian	MD	No	Yes††	India	9.2	9.4	–	–
T10	66/M	South Asian	TX	No	Yes	India	5.5	11.7	1	1.8×10^2
T11	22/M	South Asian	MD	No	Yes	India	9.9	10.9	1	8.3×10^5

*HEV, hepatitis E virus; SCR, signal/cutoff ratio; NT, nontraveler; NA, not applicable (quantitative reverse transcription PCR not done); –,not detected or tested; Neg, negative; T, traveler.
†In genome-equivalents/mL.
‡Within 2 mo before illness or visit to physician.
§Fulminant hepatic failure developed but resolved (*14*).
¶Fulminant hepatic failure developed; patient died at time of liver transplantation (*14*).
#Initial illness was meningitis.
**Asymptomatic; tested for HEV because of recent miscarriage.
††Fulminant hepatic failure developed, requiring liver transplantation; patient survived.

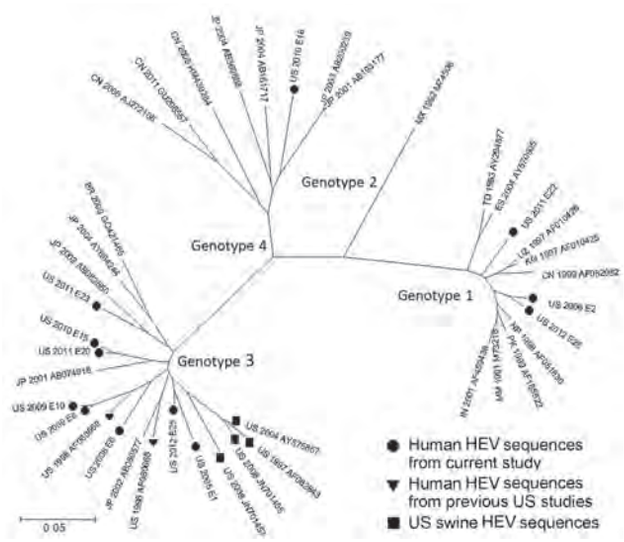

Figure. Genetic relatedness among hepatitis E virus (HEV) strains identified in hepatitis E cases, United States. Phylogenetic tree was constructed from a segment of HEV open reading frame 1 generated in MEGA5 (www.megasoftware.net) by using the neighbor-joining method. Country, year reported, and numeric or GenBank accession number assignment are denoted. Scale bar indicates genetic distance.

SOTRs was readily identifiable (most were jaundiced when test specimens were drawn), it was difficult to assess whether disease in SOTRs was at the acute or chronic stage during specimen sampling, because positivity for IgM anti-HEV or HEV RNA could reflect either stage (*3*). Thus, the case definition was kept broad to identify both stages of disease. As the study was not primarily prospective, the natural history of hepatitis E among the case-patients was largely unknown. Nevertheless, adverse outcomes could be documented for 3 case-patients, in whom fulminant hepatic failure developed.

Hepatitis E cases were found among persons who had not recently traveled abroad and those who had. Nontravelers tended to be older than travelers, a trend consistent with the finding recently reported by the Drug-Induced Liver Injury Network of 9 patients seropositive for IgM anti-HEV whose mean age was 67 years (*21*) and with similar observations in Japan and Europe (*1*,*2*,*22*). The higher proportion of anicteric persons in the nontraveler group reflects its inclusion of all SOTRs, which in Europe have been observed to have largely asymptomatic infections (*3*).

Nontravelers were infected exclusively by HEV genotype 3 strains. These strains clustered with HEV previously found in case-patients with nonimported acute hepatitis E (*9*–*12*,*14*) in the United States (Figure), suggesting that the nontravelers were infected by autochthonously circulating HEV. The similarity between HEV genotype 3 strains identified in nontravelers with those in swine (*4*) (Figure) suggests, but does not prove, HEV transmission linkage between humans and pigs (*2*). Evidence of HEV infection acquired after consumption of inadequately cooked meat and offal originating from pigs, boars, and deer has been reported from Japan (*2*) and France (*23*). Elsewhere, including the United States, evidence implicating non–travel-associated hepatitis E as a zoonosis remains weak (*24*).

The patient base from which hepatitis E cases were identified was nonselective, broad, and derived from multiple health care provider contexts. Nonetheless, data from this study were not generated from an established, systematic program of epidemiologic surveillance. Accordingly, the cases identified here may not fully represent the extent of hepatitis E in the United States. The larger number of cases among nontravelers likely reflects more persons living in the United States who do not travel abroad compared with those who do, and the many SOTRs identified with hepatitis E could be an overrepresentation resulting from increasing awareness among physicians of the predilection of the SOTR patient subpopulation to HEV infection (*3*,*15*). Future surveillance of hepatitis E may need to sample source populations from more diverse settings, such as gastroenterology/hepatology clinics (*21*), travel clinics (*6*), and the military (*7*). We recently reported findings from a study of HEV infection among immunocompromised patients other than SOTRs (*25*).

This study has provided insight into nonimported and imported hepatitis E in the United States. The nonimported form was observed to affect SOTRs, be able to lead to adverse outcomes, and be associated with infection by HEV genotype 3. The extent of nonimported hepatitis E in the United States merits further investigation, as does the role of autochthonous transmission of genotype 3 HEV strains. In clinical practice, entry of hepatitis E into the differential diagnosis of suspected hepatitis, regardless of the patient's travel history, would be appropriate.

Dr Drobeniuc is a microbiologist in the Division of Viral Hepatitis, Centers for Disease Control and Prevention. His research interests include laboratory diagnostics, assay development, quality assurance, and epidemiology relating to viral hepatitis.

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Severe Lower Respiratory Tract Infection in Early Infancy and Pneumonia Hospitalizations among Children, Kenya

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Severe lower respiratory tract infection (LRTI) in infants caused by respiratory syncytial virus (RSV) has been associated with later pneumonia hospitalization among children. To determine risk for pneumonia after RSV hospitalization in infancy, we conducted a retrospective cohort analysis of 2,813 infants admitted to a hospital in Kenya and identified readmissions for pneumonia among this group during early childhood (<60 months of age). Incidence of readmission for pneumonia was higher for children whose first admission as infants was for LRTI and who were <3 months of age than for children who were first admitted as infants for non-LRTI, irrespective of RSV status. Incidence of readmission for pneumonia with wheeze was higher for children whose first admission involved RSV compared with those who had non-RSV LRTI. Excess pneumonia risk persisted for 2 years after the initial hospitalization. Close postdischarge follow-up of infants with LRTI, with or without RSV, could help prevent severe pneumonia later in childhood.

Pneumonia is a major cause of illness and death among children <5 years of age in sub-Saharan Africa (*1*,*2*), and respiratory syncytial virus (RSV) is the most common viral cause of pneumonia and bronchiolitis in this age group (*3*,*4*). RSV infection in infancy is associated with other long-term respiratory problems (*5*–*10*) and, in one study, with pneumonia (*11*). The magnitude and duration of the increased risk for pneumonia after RSV infection are poorly defined (*12*). In addition, it is not clear whether this association is specific to RSV or whether other causes of lower respiratory tract infection (LRTI) in infancy are also associated with later pneumonia (*11*). A study in The Gambia reported an increased incidence of hospital admission for pneumonia, measurable up to 3 years after discharge (*11*).

We report results of a retrospective cohort analysis of children admitted to a rural district hospital in Kenya using data from a prospective longitudinal clinical surveillance project nested within a health and demographic surveillance system (*13*). The cohort was defined as all infants admitted to the hospital during 9 RSV seasons during 2002–2010; the infants were classified into exposure groups on the basis of the clinical features of LRTI and laboratory diagnosis of RSV at the first admission. The main outcome was readmission to a hospital for pneumonia before the age of 5 years.

Methods

Study Population

The study took place in the pediatric wards of Kilifi District Hospital (KDH), a rural hospital on the Indian Ocean coast of Kenya. Since 2001, data on all pediatric admissions and discharges of children at KDH have been prospectively collected in real time through an integrated online data management system in FileMaker Pro (FileMaker, Inc., Santa Clara, CA, USA). Starting from April 16, 2002, we linked admission records to the individual residence status of each child at the date of illness onset within the Kilifi Health and Demographic

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Surveillance System (KHDSS). The KHDSS covers a population of ≈250,000 living close to the hospital; ≈60% of pediatric admissions of children at KDH originate from KHDSS residents (*13*,*14*). The KHDSS fieldworkers enumerate births, deaths, and migrations (i.e., into, out of, and within the study area) every 4 months.

Since January 2002, continuous surveillance for RSV has been conducted among all children ≤60 months of age admitted to KDH for LRTI (*15*). A nasal specimen (nasal wash by bulb or nasopharyngeal aspirate) is collected from each patient by trained medical assistants soon after admission (*15*,*16*) and assayed for RSV antigen by immunofluorescence antibody testing (Imagen Respiratory Syncytial Virus Kit; Oxoid Ltd., Hampshire, UK; or Light Diagnostics Respiratory Viral Screen DFA Kit; Millipore, Temecula, CA, USA), according to the manufacturer's instructions.

For our study, the cohort was defined as residents of KHDSS admitted to KDH during the first year of life during RSV epidemics from April 16, 2002, through May 31, 2010. We classified the cohort into those who did or did not have LRTI on their first (index) admission. Among those with LRTI, we further subdivided the children on the basis of RSV immunofluorescence antibody test results. Children with LRTI not tested for RSV were examined in preliminary analyses and consolidated into the negative RSV test group. The 3 exposure groups in the cohort were the RSV LRTI group, the other LRTI group, and the non-LRTI group. Infants were excluded if they were admitted on the day of birth; had features of severe malnutrition (i.e., severe wasting, bipedal edema, or mid-upper arm circumference <11 cm); were born with low birthweight (<2.5 kg); had underlying congenital diseases; or remained in the hospital for >2 weeks. Evidence suggests that infants in these categories are at a higher risk for readmission and postdischarge death (*17*).

We used the linked databases of the KDH and KHDSS to determine which cohort members were subsequently readmitted to the hospital or died before their fifth birthday. The primary outcome was readmission to KDH for pneumonia. As secondary outcomes, we examined pneumonia with concurrent wheezing (a marker of hyperreactive airways), all-cause, and nonpneumonia readmissions, as well as all-cause mortality. The Kenya Medical Research Institute Scientific Steering Committee and the National Ethical Review Committee granted ethical approval for this study.

Definition of Terms

RSV epidemics were defined empirically as periods delimited by weeks in which ≥1 RSV cases were identified in our hospital surveillance and within which ≥3 RSV cases were found in any contiguous 3-week period, as described (*15*). Pneumonia was defined as history of cough or difficulty breathing and ≥1 of the following: fast breathing for age (>60 breaths/min if <2 months of age or >50 breaths/min if 2–11 months of age); lower chest wall indrawing; low oxygen saturation (<90%) by pulse oximetry; or inability to feed, prostration, or unconsciousness (*18*). LRTI was ascribed at first admission if the child met the criteria for pneumonia or if the clinician's discharge diagnosis included pneumonia, asthma, or bronchiolitis.

Statistical Analysis

Data were analyzed by using STATA version 11.2 (StataCorp, College Station, TX, USA). We used the Student *t* test, Mann-Whitney U test, χ^2 test, or Fisher exact test as appropriate. Admission to the cohort began at the date of first discharge and continued until the child reached 5 years of age, died, or migrated out of KHDSS, or until December 31, 2010, whichever was earliest. Cohort members who migrated back into the KHDSS were readmitted to the cohort at the date of in-migration.

Incidence rates were calculated as the number of outcome events among cohort members divided by the sum of child-years at risk within the cohort. Incidence rate ratios (IRRs) for pneumonia readmission between the study groups were estimated by using Poisson regression with Lexis expansion, adjusting for covariates related to the index admission (age, sex, admission to high dependency unit [HDU], geographic sublocation of residence, hospital access, hypoxia [oxygen saturation <90% by pulse oximetry], duration of hospital stay) and the follow-up period (RSV epidemics, age, presence of ≥1 readmissions for diagnoses other than LRTI). A cutoff of 43 admissions per 1,000 child-years, which was the median incidence of all pediatric admissions to the KDH in 2007, was used to create a binary variable for hospital access by administrative sublocation within the KHDSS.

A multivariable Poisson regression model was developed by using a forward stepwise procedure, rejecting variables with a p value ≥0.05 in likelihood ratio tests. Risk factors were introduced in descending order of strength of association determined from the univariate analysis. We used a robust variance estimator (Huber-White sandwich estimator) to account for within-person correlation of outcomes. We also performed time-to-event analysis with multiple-failure per person and single failure per person (censoring at first readmission with pneumonia), treating death as a competing risk.

Results

Baseline Description at Recruitment

The study recruitment period spanned 9 RSV epidemics lasting a total of 217 weeks. During these

weeks, 2,813 infants who met the eligibility criteria were admitted to the hospital; 560 had RSV LRTI, 1,140 had other LRTI, and 1,113 did not have LRTI (non-LRTI group). Of the children included in the other LRTI group, 341 (29.9%) were not tested for RSV. The baseline characteristics for children in the 3 groups are shown in Table 1.

Cohort Follow-up and Readmissions

The median durations of follow up (interquartile range) were 40.6 (21.4–57.8), 44.2 (22.0–57.6), and 43.9 (20.6–57.1) months for the RSV LRTI, other LRTI, and non-LRTI groups, respectively. Nine children initially in the non-LRTI group and 16 in the other LRTI group were readmitted with RSV LRTI within first year of life, leading to their crossover to the RSV LRTI group, starting from the date of discharge for the readmission.

The RSV LRTI, other LRTI, and non-LRTI groups contributed 1,781.9, 3,693.8, and 3,550.0 child-years of observation (cyo), respectively; the number of associated readmissions was 231, 419, and 337, respectively. Discharge diagnoses are shown in online Technical Appendix Table 1 (wwwnc.cdc.gov/eid/article/1/12-0940-Techapp/.pdf). Pneumonia accounted for 131 (57%) readmissions for the RSV LRTI group, 228 (54%) for the other LRTI group, and 119 (36%) for the non-LRTI group. The numbers of children with only 1 readmission for pneumonia were 58, 173, and 82 for the RSV LRTI, other LRTI, and non-LRTI groups, respectively; the numbers with ≥2 readmissions were 27, 34, and 15, respectively. A total of 62 (2.2%) children were admitted to the hospital during the follow-up period with laboratory-confirmed RSV infections, 12 (2.1%) in the RSV LRTI group, 17 (1.5%) in the other LRTI group, and 33 (3.0%) in the non-LRTI group. Invasive bacterial pathogens were detected in 8 (1.4%), 12 (1.1%), and 15 (1.3%) children in these groups, respectively.

Comparison of Readmission Rates between Exposure Groups

Adjusted IRRs comparing rehospitalization for pneumonia by exposure groups are shown in Table 2. The rate of readmission for pneumonia in the RSV LRTI group did not differ from that in the other LRTI group (IRR 1.14, 95% CI 0.85–1.53), even after excluding children who were not tested for RSV (IRR 0.99, 95% CI 0.73–1.34). We combined data for the RSV and other LRTI groups to create an all-LRTI group, consisting of all children with prior exposure to any LRTI; the incidence rate for readmission for pneumonia among these children was significantly higher than for those not exposed to LRTI (non-LRTI group) ($p<0.001$). The observed effect was modified by age at time of exposure among children whose index LRTI admission occurred at age <3 months (IRR 2.83, 95% CI 1.93–4.15) versus >3 months (IRR 1.39, 95% CI 0.99–1.96). An effect modification by age at the index admission for all LRTI infants was also observed for all-cause readmissions but not for nonpneumonia readmissions (Table 2). The incidence rate relative to non-LRTI was 1.57 (95% CI 1.21–2.04) for children whose first admission occurred in the first 3 months of life and 1.04 (95% CI 0.84–1.30) for older children. Results from the time to first pneumonia readmission analysis accounting for death as a competing risk did not change the interpretation of the associations between LRTI exposure and later pneumonia readmissions; hence, only the multiple-failure regression results are reported.

Using non-LRTI children as the baseline group, IRRs for pneumonia with wheeze were 5.37 (95% CI 2.66–10.83) in the RSV LRTI group and 3.50 (95% CI 1.77–6.94) in the other LRTI group. These associations were not modified by age at first admission. IRR for pneumonia with wheeze was 1.53 (95% CI 0.92–2.54) in the RSV LRTI group compared with the other LRTI group; excluding children whose RSV status was not known from the other LRTI group lowered IRR to 1.32 (95% CI 0.78–2.26). The mortality rate was

Table 1. Baseline characteristics of children in study of pneumonia hospitalizations after severe LRTI in infancy, by study group, at time of first admission in Kilifi District Hospital, coastal Kenya, April 16, 2002–May 31, 2010*

Characteristics	Initial hospitalization		
	RSV LRTI, n = 560	Other LRTI, n = 1,140	Non-LRTI, n = 1,113
Male	296 (52.9)	646 (56.7)	607 (54.5)
Median age, mo (IQR)	**3.7 (1.9–6.6)**	4.6 (2.1–7.7)	4.7 (0.3–8.6)
Children age ≤3 mo	241 (43.0)	**406 (35.6)**	489 (43.9)
Median hospital stay, d (IQR)	**4 (3–5)**	**3 (2–5)**	4 (2–6)
Malaria†	9 (1.6)	**114 (10.0)**	147 (13.2)
Gastroenteritis	34 (6.1)	**140 (12.3)**	338 (30.4)
Pneumonia with wheeze	95 (17.0)	125 (11.0)	NA
Bacteremia, no./n (%)	**7/545 (1.3)**	33/1,109 (3.0)	33/1,053 (3.1)
Admission to high-dependency unit	**18 (3.2)**	89 (7.8)	79 (7.1)
Good hospital access	326 (59.5)	621 (55.4)	636 (58.6)
Hypoxia	**44 (7.9)**	**95 (8.3)**	17 (1.5)

*Values are no. (%) except as indicated. **Boldface** indicates statistical significance in the respective group relative to the non-LRTI group. RSV, respiratory syncytial virus; LRTI, lower respiratory tract infection; IQR, interquartile range; NA, not applicable.
†Indicates blood slide testing positive for malaria parasites.

Table 2. Results of multivariable Poisson regression analyses for hospital readmission diagnoses and all-cause mortality among children initially hospitalized during infancy at Kilifi district hospital, coastal Kenya, April 16, 2002–May 31, 2010*

	IRR (95% CI)				
Risk factor	Pneumonia†	Pneumonia with wheeze‡	All readmissions	Nonpneumonia	All-cause mortality
All LRTI§					
Non-LRTI	Referent				
Patient age ≤3 mo	2.83 (1.93–4.15)	NS	1.57 (1.21–2.04)	0.84 (0.69–1.02)	NS
Patient age >3 mo	1.39 (0.99–1.96)		1.04 (0.84–1.30)		
LRTI					
Non-LRTI	Referent				
RSV LRTI	NS	5.37 (2.66–10.83)	NS	NS	0.42 (0.20–0.90)
Other LRTI		3.50 (1.77–6.94)			1.09 (0.70–1.69)
Patient age					
≤3 mo at first admission	Referent				
>3 mo at first admission	NS	1.31 (0.79–2.15)	NS	1.38 (1.11–1.71)	0.88 (0.57–1.35)
Access to hospital					
Poor	Referent				
Good	0.80 (0.62–1.02)	0.58 (0.35–0.98)	0.75 (0.63–0.89)	0.74 (0.60–0.91)	1.76 (1.16–2.67)
Length of hospital stay, d					
≤7 d	Referent				
>7 d	1.31 (0.88–1.95)	NS	1.21 (0.94–1.56)	1.11 (0.85–1.46)	2.45 (1.52–3.93)
Admitted to HDU					
No	Referent				
Yes	0.48 (0.27–0.85)	NS	0.64 (0.44–0.94)	0.82 (0.52–1.29)	NS
Age at follow-up, mo					
0–11	Referent				
12–23	0.56 (0.45–0.68)	0.54 (0.35–0.82)	0.67 (0.58–0.77)	0.80 (0.65–0.99)	0.29 (0.17–0.49)
24–36	0.20 (0.14–0.28)	0.28 (0.13–0.60)	0.33 (0.27–0.41)	0.50 (0.39–0.65)	0.11 (0.05–0.25)
48–59	0.05 (0.03–0.09)	0.01 (0.002–0.10)	0.13 (0.10–0.17)	0.23 (0.17–0.30)	0.12 (0.06–0.23)
Admission for non-LRTI					
No	Referent				
Yes	1.92 (1.49–2.47)	1.44 (0.84–2.50)	NS	NS	2.03 (1.32–2.67)
Readmission					
During Jan–Jun	Referent				
During Jul–Dec	NS	NS	NS	NS	0.60 (0.39–0.93)

*Risk factors refer to state at the time of first admission, except the last 3 variables (age group in months, occurrence of ≥1 non-LRTI admissions, and readmission time), which refer to calendar time during follow-up. IRR, incidence rate ratio; LRTI, lower respiratory tract infection; NS, not statistically significant and thus excluded from the final model for the specified outcome; RSV, respiratory syncytial virus; HDU, high dependency unit.
†Defined as history of cough or difficulty breathing and >1 of the following: fast breathing for age (>60 breaths/min if <2 months of age or >50 breaths/min if 2–11 months of age); lower chest wall indrawing; low oxygen saturation (<90%) by pulse oximetry; or inability to feed, prostration, or unconsciousness.
‡Pneumonia with concurrent wheeze.
§Combined group of children with RSV and other LRTI. IRRs (95% CIs) for readmission with pneumonia, pneumonia with wheeze, any readmission, nonpneumonia, and all-cause mortality comparing RSV LRTI vs. other LRTI group are 1.14 (0.85–1.53), 1.53 (0.92–2.54), 1.10 (0.89–1.37), 1.07 (0.82–1.39), and 0.39 (0.18–0.82), respectively.

significantly lower for RSV-exposed children compared with the non-LRTI (IRR 0.42, 95% CI 0.20–0.90) and other LRTI (IRR 0.39, 95% CI 0.18–0.82; p = 0.013) groups. The mortality rate increased in areas with good access to the hospital (IRR 1.76, 95% CI 1.16–2.67), and incidence rate of subsequent pneumonia after admission to HDU decreased compared with other categories (IRR 0.48, 95% CI 0.27–0.85) (Table 2).

Rates of readmission were highest immediately after discharge for all groups, 6/1,000 cyo for the non-LRTI group and 13–15/1,000 cyo for the other LRTI and RSV LRTI groups, and decreased to ≤2/1,000 cyo at 18–30 months after discharge (online Technical Appendix Figure). The differential in the incidence of pneumonia readmissions between LRTI groups and the non-LRTI group over time after discharge is shown as IRRs in Figure 1 and time-to-event profiles in Figure 2. The rate of readmission for pneumonia was higher for the all-LRTI group compared with that for the non-LRTI group up to 2 years after discharge (log-rank test p value <0.001) (Figure 1). The profiles for readmission with pneumonia (Figure 2, panel A) and pneumonia with wheeze (Figure 2, panel B) and for postdischarge death (Figure 2, panel C) show the associations reported in Table 2 to be sustained primarily during the first 12–24 months after discharge.

Discussion

Using detailed hospital-based data linked to a closely monitored population, we found no difference in the incidence of pneumonia hospitalization among children previously admitted for RSV-associated LRTI in infancy compared with those admitted for non-RSV associated LRTI. However, we did find elevated incidence of readmission for pneumonia among children admitted as infants for LRTI, with or without RSV diagnosis, compared with children admitted as infants for a non-LRTI

condition. The magnitude of this association was highest among children admitted in the first 3 months of life and decreased to nonsignificant levels in children ≥3 months of age at the first admission. Although the association between LRTI during infancy and readmission for pneumonia was unaffected by RSV status at first admission, the association between readmission for pneumonia with wheeze was greater among children whose previous admission was for RSV LRTI (5-fold) compared with children whose previous admission was for LRTI without RSV (3-fold). Only children who were admitted for LRTI at <3 months of age had higher incidence of all-cause readmission compared with those admitted for non-LRTI. The association was lost with no effect modification by age when pneumonia admissions at the index admission were excluded; this finding indicates that the later pneumonia was the main driver of the increased incidence of subsequent readmissions and the observed effect modification by age at index admission.

Although this study involves a retrospective analysis, it is based on a large, unique dataset from sub-Saharan Africa in which detailed and consistent data were collected prospectively. KDH is the main inpatient facility in this rural district of Kenya. Previous reports indicate a distance decay in access to KDH, but this would only influence the relative incidence rates between study groups if there was a differential in access by study groups (*14*,*15*). Although Cox regression would have provided a more flexible analysis method, the proportional hazards assumption was violated, and we therefore used Poisson regression with Lexis expansion. Alternative analyses accounting for death as a competing risk yielded no qualitative differences in the study results. To account for the time-dependent incidence (seasonal changes) of exposure to LRTI pathogens, we set the analysis time based on the calendar time in the survival analysis. Seasonal variation in exposure was also minimized by restricting cohort recruitment to RSV epidemic periods only.

Our findings are consistent with the results of similar studies in The Gambia (*11*,*19*) and elsewhere (*6*,*9*,*11*,*20*,*21*). Weber et al. reported a 3-fold increase in incidence of admission for pneumonia or wheezing in children with prior exposure to RSV infection compared with a community of age-matched neighborhood children (*11*). Their study suggested that the duration of increased risk for pneumonia after RSV LRTI in infancy waned by the end of the second year after discharge, a finding duplicated in our study. However, that study did not explore the differential incidence of pneumonia after non-RSV LRTI, nor did it explore the influence of age at first episode of LRTI. Our study also used a hospital-based comparison group for which risk for readmission with pneumonia might have been elevated.

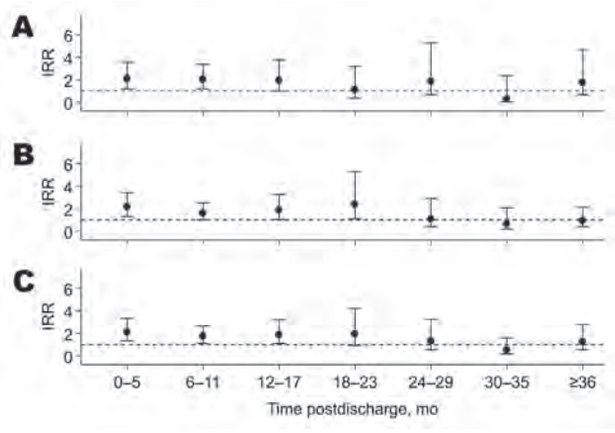

Figure 1. Incidence rate ratios (IRR) for readmission with pneumonia over follow-up time for each of 3 comparisons among children initially admitted during infancy to Kilifi District Hospital, coastal Kenya, April 16, 2002–May 31, 2010. A) Respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) versus non-LRTI group; B) other LRTI versus non-LRTI group; C) all LRTI (RSV and other LRTI combined) versus non-LRTI group. Error bars indicate 95% CIs.

Prospective studies in Europe and the United States reported that the effects of RSV in infancy on respiratory sequelae decreased sharply during the first year of follow-up and that, by 5 years after RSV infection, the association is insignificant (*11*,*20*,*21*). Other studies have reported persistence of increased respiratory disease up to 7 years (*22*) and 11 years (*8*) after RSV infection but not at 18 years after infection (*5*).

A second observational, age-matched, case–control study in The Gambia examining later lung problems in children after childhood (<5 years of age) admission for severe pneumonia reported inconclusive results (*19*). Even though the odds of lung disease were higher among the childhood pneumonia case-patients compared with controls (odds ratio 2.93, 95% CI 0.69–12.48), the study had severe limitations, including small sample size and potential bias (only 68 of 190 possible cases were traced).

Our findings suggest that, in addition to RSV infection, other etiologies of LRTI are associated with subsequent hospital admissions for pneumonia or wheeze. We offer 2 possible explanations for this. First, children with an inherent predisposition to subsequent pneumonia or wheezing episodes, such as those with smaller airways (*23*–*26*) or with atopy (*27*), may also be more susceptible to viral LRTI in early infancy. Second, viral LRTI in early infancy could lead to structural lung damage or immune paresis that causes further pneumonia episodes with or without wheeze. The latter explanation has been reported for reactive airway disease (mainly manifested as wheeze) in relation to RSV predisposition (*28*–*32*).

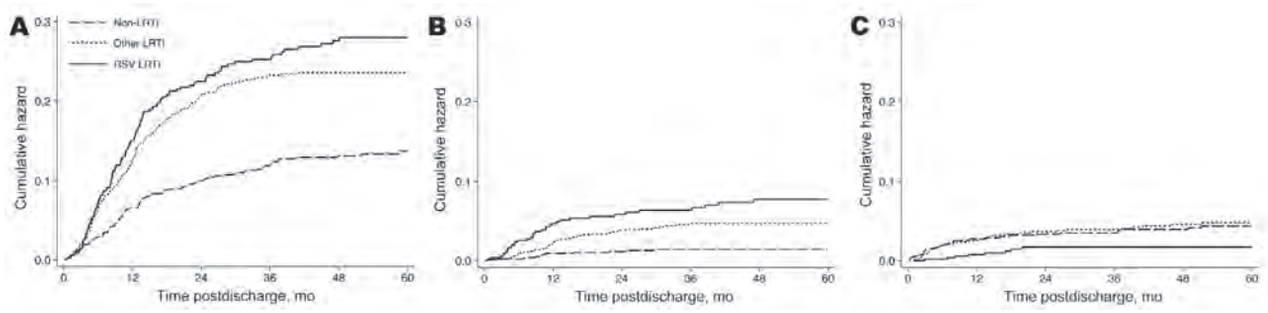

Figure 2. Probability over time of A) readmission for pneumonia, B) readmission for pneumonia with wheeze, and C) death for children with prior respiratory syncytial virus lower respiratory tract infection (LRTI) (solid line), other LRTI (short dashed line), and non-LRTI (long dashed line) during infancy who were hospitalized in Kilifi District Hospital, coastal Kenya, April 16, 2002–May 31, 2010.

In our study, RSV infection appeared to protect against death, but this conclusion is best explained in reverse. In the comparison group, children admitted to a hospital for conditions other than RSV were more likely to die subsequently than were children admitted for RSV. However, unlike some other viruses that cause severe conditions, RSV causes a highly infectious disease that affects most children and does not associate specifically with markers of chronic ill health. A similar phenomenon has been observed with malaria parasitemia, which is associated with a reduced postdischarge mortality rate when compared with admissions for other conditions (*17*).

Findings of an increased mortality rate among children residing in areas with good access to KDH, as well as decreased incidence of subsequent pneumonia following admission to HDU, were unexpected and counterintuitive. Good access was mainly in urban and periurban administrative areas of Kilifi; because urbanization is associated with high HIV prevalence, some of the access-to-care effect may be attributable to HIV/AIDS, but our data lacked HIV results for all admissions to check this effect. The protective association between HDU treatment and later pneumonia may be explained because the HDU has no ventilators and mainly admits children with a nonrespiratory severe illness and because nonrespiratory disease does not have an association with later pneumonia.

We report that hospitalizations for severe LRTI in early infancy in Kenya are associated with increased risk for subsequent pneumonia. The phenomenon has been observed for RSV-associated severe LRTI but not for non-RSV severe LRTI. This raises questions about the underlying cause and, on a practical level, alerts clinicians that a child with LRTI in the first 3 months of life is at risk for readmission with severe respiratory disease over the period of 1–2 years after discharge. Parents of children with LRTI-related hospital admissions during infancy should be advised to be vigilant in the care of the child and to seek medical advice rapidly in the event of further respiratory symptoms. An effective outpatient follow-up for these children throughout early childhood might also be warranted. Larger cohorts and probe studies using interventions against LRTI in infancy (e.g., vaccines) will be pivotal for confirming the causality and the magnitude of the associations observed here and for determining the specificity of the infectious etiologies associated (or not associated) with later episodes of pneumonia.

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Plague Outbreak in Libya, 2009, Unrelated to Plague in Algeria

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After 25 years of no cases of plague, this disease recurred near Tobruk, Libya, in 2009. An epidemiologic investigation identified 5 confirmed cases. We determined ribotypes, *Not*1 restriction profiles, and IS*100* and IS*1541* hybridization patterns of strains isolated during this outbreak. We also analyzed strains isolated during the 2003 plague epidemic in Algeria to determine whether there were epidemiologic links between the 2 events. Our results demonstrate unambiguously that neighboring but independent plague foci coexist in Algeria and Libya. They also indicate that these outbreaks were most likely caused by reactivation of organisms in local or regional foci believed to be dormant (Libya) or extinct (Algeria) for decades, rather than by recent importation of *Yersinia pestis* from distant foci. Environmental factors favorable for plague reemergence might exist in this area and lead to reactivation of organisms in other ancient foci.

Plague is a zoonosis caused by the bacillus *Yersinia pestis*. Rodents are the reservoir and fleas are the vector of this organism. Humans most often become infected by an infectious fleabite, which leads to development of a bubonic form of plague (*1*). If the bacillus reaches the lungs, the patient will expel the bacteria while coughing, causing another clinical form: pneumonic plague, which is directly transmissible from person to person. Without prompt and efficient treatment, the case-fatality rate is 40%–70% for bubonic plague and ≈100% for pneumonic plague (*1*).

The plague bacillus is believed to have originated <20,000 years ago in central Asia (*2*), from which it has spread on multiple occasions and caused 3 well-documented pandemics (*1*). The first pandemic, known as Justinian's plague, reached Africa and then Europe during the sixth century. The second pandemic struck the countries surrounding the Mediterranean in 1348 and then spread rapidly throughout Europe. The third pandemic started in Hong Kong in 1894 and reached previously unscathed territories worldwide. These 3 pandemics were extremely devastating and killed millions of persons. Because of identification of the causative agent (*3*), the reservoir, and the vector of the disease at the end of the 19th century (*4*) and then the availability of effective antimicrobial drugs, human illness and death caused by plague have been considerably reduced since the middle of the 20th century.

However, the disease has not been eradicated. Plague-endemic foci persist in Africa (the most affected continent), Asia, South America, and North America (*5*). Moreover, since the beginning of the 1990s, plague outbreaks have recurred in countries where no cases were reported for decades, and where the disease was believed to have been eliminated. Among the most conspicuous examples is that of India, which experienced a large outbreak of pneumonic plague in 1994 after 30 years with no reports of plague cases (*6*). Another striking example of plague reemergence is that of Algeria, where the most recent cases were reported in the 1940s. After an absence of >50 years, the disease reappeared in 2003 in a village south of Oran (*7*,*8*); and then in 2008, in the Laghouat area (*9*) (Figure 1). Whether these outbreaks were caused by reimportation of the disease from other countries or by reactivation of organisms in a local quiescent plague focus could not be formally established.

Neighboring Libya experienced several plague outbreaks during 1913–1920, the largest of which resulted in 1,449 deaths in Benghazi in 1917 (*10*). Other plague epidemics of lower amplitude occurred in 1972, 1976, 1977, and 1984. After an apparent absence of 25 years, plague cases recurred in June 2009 near Tobruk, Libya, close to the border with Egypt (Figure 1). A total of 5 plague cases were confirmed, 3 of which occurred in 1 family and the other 2 in patients living in the same district (*11*,*12*). An even more recent plague epidemic that had ≥21 suspected

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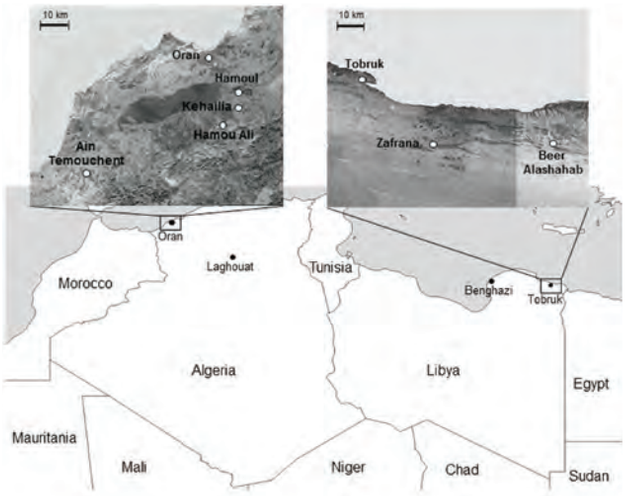

Figure 1. Locations of plague outbreaks in Oran, Algeria, and Tobruk, Libya. Upper panels show regions around Oran and Tobruk where plague cases were found.

cases was reported in May 2011 in the city of Tobruk (*12*). An investigation of this epidemic did not confirm the plague etiology, but the political troubles resulting from the onset of the revolution in February made this investigation difficult.

The purpose of this study was to obtain insights into the origin of the outbreak that occurred near Tobruk in 2009. We analyzed *Y. pestis* strains that were isolated from patients during this epidemic and determined whether links exist to the recent resurgence of plague in Algeria.

Materials and Methods

Bacterial Strains Isolation and Characterization

The *Y. pestis* strains used in this study are shown in the Table. *Y. pestis* strains were isolated from biological specimens after streaking them on cefsulodin-irgasan-novobiocin plates (Merck, Schaffhausen, Switzerland) and injection of bacteria into mice. Phenotypic characterization of strains was determined by biochemical reactions on API 20E and API 50CH strips (bioMérieux, Marcy l'Etoile, France) incubated at 28°C, lysis by a *Y. pestis*–specific bacteriophage, reduction of nitrates, and glycerol fermentation. For further analyses, bacteria were grown in Luria-Bertani broth or on Luria-Bertani agar plates containing 0.002% hemin for 24–48 h at 28°C.

Molecular Typing

Total genomic DNA was extracted by using a Gentra Puregene Cell Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Ribotyping was performed as described (*13*) after digestion of genomic DNA with *Eco*RI or *Eco*RV restriction enzymes for 30 min at 37°C. Each profile was classified according to the scheme of Guiyoule et al. (*13*).

Insertion sequence–restriction fragment length polymorphism (IS-RFLP) analysis was performed as described (*14*). DNA samples were digested with *Eco*RI (IS*100*-RFLP) or *Hin*dIII (IS*1541*-RFLP) for 30 min at 37°C before being loaded onto 0.8% agarose gels and subjected to electrophoresis for 24 h. The IS fingerprints were analyzed by using BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium) as described (*14*). Two IS profiles were considered identical when their percent similarity was >98%.

For pulsed-field gel electrophoresis (PFGE), bacterial genomic DNA was prepared in agarose plugs as described (*15*) and digested with 50 U of *Not*I endonuclease in 200 μL of the corresponding buffer for 3 h at 37°C. Electrophoresis was performed as described (*16*), except that the pulse time ranged from 4 s to 8 s over 42 h in a buffer maintained at 14°C.

Results

Plague Outbreak in Libya

On June 9, 2009, three patients 14, 13, and 4 years of age were admitted to Tobruk Central Hospital because of

Table. Fifteen strains of *Yersinia pestis* used for analysis of plague outbreaks in Libya, 2009, and Algeria, 2003

Strain	Biovar	Country	Place	Year of isolation	Source
IP1973	Medievalis	Libya	Eltarsha	2009	Libyan health authorities
IP1974	Medievalis	Libya	Alashahab	2009	Libyan health authorities
IP1975	Medievalis	Libya	Zafrana	2009	Libyan health authorities
IP516	Medievalis	Iran	Kurdistan	1948	Yersinia Research Unit (Institut Pasteur)
IP519	Medievalis	Iran	Kurdistan	1951	Yersinia Research Unit (Institut Pasteur)
IP562	Medievalis	Iran	Kurdistan	1947	Yersinia Research Unit (Institut Pasteur)
IP564	Medievalis	Iran	Kurdistan	1948	Yersinia Research Unit (Institut Pasteur)
IP565	Medievalis	Turkey	Unknown	1952	Yersinia Research Unit (Institut Pasteur)
IP1860	Orientalis	Algeria	Kehailia	2003	University Hospital, Oran
IP1861	Orientalis	Algeria	Hama Ali	2003	University Hospital, Oran
IP1862	Orientalis	Algeria	Hamoul	2003	University Hospital, Oran
IP1863	Orientalis	Algeria	Ain Temouchent	2003	University Hospital, Oran
IP1864	Orientalis	Algeria	Ain Temouchent	2003	University Hospital, Oran
IP1866	Orientalis	Algeria	Unknown	1944	Yersinia Research Unit (Institut Pasteur)
IP1867	Orientalis	Algeria	Unknown	1945	Yersinia Research Unit (Institut Pasteur)

a severe infectious syndrome. All 3 persons were members of the same family of nomads leaving in Eltarsha, 30 km south of Tobruk. The 13-year-old patient (patient 1) had a septicemic syndrome 2 days after admission and died on June 11 despite intensive care. His 14-year-old brother (patient 2) had a tender left cervical lymph node and a fever of 39.5°C. He received ciprofloxacin and doxycycline before being transferred to Benghazi Hospital on June 11, and he recovered. His 4-year-old sister (patient 3) had signs of severe infection with no visible bubo. She received cefotaxime and metronidazole and then gentamicin before being transferred to Benghazi Hospital, and she recovered. Their father reported having axillary lymphadenitis and indicated that 2 or 3 sudden deaths had occurred in the previous 2 months in the region.

On the basis of clinical manifestations and previous plague cases in the Tobruk area, these 3 patients were considered to have contracted plague. All patients from this area with an infectious syndrome were reported as having suspected cases of plague. All but 1 of these patients received gentamicin at Tobruk Hospital and no additional deaths occurred. On June 13, the Libyan authorities reported 13 cases of plague to the World Health Organization (WHO) and requested technical assistance.

A joint investigation of the outbreak led by WHO and the Libyan National Center for Infectious Disease Prevention and Control concluded that the number of plague cases was overestimated, but it identified 2 additional probable cases. These cases were in a 20-year-old woman (patient 4) and a 24-year-old woman (patient 5) who had an infectious syndrome and a painful inguinal lymph node and were admitted to the Tobruk Hospital on June 16 and 18, respectively. Patient 4 lived in Beer Alashahab and patient 5 lived in Zafrana (Figure 1), ≈60 and 30 km from Eltarsha, respectively. Control measures (chemoprophylaxis of contact persons, insecticide treatment, and rodent control) were implemented, and no additional cases were reported.

Bacteriologic Findings

In Benghazi, blood samples collected from patients 3, 4, and 5 contained gram-negative bacteria resembling *Yersinia* spp. These blood samples and serum samples from all 5 patients and a bubo aspirate from patient 4 were sent to the WHO Collaborating Center at the Institut Pasteur in Antananarivo, Madagascar, for further analyses. F1 dipstick test (*17*) results were positive for samples from all 5 patients. A *Y. pestis* strain was isolated from the blood of patients 3 and 5 and from the bubo aspirate of patient 4, thus confirming the etiology of this outbreak. These 3 strains were then sent to the WHO Collaborating Center at the Institut Pasteur in Paris, France, for further characterization. On the basis of glycerol fermentation and nitrate reduction, the 3 strains were assigned to *Y. pestis* biovar Medievalis (*18*).

Molecular Characteristics of Strains from Libya

The 3 strains exhibited identical *Not*I PFGE profiles (Figure 2, panel A), and all had ribotype O (*Eco*RI.4 + *Eco*RV.5) according to Guiyoule et al. (*13*) and identical IS*100* and IS*1541* hybridization patterns (Figure 3). These results are consistent with a single *Y. pestis* strain as the origin of the cases that occurred in distinct places in the Tobruk area.

Ribotype O is commonly associated with biovar Medievalis strains (*13*). When the IS*100* and IS*1541* profiles were combined (2IS-RFLP), the isolates from Libya clustered with other biovar Medievalis strains (Figure 4) in our database (*14*). Moreover, the chromosomal location of some IS*100* sequences determined by PCR was typical of this biovar (*19*). Therefore, our results demonstrate unambiguously that the *Y. pestis* strain that caused the plague outbreak in Libya in 2009 belongs to the

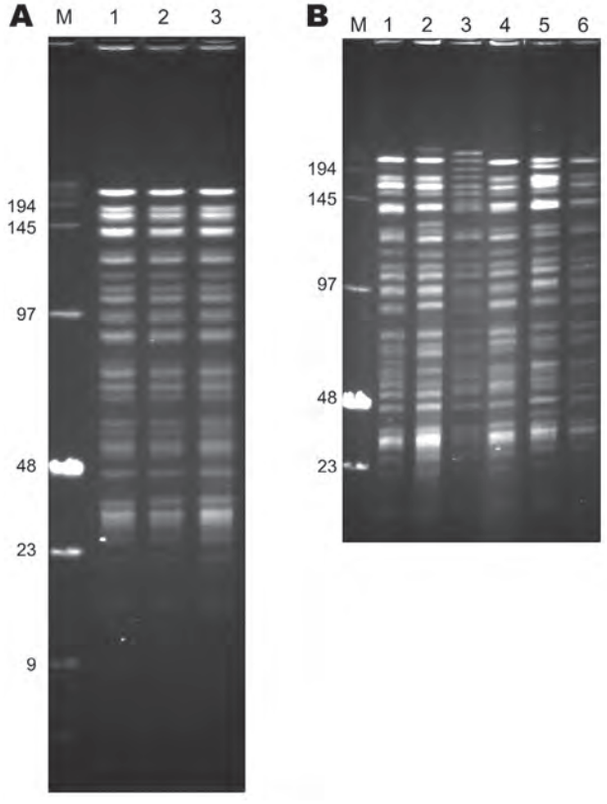

Figure 2. *Not*I pulsed-field gel electrophoresis patterns of *Yersinia pestis* strains of biovar Medievalis obtained during plague outbreak in Libya, 2009. A) Pattern of three 2009 isolates from Libya. Lane M, low-range DNA marker (New England Biolabs, Ipswich, MA, USA); lane 1, IP1973; lane 2, IP1974; lane 3, IP1975. B) Comparison of the pattern of 1 isolate from Libya with those of other biovar Medievalis strains. Lane M, low-range DNA marker (New England Biolabs); lane 1, IP516 (Kurdistan); lane 2, IP519 (Kurdistan); lane 3, IP565 (Turkey); lane 4, IP1975 (Libya); lane 5, IP562 (Kurdistan); lane 6, IP564 (Kurdistan). Values on the left are in kilobases.

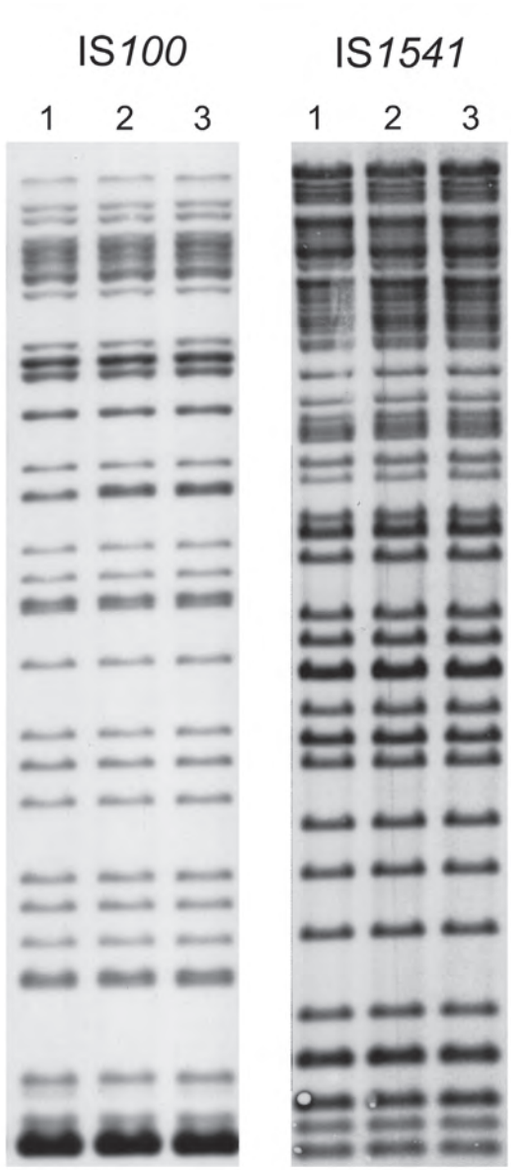

Figure 3. Insertion sequence–restriction fragment length polymorphism profiles of 3 *Yersinia pestis* strains obtained during plague outbreak in Libya, 2009. Genomic DNA of strains IP1973 (lane 1), IP1974 (lane 2), and IP1975 (lane 3) were hybridized with an IS*100* (after *Eco*RI digestion) or an IS*1541* probe (after *Hind*III digestion).

biovar Medievalis lineage, a lineage typical for strains that originated in central Asia.

The 2IS-RFLP dendrogram suggested that strains from Libya were closely related to but different from those from Kurdistan and more distantly related to the biovar Medievalis strain from Turkey (Figure 4). A comparison of the *Not*I PFGE profile of an isolate from Libya with those of 5 other biovar Medievalis strains confirmed this observation: all isolates had similar but different profiles (Figure 2, panel B), and the similarity was more pronounced with strains from Kurdistan than with the strain from Turkey. Thus, strains from the 2009 outbreak in Libya are genetically close to those isolated in the Iranian part of Kurdistan.

Y. pestis Strains that Caused the Plague Outbreak in Algeria in 2003

Because *Y. pestis* strains were isolated from patients during the outbreak that occurred in the region of Oran in 2003 (*7*) (Figure 1), we performed the same analyses on these strains and compared them with isolates from Libya. Biochemical characterization of the 5 strains from Algeria indicated that they belonged to biovar Orientalis. They were of ribotype B (*Eco*RI.1 + *Eco*RV.2), which is found only in biovar Orientalis strains (*13*). Their IS*100* + IS*1541* profiles also included them in the biovar Orientalis group (Figure 4). Therefore, the strains that caused the 2003 plague outbreak in Algeria belong to the biovar Orientalis lineage.

Four of these strains had identical IS*100* + IS*1541* profiles, but the IS*1541* profile of the strain from Hamoul (IP1862) displayed 1 additional band. The *Not*I PFGE patterns of the 5 strains from Algeria were highly similar but differed by a few bands (Figure 5). The 2 strains from Ain Temouchent (IP1863 and IP1864) had an identical *Not*I pattern, which was different from that of the strain from Hamoul (IP1862), which also slightly differed from the *Not*I profile of the strains from Kehailia (IP1860) and Hama Ali (IP1861). Thus, these results suggest that the 2003 outbreak in Algeria was caused by closely related, but not identical, strains.

Two *Y. pestis* strains isolated in Oran in 1944 and 1945 from bubonic plague patients were available in our collection and were used for comparison with the 2003 strains. These 2 more ancient isolates also belonged to biovar Orientalis. However, the strain isolated in 1945 (IP1867) had ribotype E, a ribotype found in strains from Saigon, Vietnam. This strain also clustered with an isolate from Saigon (IP532) in the 2IS-RFLP dendrogram (Figure 4), which suggested an epidemiologic link between these 2 foci. In contrast, the strain isolated in 1944 (IP1866) had ribotype B and was found in the same cluster in the 2IS-RFLP dendrogram as that containing the 2003 strains from Algeria (Figure 4). This cluster grouped all other strains from northern Africa (Morocco and Senegal). These data support a local or regional origin of the strains that caused the 2003 plague outbreak in Algeria.

Discussion

After decades of no plague cases, human plague cases have recently recurred in countries surrounding the Mediterranean Sea. New cases occurred in Saudi Arabia in 1994 (*20*) where no human cases of plague had been

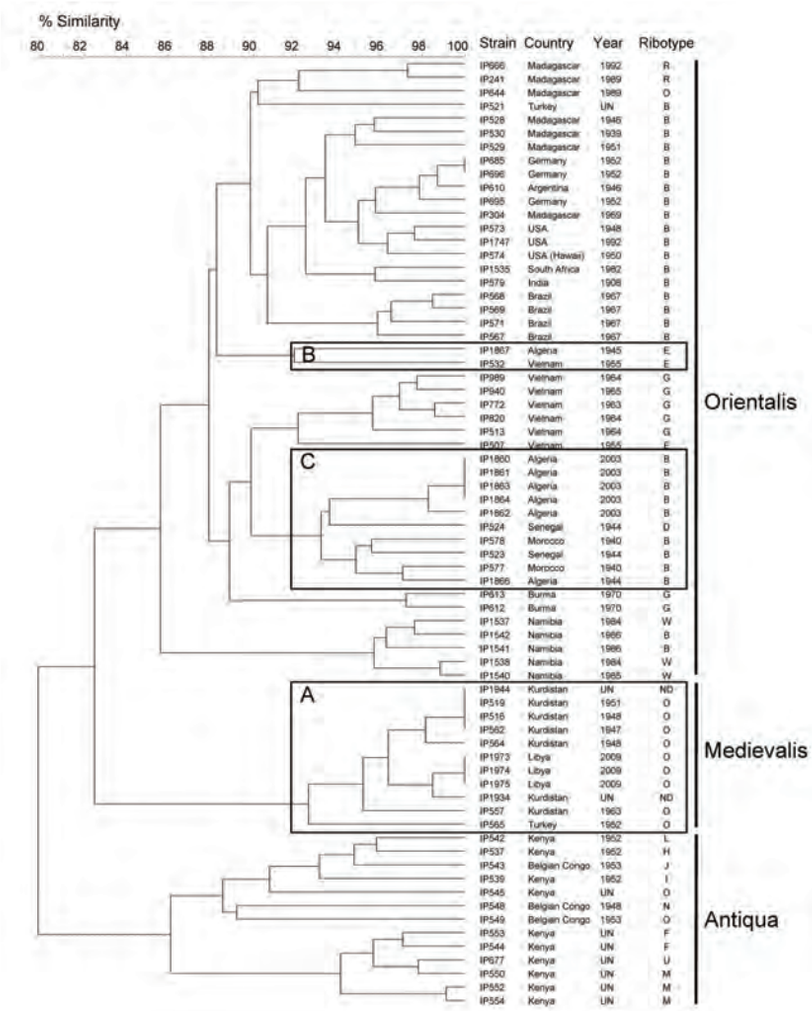

Figure 4. IS100 and IS*1541* restriction fragment length polymorphism patterns of 70 *Yersinia pestis* isolates of worldwide origin. A) Medievalis branch. B), ancient strain from Algeria (IP1867); C) other strains from Algeria and various isolates from Africa. The dendrogram was constructed by using the unweighted pair group method with arithmetic mean clustering analysis and a position tolerance of 1.8%. Biovar is shown on the right. UN, unknown; ND, not determined.

reported for ≥40 years, in Jordan in 1997 (*21*), where no cases had been reported during the past 70 years, in Algeria in 2003, where no cases had been reported for 50 years (*7*), and more recently in Libya in 2009, where the last plague case was reported 25 years ago. Recent reappearance of plague cases in this area could have been caused by regional spread of organisms from a single focus, reimportation of strains from distant areas by land or sea transportation, or reactivation of the organism in local foci that had been apparently dormant for years.

Analysis of the 3 strains isolated in Libya in 2009 showed that they were identical by 2IS-RFLP and PFGE, which suggested that a single strain caused this outbreak and that a single focus was the source of infection for different human patients. Phenotypic and genetic analyses indicated that this strain belongs to the biovar Medievalis lineage. Thus, as for other strains of this lineage, the strain from Libya most likely originated in central Asia. To our knowledge, no biovar Medievalis strains have been reported in Africa. However, this finding might reflect a lack of reporting rather than a true absence of these strains in countries in Africa near Asia.

Camel caravans travel through central Asia and the Middle East and consumption of infected camel meat were shown to be responsible for human plague cases in Libya in 1976 (*22*), in Saudi Arabia in 1994 (*20*), in Jordan in 1997 (*21*), and in Afghanistan in 2007 (*23*). Thus, infected camels could have been a means of importing new *Y. pestis* strains into Libya. However, because camels are highly susceptible to plague, it is unlikely that sick animals could travel long distances. Furthermore, dead rats were found in the vicinity of a sick camel in Libya (*22*), *Y. pestis* strains were isolated from rodents and fleas in the corral where a camel died of plague in Saudi Arabia (*20*), and dogs had antibodies against the plague bacillus in Jordan (*21*). These observations suggest that plague was already present in these countries and that camels were not the mode of transport.

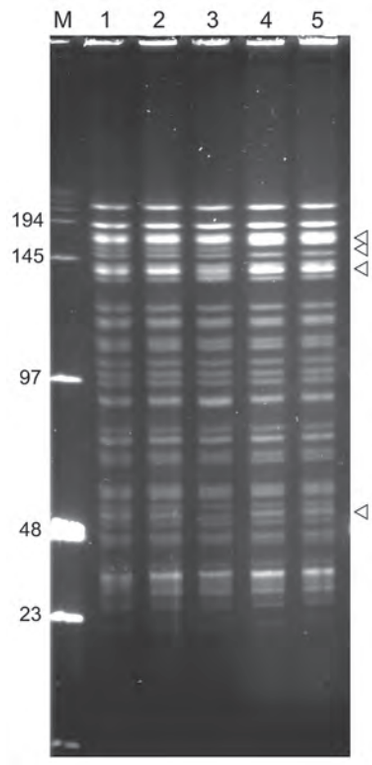

Figure 5. *Not*I pulsed-field gel electrophoresis patterns of *Yersinia pestis* isolates from plague outbreak in Algeria, 2009. Lane M, low-range DNA marker (New England Biolabs, Ipswich, MA, USA); lane 1, IP1860 (Kehailia); lane 2, IP1861 (Hama Ali); lane 3, IP1862 (Hamoul); lane 4, IP1863 (Ain Temouchent); lane 5, IP1864 (Ain Temouchent). Values on the left are in kilobases. Arrowheads indicate positions of variable bands.

Resurgence of plague in Libya in 2009 could most likely be attributed to reactivation of established and permanent local plague foci resulting from ancient importation from central Asia. Strengthening this hypothesis are the numerous plague outbreaks that occurred in Libya during the 20th century. In the Tobruk area, human cases were reported in 1976–1977 and in 1984 (*24*). This finding and the fact that the recent epidemic involved persons living 30–60 km from each other are highly evocative of reactivation of organisms in a local plague focus in 2009. Comparison of strains from past epidemics in Libya with the strain from 2009 would have helped answer this question, but such strains were not available in our collection.

The sudden resurgence of plague cases in the Tobruk region >2 decades after the last reported case might be linked to unusual climatic conditions. The outbreak was preceded by a particularly humid winter, which favored flea proliferation, and exceptionally good harvests, which supported rodent multiplication. The effect of climatic changes on human plague has been documented (*25*,*26*) and further emphasizes the need to take into consideration the effect of global warming on infectious diseases that have a nonhuman reservoir.

Although Libya and Algeria have a common border, our results demonstrate that the plague outbreaks that occurred recently in Algeria were not caused by spread of organisms from the focus in Libya. Phenotypic and genetic analyses of 5 strains isolated from patients in Algeria in 2003 demonstrated that they belong to biovar Orientalis. Similarly, the strains that caused the plague episode in Laghouat (550 km south of Algiers) in 2008 had a multispacer sequence type typical for biovar Orientalis strains (*9*). This lineage is distinct from the biovar Medievalis lineage of strains from Libya. Thus, the plague foci in Algeria and Libya are not linked.

Several plague outbreaks in Oran have been attributed to importation of infected rodents or fleas by marine shipping, e.g., during World War II military operations (*27*). Ribotyping and 2IS-RFLP analyses of the *Y. pestis* strain isolated from a bubonic plague patient in the Oran area in 1945 fully support this point and suggest that this strain was imported from southern Vietnam (Saigon). In contrast, *Y. pestis* isolates from 1944 and from 2003 in Oran cluster together by 2IS-RFLP. They also cluster with the other strains from northern Africa (Morocco and Senegal) isolated in the 1940s. These results are consistent with reactivation of organisms in a local or regional plague focus and argue against importation of infected materials or animals from a distant plague-infected region.

Also arguing for existence of an active local reservoir was detection of *Y. pestis* DNA in fleas collected near Oran 1–2 years after the 2003 outbreak (*8*) and in native rodents trapped in the Laghouat area a few months after the 2008 plague cases (*9*). The fact that 3 similar but distinct *Not*I patterns were observed among the 5 *Y. pestis* strains isolated in Oran in 2003 also argues against importation of a foreign strain and suggests emergence of variants from a local common ancestor. If true, this suggestion would also imply that it was not organisms in 1 focus but organisms in several adjacent foci that were reactivated at the same time in the Oran region. Climatic and environmental factors may have played a critical role in this resurgence because they have been shown to be predictors of human risk for exposure to plague in other foci in Africa (*28*).

Our results indicate that adjacent but independent plague foci coexist in Algeria and Libya. Plague outbreaks that occurred in these 2 countries are most likely the result of reactivation of organisms in local foci that were believed to be dormant (Libya) or extinct (Algeria). Recent reemergence of these independent foci suggests that climatic and environmental changes in northern Africa may be favorable for the *Y. pestis* epidemiologic cycle. Thus, other countries in northern Africa that have had plague foci may also be at risk for plague outbreaks in the near future.

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Phylogenetic and Ecologic Perspectives of a Monkeypox Outbreak, Southern Sudan, 2005

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Identification of human monkeypox cases during 2005 in southern Sudan (now South Sudan) raised several questions about the natural history of monkeypox virus (MPXV) in Africa. The outbreak area, characterized by seasonally dry riverine grasslands, is not identified as environmentally suitable for MPXV transmission. We examined possible origins of this outbreak by performing phylogenetic analysis of genome sequences of MPXV isolates from the outbreak in Sudan and from differing localities. We also compared the environmental suitability of study localities for monkeypox transmission. Phylogenetically, the viruses isolated from Sudan outbreak specimens belong to a clade identified in the Congo Basin. This finding, added to the political instability of the area during the time of the outbreak, supports the hypothesis of importation by infected animals or humans entering Sudan from the Congo Basin, and person-to-person transmission of virus, rather than transmission of indigenous virus from infected animals to humans.

Monkeypox is caused by a member of the genus *Orthopoxvirus*, first identified as the cause of disease in captive cynomolgus monkeys in 1959 (*1*). Twelve years later, the virus was identified as the cause of smallpox-like disease in humans (*2*). Although monkeypox virus (MPXV) can infect a wide variety of animal species when experimentally introduced, it is currently unknown which species are directly involved in its natural transmission cycle and whether ≥1 species are responsible for MPXV perpetuation in nature (*3*). Multiple events of human-to-human transmission have been reported, but sustained MPXV infection cycles among humans have not been documented (*4*–*6*). Likos et al. (*7*) investigated phylogenetic relationships between MPXV isolates by examining 5 whole-genome sequences. That analysis confirmed the existence of 2 distinct groups suggested by previous studies (*8*–*10*): the first group contained isolates from the Congo Basin (Congo Basin clade), and the second group included isolates from countries in western Africa. Differences in epidemiologic and clinical features between MPXV isolates (e.g., higher rates of illness and death of the Congo Basin clade) support the differentiation between these 2 clades.

In 2005, an outbreak of monkeypox among humans was reported from Unity State, Sudan (now South Sudan) (*4*); 19 cases were identified (*5*). Monkeypox cases among humans derived from contact with native animals have been reported in central and western Africa only; thus, this outbreak in Sudan could represent, if zoonotic transmission is confirmed, endemic transmission of monkeypox outside the recognized geographic range of the disease (*7*,*11*). Preliminary genetic and serologic analyses and epidemiologic investigations of the 2005 outbreak in Sudan showed ecological and genetic differences between the causative agent of this outbreak and of those that caused central and western African monkeypox outbreaks, and suggested that it could potentially be a novel virus (*5*). However, evidence

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indicating that the outbreak resulted from local virus transmission from wildlife to humans has not been presented.

Ecological niche modeling (ENM) has been used in the study of the ecological characteristics and distribution of a variety of diseases, such as dengue fever (*12*), leishmaniasis (*13*), plague (*14*,*15*), tularemia (*14*,*16*), West Nile virus infection (*17*), avian influenza (*18*,*19*), filovirus infections (*20*,*21*), and monkeypox (*22*–*24*). ENM is used as a tool for analyzing and identifying ecological requirements for the transmission of diseases and for localizing the geographic areas in which these requirements are met. When applied to human cases of monkeypox, ENM has enabled detection of an environmental signal common to all reported cases, which successfully predicts the range of the 2 recognized clades of monkeypox throughout the humid lowland forest regions of Africa (*23*). The area where the 2005 outbreak occurred represents a drier climate, and the dominant vegetation is substantially different from that in areas where monkeypox viruses from either of the 2 clades have been reported. Furthermore, Sudan has not been recognized as an area of potential favorability for MPXV transmission by previous ENM analyses.

To examine 2 hypotheses about the origin of the virus that caused the outbreak in Sudan, we explored genetic and ecological evidence from the 2005 Sudan outbreak and compared this evidence with what is currently understood about viruses in the 2 recognized clades of MPXV. The first hypothesis is that there was a previously unrecognized MPXV strain circulating naturally in the area of the outbreak; the second hypothesis is that the virus was imported into the area from a place where monkeypox is endemic. We used 2 independent lines of investigation: 1) the genetic characterization of the virus isolates from Sudan (Sudan isolates 1 and 2) and their comparison with previous isolates of MPXV from various regions of Africa by using phylogenetic analysis and 2) the generation of ecological niche models and characterization of ecological factors associated with monkeypox virus transmission on the basis of reported human cases in central and western Africa, including the assessment of environmental suitability for MPXV transmission among the Sudan localities.

Materials

Genetic Analysis

In addition to using the MPXV isolates used in the phylogenetic analysis by Likos et al. (*7*), we included 6 more isolates: 2 of these isolates correspond to strains that cause monkeypox outbreaks in laboratory animals (Copenhagen and Walter Reed) (*24*), 1 from Sierra Leone (*24*), 1 from Yandongi in the Democratic Republic of Congo (DRC), and 2 from the Sudan outbreak during 2005. The latter 2 isolates were found to be identical after alignment and were regarded as 1 genome, MPXV_Nuria_Sudan_2005, during our analysis (Table 1, Figure 1). In total, 11 isolates were used in the phylogenetic analysis.

ENM

Human Case Data

We reviewed the reported human monkeypox cases in Africa, which were georeferenced at the patient's residence village by using digital versions of 1:250,000 Joint Operational Graphic (www.map-reading.com/appendd.php) topographic maps from DRC and GEOnet Names Server (http://earth-info.nga.mil/gns.html/index.html) in tandem with detailed case information from the original reports, and following georeferencing procedures from MaNIS (*25*). Details of these procedures are provided in greater depth in a separate publication (*26*). The geographic coordinates of exposure locations for each case and its associated uncertainty were summarized in a database from which we selected all unique localities with the highest geographic confidence (small spatial uncertainty). Our final database contained 116 unique occurrence localities for Congo Basin and West African clades (Figure 2). Human monkeypox cases during the 2005 outbreak in Sudan were reported from 4 villages: Nuria, Bentiu, Rubkona, and Modin (Figure 1).

Environmental Datasets

We used 7 low-correlated bioclimatic variables from Worldclim (http://www.worldclim.org/) at a spatial resolution of 2.5 km to train the ecological niche models (*27*,*28*). These variables included annual mean temperature, mean diurnal range, maximum temperature of the warmest month, minimum temperature of the coldest month, annual precipitation, precipitation of the wettest month, and precipitation of the driest month.

Methods

Genetic Analysis

Sequencing and Alignment

We used previously described Sanger sequencing methods to sequence the genomes of 2 MPXV isolates collected in southern Sudan (MPXV_Nuria_Sudan_2005, 1 and 2) and an isolate from northern DRC (MPXV_Yandongi_DRC_1986), isolated from a scab collected from an 8-month-old boy. An alignment was created from complete genome sequences from 11 MPXV isolates and cowpox virus Grisham (CPXV_GRI) by using MAFFT version 6 (http://mafft.cbrc.jp/alignment/server/) (*29*). All columns containing gaps were then removed. Consequently, the insertion/deletion region (bp 188854–199543) in MPXV_

Table 1. Virus isolates included in the phylogenetic analysis.of monkeypox outbreak, southern Sudan, 2005*

Isolate	Isolate no.	Source	GenBank accession no.	Clade
Copenhagen 1958	1	Cynomolgus monkey/laboratory	AY753185	West African
Walter Reed 1961	2	Cynomolgus monkey/laboratory	AY603973	West African
Liberia 1970	3	Human/wild	DQ011156	West African
Sierra Leone 1970	4	Human/wild	AY741551	West African
USA/Ghana 2003_039	5	Human/wild	DQ011157	West African
USA/Ghana 2003_044	6	Prairie dog/wild	DQ011153	West African
Impfondo RoC 2003	7	Human/wild	DQ011154	Congo Basin
Mindembo Zaire 1979	8	Human/wild	DQ011155	Congo Basin
Nuria Sudan 2005	9	Human/wild	KC257459	Congo Basin
Yandongi DRC 1986	10	Human/wild	KC257460	Congo Basin
Sankuru Zaire 1996	11	Human/wild	NC_003310	Congo Basin

*DRC, Democratic Republic of Congo.

Nuria_Sudan_2005 sequence from Sudan isolates 1 and 2 was removed (Figure 3). The final alignment was 189,830 bp and was identical in the 2 Sudan isolates; thus, only 1 isolate was used in the analyses.

Phylogenetic Analysis

A maximum clade credibility tree was generated from the sequence matrix by using MrBayes (*30*,*31*) under the general time reversible + proportion invariant + Γ model

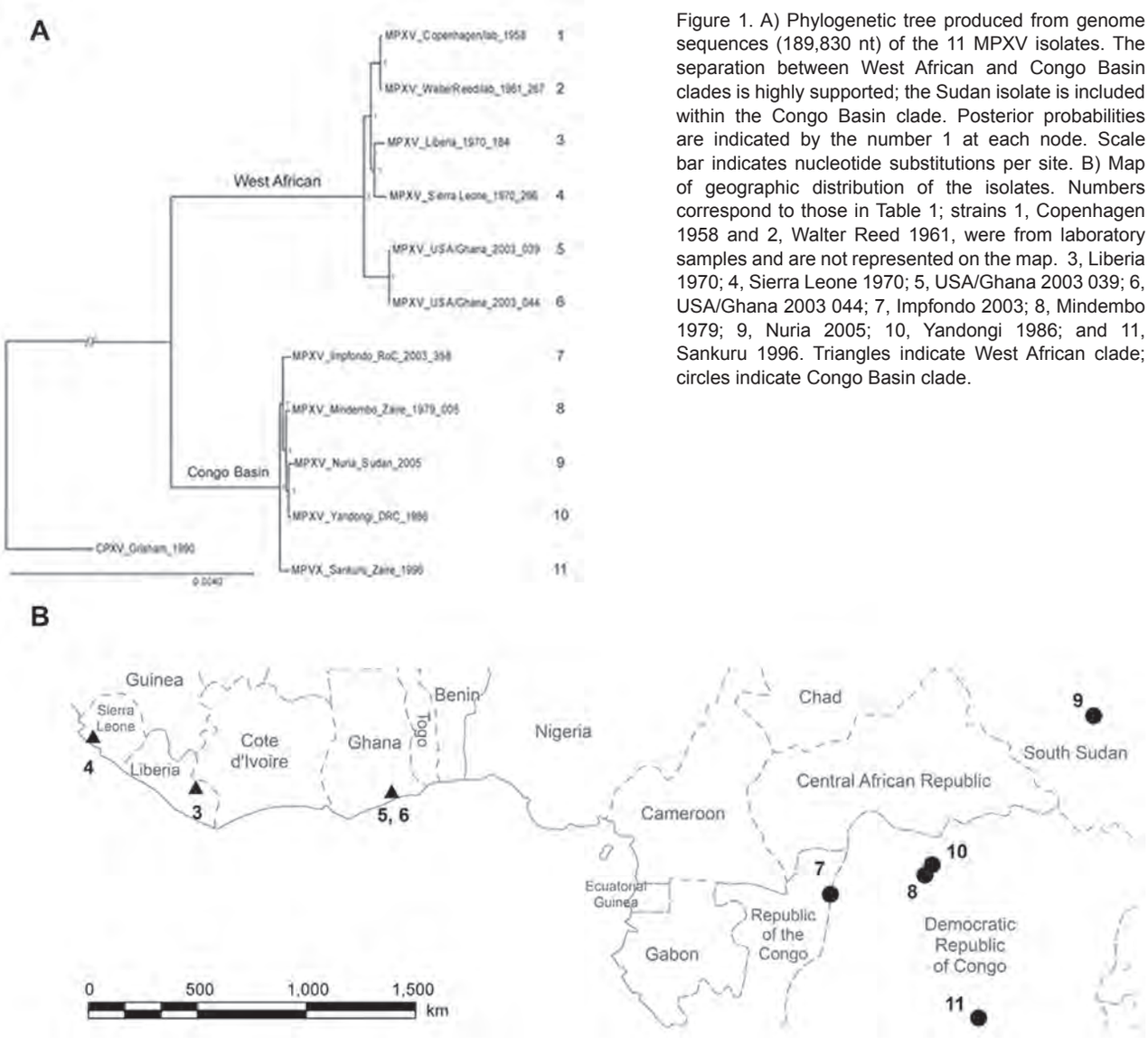

Figure 1. A) Phylogenetic tree produced from genome sequences (189,830 nt) of the 11 MPXV isolates. The separation between West African and Congo Basin clades is highly supported; the Sudan isolate is included within the Congo Basin clade. Posterior probabilities are indicated by the number 1 at each node. Scale bar indicates nucleotide substitutions per site. B) Map of geographic distribution of the isolates. Numbers correspond to those in Table 1; strains 1, Copenhagen 1958 and 2, Walter Reed 1961, were from laboratory samples and are not represented on the map. 3, Liberia 1970; 4, Sierra Leone 1970; 5, USA/Ghana 2003 039; 6, USA/Ghana 2003 044; 7, Impfondo 2003; 8, Mindembo 1979; 9, Nuria 2005; 10, Yandongi 1986; and 11, Sankuru 1996. Triangles indicate West African clade; circles indicate Congo Basin clade.

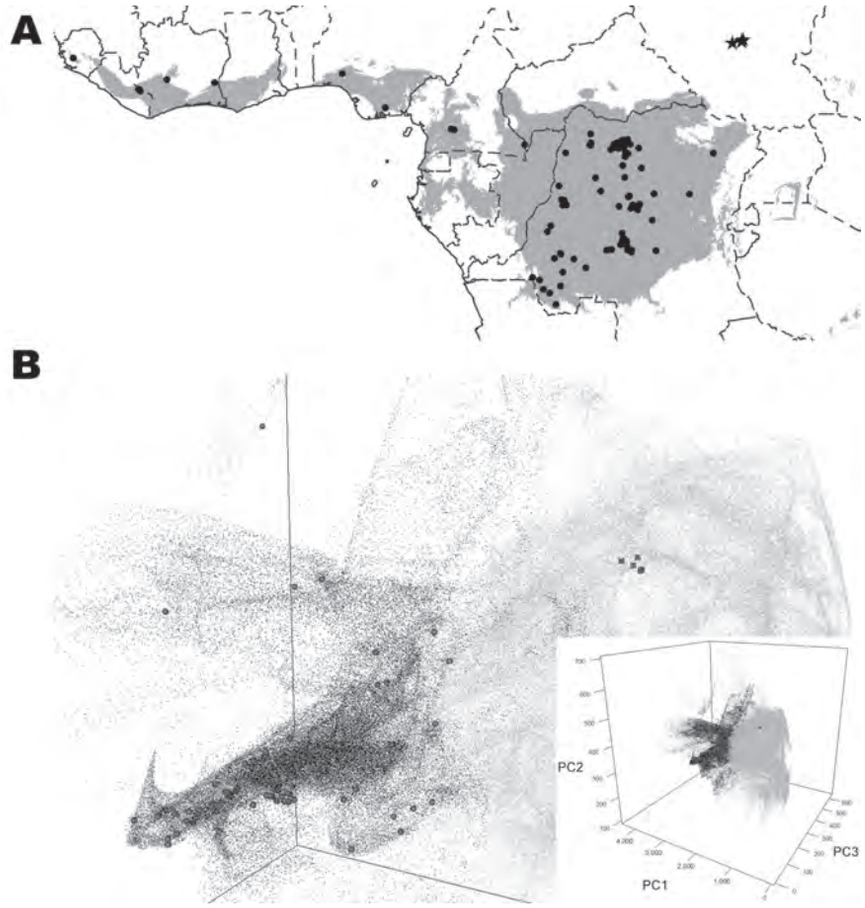

Figure 2. A) Predicted geographic distribution of suitable environments for monkeypox virus transmission on the basis of the Maxent algorithm (www.cs.princeton.edu/~schapire/maxent/). Gray shading represents suitable environmental conditions identified by the algorithm; circles indicate localities of human monkeypox cases used to build the ecological niche models. Stars indicate localities reported during the human monkeypox outbreak in southern Sudan in 2005. B) Scatterplot using the first 3 principal components (PC1, PC2, and PC3) of the environmental variables in sub-Saharan Africa. Gray dots, environmental conditions in the entire area; black dots, suitable conditions identified by the ecological niche models; circles, human monkeypox case localities; squares, localities where monkeypox was reported in southern Sudan. Inset shows scatterplot scale.

with the following settings: lset nst = 6, rates = invgamma, mcmc ngen 5,000,000, sample freq = 1,000, nchains = 4, startingtree = random, burnin = 500. The model was chosen because it allows for variable base frequencies, rate variation among sites, and a proportion of invariable sites within the matrix. No priors were specified because the default priors are expected to work well for most analyses and parameters are then estimated from the data assuming no prior knowledge of their values. The final average standard deviation of split frequencies for the 2 runs was 0.00000, demonstrating convergence.

ENM Procedures

We used 2 algorithms, Genetic Algorithm for Rule-Set Production (GARP [http://openmodeller.sourceforge.net/index.php?option=com_content&task=view&id=8&itemid=4]) and Maxent (www.cs.princeton.edu/~schapire/maxent/), to create ecological niche models of monkeypox transmission by using localities where the 2 recognized clades (Congo Basin and West African) were identified in human samples. GARP is based on a genetic algorithm and is used to find a set of rules describing nonrandom associations between localities where disease transmission has been reported and environmental conditions in those localities (*32*,*33*). These rules are built and selected through an iterative process of creation, evaluation, modification, and inclusion or exclusion of rules that follow 4 basic forms (bioclimatic, atomic, negated, and logistic regression); this process stops when a maximum number of iterations (1,000) is met or an optimization parameter changes by <1% from 1 generation to the next. We tested 500 models by using Desktop GARP (www.nhm.ku.edu/desktopgarp/index.html). We used 50% of points for training the model and 50% to test it. We then selected 50 models by using the best subset option (20% of soft omission threshold distribution and 50% of commission threshold distribution); all other parameters were left as default values. The 50 selected models were then combined. The predicted area was defined as a combination of those areas with higher or equal model coincidence value than the lowest model coincidence value for the human case-patient localities.

Maxent is used to estimate a probability distribution by comparing the environmental conditions at localities in which disease transmission was recorded with environmental conditions across the landscape (*34*,*35*). We used the maximum entropy principle calculation of the program

to find this estimated distribution; thus, the result was the closest to a uniform distribution with a mean that was closest to the observed mean value from known occurrences, achieved by a regularization parameter (β). We used the default values for all parameters; the predicted area was then selected by using a lowest probability threshold value to differentiate between suitable and nonsuitable areas (*36*).

Principal Component Analysis

By using the 7 environmental variables described, we performed a principal component analysis to describe the environmental variability in sub-Saharan Africa and identify those conditions in which monkeypox cases in humans have been recorded and those conditions identified as suitable by ENM analysis. We used the principal components tool in ArcGIS 10 (Esri, Redlands, CA, USA) to calculate the principal components and used the rgl package 0.92.798 (http://ftp.osuosl.org/pub/cran/) for R 2.13.1 (*37*) to visualize the environmental conditions at the localities where monkeypox had been reported, in the areas predicted by ENM algorithms to be suitable for monkeypox transmission, and in the localities of the Sudan outbreak in 2005 in association with the conditions in sub-Saharan Africa. Additionally, we calculated basic statistical parameters (mean, maximum, minimum, and SD) for each variable within the area predicted by the ENM algorithms, which, in turn, were compared with the environmental conditions found at the locations proposed as transmission sites in Sudan.

Results

Genetic Analysis

We used a maximum clade credibility tree, which displayed high support at all nodes (Figure 1). Six isolates were grouped within the clade identified in western Africa: Liberia, Sierra Leone, 2 isolates from Ghana, and 2 isolates acquired from primate colony outbreaks and subsequently extensively passaged in cell culture (Copenhagen and Walter Reed). The remaining 5 isolates were grouped within the Congo Basin clade, including the 2 isolates obtained during the Sudan outbreak in 2005 that were processed and combined for analysis. The isolate from Sudan was most closely related to isolates from Yandongi and Mindembo, located in north central DRC. Of isolates examined, these were from locations nearest to Sudan (Figure 2).

Figure 3 shows a genotypic map of a unique indel region in the right side of the MPXV_Nuria_Sudan_2005 genome that represents a large inverted duplication originating from the left end. This unusual (10.8 kbp) duplication found in Sudan isolates 1 and 2 is composed of open reading frames of several host immune modulator genes (MPXV_Zaire_1979–005 open reading frames 5–16) and some fragments of the inverted terminal repeats. This duplication was partially lost in 1 of the 2 isolates after the second BSC40 tissue culture passage. Additional sequence variations between Sudan isolates 1 and 2 occurred at 4 locations (these variations were also excluded from the phylogenetic analysis because they created gaps that were

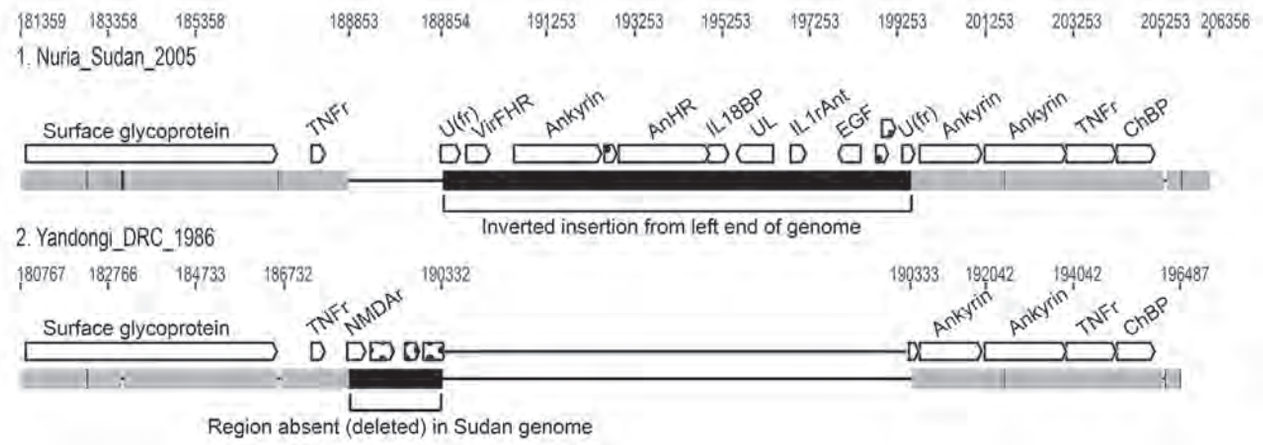

Figure 3. Comparison of a right-end segment from genomes of monkeypox virus (Nuria Sudan 2005 and Yandongi DRC1986. Numbers above genome map are nucleotide positions. Gray boxes represent DNA sequence identity in the 2 genomes; black represents differences. The 2 large black boxes illustrate the insertion/deletion event found in Sudan isolates 1 and 2. A region from the left end of the genome has been inserted where a portion of the right end (shown in Yandongi) has been deleted. Thin black horizontal lines represent gaps in the alignment. Open reading frames are shown in white. Open reading frame names were assigned with reference to MPXV genomes available at the Poxvirus Bioinformatics Resource Center (www.poxvirus.org). Segment boxes with dots indicate unknown genome sequences; TNFr, tumor necrosis factor receptor; U(fr), unknown fragment; VirFHR, virulence factor host range; AnHR, ankyrin host range; IL18BP, interleukin 18 binding protein; UL, ubiquitin ligase; IL1rAnt interleukin 1 receptor antagonist; EGF, epidermal growth factor; ChBP, chemokine binding protien; NMDAr, *N*-methyl D-aspartate receptor-like protein.

Table 2. Nucleotide differences and distances between genome sequences (189,830 nt) of monkeypox virus isolates, 1958–2005*

Isolate	Copenhagen 1958	Liberia 1970	Sierra Leone 1970	Walter Reed 1961	USA/ Ghana 039 2003	Sankuru Zaire 1996	Impfondo RoC 2003	Mindembo Zaire 1979	Yandongi DRC 1986	Sudan 2005
Copenhagen 1958	–									
Liberia 1970	74	–								
Sierra Leone 1970	81	67	–							
Walter Reed 1961	7	73	80	–						
USA/Ghana 039 2003	130	138	143	129	–					
Sankuru Zaire 1996	1,000	1,007	1,017	1,001	1,029	–				
Impfondo RoC 2003	1,005	1,012	1,022	1,006	1,034	59	–			
Mindembo Zaire 1979	998	1,005	1,015	999	1,027	56	43	–		
Yandongi DRC 1986	1,003	1,009	1,020	1,004	1,032	55	44	34	–	
Sudan 2005	1,012	1,018	1,029	1,013	1,041	66	55	34	21	–

*Dashes indicate 100% identity points; blank cells indicate identical values to those below the diagonal; shaded cells indicate within-clade variation; raw number of nucleotide differences between pairs of isolates. DRC, Democratic Republic of Congo.

removed during the alignment): at nt position 10838, Sudan isolate 2 has additional copies of a repeat TTAGA (this variation is in the inverted terminal repeat and so is also reflected at the right end of the genome); at nt 20935, Sudan isolate1 has an additional T in a homopolymer string; at nt 138827, Sudan isolate 1 has an additional ATC repeat; at nt 179133, Sudan isolate 1 has a repeat of 23[ATATACATT] not present in Sudan 2. One of these 4 variations occurs in a coding region. The additional ATC repeat is found in the P4c precursor gene where it codes for an additional aspartic acid residue. The complete genome sequence, absent the full inverted terminal repeat regions and hairpin ends, of the Sudan 1 isolate was 206,346 nt.

A comparison of the 189,830-nt alignment data (Table 2) revealed no nt differences between Sudan isolates 1 and 2, 21 nt differences between MPXV_Nuria_Sudan 2005 and MPXV_Yandongi_DRC_1986, and 34 nt differences between Sudan and Mindembo. Notably, isolates from Yandongi and Mindembo were collected in 1986 and 1979, respectively (19 and 26 years before the monkeypox outbreak in Sudan), and there are 34 nt differences between these 2 isolates. We compared data from the monkeypox outbreaks in Yandongi, Sankuru, and Impfondo and found 55 nt differences between isolates from Yandongi and Sankuru and 44 between those from Yandongi and Impfondo; locations in these pairs are separated by a geographic distance less than that between Sudan and Yandongi. Consequently, the genetic distance between Sudan and Yandongi isolates is low in comparison with other pairs, especially considering the greater number of years between collection dates of the 2 isolates and greater geographic distance.

Ecological Niche Models

The areas with suitable environmental conditions obtained from the GARP and Maxent models were similar; thus, we present only the results from Maxent (Figure 2). We used Maxent probability >0.0903047 and GARP model coincidence ≥34 to determine the areas predicted by the model because these are the lowest probability and coincidence values at the localities used to train the models (i.e., omission error = 0). Reported cases from southern Sudan do not fall within the suitable areas predicted by the ENM algorithms (Figure 2).

Table 3 summarizes the environmental conditions found in the areas predicted as suitable by the ecological niche models for all 7 variables. In general, monkeypox cases in humans in Sudan were reported from areas with higher mean temperatures, lower annual precipitation, and higher temperature ranges than those from areas with indigenous monkeypox occurence. Annual precipitation values for the localities in Sudan are lower than suitable values predicted by Maxent and are at the drier end of values predicted by GARP; furthermore, values for precipitation of the driest month for Sudan localities reach 0 mm, although neither algorithm predicts such conditions to be suitable for MPXV transmission. Annual mean temperatures of Sudan localities are higher than mean values from ENM algorithm predictions but slightly lower than the maximum values from the models. Maximum temperature of the warmest month is consistently higher in Sudan than in either ENM prediction.

The first 3 components describe >99% of the environmental variability in sub-Saharan Africa on the basis of the 7 selected variables (principle component [PC]1 = 97.47%, PC2 = 1.35% and PC3 = 0.84%). Figure 2 shows the distribution within sub-Saharan environments, historic MPXV case localities, and localities corresponding to the 2005 Sudan monkeypox outbreak. The latter localities fall outside the suitable environmental conditions for MPXV transmission predicted by ENMs.

Discussion

Our phylogenetic analysis strongly supports the existence of distinct clades from the Congo Basin and western African, and all subclades were well supported. Sudan isolates 1 and 2 are imbedded within the Congo Basin MPXV clade, specifically within a northern DRC subclade. However, we did not have samples from the northernmost

Congo Basin forest, which is closer to Sudan. In comparison of the position of the isolates from the 2005 Sudan outbreak with that of other isolates from Congo Basin during 1986–2003, the former cannot be distinguished as a new strain of MPXV on the basis of these phylogenetic analyses. Furthermore, the isolates most closely related to the Sudan isolate are from Yandongi and Mindembo DRC, suggesting that the virus obtained during the 2005 outbreak probably originated from northern DRC (Figure 1).

The Sudan isolates uniquely duplicate a 10.8-kb sequence that represents a single mutation event. Given the overall similarity to the Congo Basin isolates, this single duplication is not considered sufficient evidence to suggest an independent evolutionary trajectory. Formenty et al. (*5*) proposed that the Sudan virus was novel among Congo Basin isolates because of this large duplication of genetic information not seen in other monkeypox viruses sequenced to date. The changes seen in the 4 regions between the 2 sequenced Sudan isolates were not seen in the sequenced monkeypox isolates from the 2003 US outbreak of monkeypox. Further genetic analyses could help clarify epidemiologic details through examination of genetic variations accumulated during a single outbreak, but these analyses are beyond the scope of the current study.

The long-term maintenance and transmission of a virus in wildlife would presumably require genotypic adaptations to susceptible hosts, which in turn are adapted to the environmental characteristics of a particular region. Although there are some differences between the Sudan MPXV and other Congo Basin viruses, these differences are well within the limits of variation seen within the Congo Basin clade. The 2 recognized MPXV clades (West Africa and Congo Basin clades) have been described in areas in which the dominant ecosystem is tropical rainforest. Although there are slight habitat differences between the MPXV ranges within western Africa and the Congo Basin, the grassland environmental characteristics and habitat descriptions at the outbreak localities in Sudan are dramatically different and do not fit the expected suitable environmental conditions on the basis of current knowledge of the 2 MPXV clades. This observation is supported by the ENMs (Figure 2), in which the 2005 outbreak localities are not identified as suitable for MPXV transmission and life cycle maintenance.

The 2 possible explanations for the source of the virus that caused the 2005 monkeypox outbreak in Sudan are 1) the existence of conditions permitting the long-term maintenance of MPXV in wildlife within the area where this outbreak occurred and transmission of the virus from reservoir hosts into humans; and 2) the importation of MPXV into the outbreak area by an infected human or animal. The first hypothesis cannot be supported by the results from ENMs and the criteria of ecological niche conservatism between genetically differentiated taxa, which demonstrate that genetic differentiation occurs faster than ecological differentiation (*38*). On the basis of the ecological differences described in this study, we would expect indigenous isolates from Sudan to have high genetic differentiation when compared with isolates from MPXV clades found in West Africa and Congo Basin. Our genetic analysis, however, groups Sudan isolates 1 and 2 within the Congo basin clade; therefore, we consider the hypothesis of an indigenously acquired infection to be unlikely.

Genetic similarity between isolates from DRC and the sample obtained from the 2005 Sudan MPXV outbreak support the second hypothesis, importation of the virus. Additionally, human nomadic events, displacement and

Table 3. Ecological analyses of monkeypox outbreak, southern Sudan, 2005*

Locality	Value	AMT, °C	MDR, °C	MTWM, °C	MTCM, °C	AP, mm	PWM, mm	PDM, mm
Sub-Saharan Africa	Mean	23.60	13.052	33.309	13.060	978.17	195.34	9.23
	Min	–3.0	2.7	3.5	–8.6	8	3	0
	Max	31.9	20.8	42.5	23.3	4552	1157	165
	SD	3.525	2.459	3.933	5.589	569.75	97.94	18.54
Sudan	Mean	27.4	13.925	37.3	17.525	821	193.25	0
	Min	27.3	13.9	37.1	17.5	815	192	0
	Max	27.6	14.0	37.7	17.6	829	195	0
	SD	0.141	0.05	0.282	0.05	5.83	1.25	0
SPA GARP	Mean	24.34	10.59	31.36	17.55	1,680.45	253.6	32.86
	Min	16.1	7.4	24.5	5.9	803	135	3
	Max	27.80	15.4	36.9	21.9	2902	699	137
	SD	1.392	1.156	1.63	2.170	263.133	54.746	28.442
SPA Maxent	Mean	24.74	10.24	31.32	18.43	1,745.54	251.6	38.26
	Min	13.7	5.4	19.4	7.8	941	157	1
	Max	27.8	14.5	37.3	23.0	3303	798	137
	SD	1.224	1.085	1.41	1.927	254.025	60.917	29.946

*Deviation values for climatic environmental variables within the study region (sub-Saharan Africa); at georeferenced points of human monkeypox occurrences in Sudan; and within the suitable predicted areas for monkeypox identified by each algorithm, GARP (Genetic Algorithm for Rule-Set Production [http://openmodeller.sourceforge.net/index.php?option=com_content&task=view&id=8&itemid=4] and Maxent [www.cs.princeton.edu/~schapire/maxent/]). AMT, annual mean temperature; MDR, mean diurnal range; MTWM; maximum temperature of the warmest month; MTCM, minimum temperature of the coldest month; AP, annual precipitation; PWM, precipitation of the wettest month; PDM, precipitation of the driest month; Min, minimum; Max, maximum; SPA, suitable predicted areas for monkeypox transmission.

repatriation of Sudanese residents, were characteristic of southern Sudan and northeastern DRC during the time of the outbreak (*39*). Members of the Lord's Resistance Army fled from Sudan into the Oriental Province of DRC persecuted by the Uganda People's Defense Forces during September 2005 and were reported to have left the area by October (http://www.irinnews.org/report.aspx?reportid = 57083). In January of that year, the Comprehensive Peace Agreement was signed, representing the end of a civil war that started in 1983 between northern and southern Sudan and giving autonomy to southern Sudan until 2011, when a referendum on independence was held, resulting in the recognition of South Sudan as a country. The end of the civil strife in Sudan precipitated the return of refugees who had sought assistance in neighboring countries (including DRC). Some sources reported the spontaneous return of thousands of persons from southern Sudan to their homeland in 2005 (http://reliefweb.int/node/198511). Whether the movements of these persons are linked to the cases of monkeypox in Sudan may never be known with certainty, but the circumstances could have facilitated the importation of the disease by translocation of an infected animal or person from DRC.

An MPXV endemic to Sudan should reflect its adaptation to different hosts and ecological environments with respect to the currently known areas where the disease is endemic in the form of genetic divergence. Given the dramatic difference in ecology between the region surrounding Nuria, Sudan, and historic points of MPXV occurrence, the genomic comparisons between the Sudan isolate and other strains of MPXV would be expected to reveal genetic divergence as great as or even exceeding that observed between the 2 currently recognized MPXV clades in western and central Africa. However, our data indicate that the Sudan MPXV isolates and an MPXV isolate from Yandongi in north-central DRC are genetically similar to each other, even though they were collected 19 years apart from ecologically disparate and geographically discrete locations. For reference, these isolates from Yandongi and Sudan were more genetically similar to each other than the Yandongi and Mindembo DRC isolates (Figure 1), that were the geographically and temporally closest isolates studied.

Further serologic surveys of human, animal, or both populations in Sudan could provide useful evidence in the investigations of the origin of the virus that caused this outbreak in Sudan. In addition, increased disease surveillance, ecological studies, and further characterization of the variability within and between clades will improve our understanding of the natural history of MPXV. Further epidemiologic studies to identify the sources and potential risks of MPXV infection in localities inside and outside the areas in which the disease is known to occur are clearly warranted.

Dr Nakazawa is an Oak Ridge Institute for Science and Education fellow in the Poxvirus Program at the Centers for Disease Control and Prevention, Atlanta. His research interests include application of ecological niche concepts and modeling tools to study distributional patterns of species, biodiversity, and infectious diseases among wildlife and humans.

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Rift Valley Fever, Sudan, 2007 and 2010

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To elucidate whether Rift Valley fever virus (RVFV) diversity in Sudan resulted from multiple introductions or from acquired changes over time from 1 introduction event, we generated complete genome sequences from RVFV strains detected during the 2007 and 2010 outbreaks. Phylogenetic analyses of small, medium, and large RNA segment sequences indicated several genetic RVFV variants were circulating in Sudan, which all grouped into Kenya-1 or Kenya-2 sublineages from the 2006–2008 eastern Africa epizootic. Bayesian analysis of sequence differences estimated that diversity among the 2007 and 2010 Sudan RVFV variants shared a most recent common ancestor circa 1996. The data suggest multiple introductions of RVFV into Sudan as part of sweeping epizootics from eastern Africa. The sequences indicate recent movement of RVFV and support the need for surveillance to recognize when and where RVFV circulates between epidemics, which can make data from prediction tools easier to interpret and preventive measures easier to direct toward high-risk areas.

Rift Valley fever (RVF) is a mosquito-borne viral disease that typically occurs in various areas of sub-Saharan Africa, where virus activity varies from a low-level enzootic cycle to explosive outbreaks covering large areas. Periodically, Rift Valley fever virus (RVFV) spreads to other areas, including northward into Egypt in 1977 and eastward across the Red Sea into Saudi Arabia and Yemen in 2000 (*1*–*7*). How RVFV travels is unclear but probably involves movement of infected livestock or mosquitoes.

Flooding and filling of shallow depressions (*damboes*) during unusual weather events create ideal conditions for emergence of RVFV-infected mosquitoes. The primary vector is *Aedes* spp. floodwater mosquitoes, which transmit the virus transovarially, so infected mosquito eggs lay dormant, ready to hatch when surface water levels rise. The infected mosquitoes feed on livestock, causing high viremia, and provide a way for RVFV to 1) infect secondary vector mosquito species (e.g., *Culex* spp. mosquitoes), which can further transmit the virus to other animals and humans, and 2) infect humans who contact infected animal tissues and blood (*8*,*9*).

RVF in livestock can devastate agricultural communities; it causes almost 100% mortality rates among young animals and high abortion rates among livestock. Most RVF in humans is asymptomatic or a mild febrile illness; only 1%–2% of cases progress to more severe disease, such as acute hepatitis, encephalitis, retinitis, and/or a hemorrhagic syndrome; case-fatality rates among hospitalized patients reach 10%–20% (*10*–*12*). When outbreaks cover a wide geographic area, hundreds of thousands of livestock are affected, leading to tens of thousands of human infections and hundreds of hospitalizations.

The first reported outbreak of RVF in Sudan occurred in 1973 in sheep and cattle in White Nile State; shortly thereafter, RVFV was isolated from a herd of cattle in northern Khartoum (*2*,*3*,*13*). Serologic surveys have detected RVFV antibodies in domestic livestock (*14*,*15*) and in humans from different Sudanese states, including Nile, Khartoum, Kassala, El Gezira, Sennar, and White Nile (*16*–*18*). A recent seroepidemiologic survey reported a high prevalence of RVFV IgG among febrile patients admitted to New Halfa Hospital in Kassala State (*19*). New Halfa is an extensively irrigated agricultural area ≈500 km east of Khartoum. Although the presence of IgG does not indicate recent infection, it does suggest considerable circulation of RVFV in the area at some time in the past.

In late fall and early winter 2007, a large outbreak of RVF, characterized by abortion storms among domestic livestock and febrile hemorrhagic illness in humans, was reported in several Sudanese states (*20*). Estimates suggested ≈75,000 human infections, similar to the number estimated to have occurred in Kenya around this time (*21*). The clinical

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descriptions of severe RVF cases from the 2007 outbreak are similar to those reported from earlier outbreaks (*22*–*24*). Several human RVF cases also were reported in 2010 from the El Gezira State after an increase in abortions among ewes and does; however, the outbreak appears to have been limited geographically, and little information is available about the outbreak (I.E. Aradaib, pers. comm.)

The large, well-documented RVF outbreak in Kenya and Tanzania in 2006–2007, with subsequent spread to Madagascar by 2008 (*1*,*8*,*25*–*30*), was characterized as a steady expansion of many circulating strains of RVFV from 2 sublineages (Kenya-1 and Kenya-2) rather than the introduction and spread of a single strain (*5*,*25*,*26*,*31*). However, to our knowledge, no information is available about the genetic lineages of RVFV strains circulating in Sudan during this time and in subsequent years. The purpose of this study, therefore, was to generate whole-genome sequences of RVFV associated with the 2007 and 2010 RVFV outbreaks in Sudan. Comparison of these sequences with those derived from known strains from eastern Africa and Egypt may provide insight into the origins of Sudan RVFV strains and whether recent outbreaks in Sudan resulted from single or multiple virus introductions.

Materials and Methods

Case Definitions

A suspected RVF case was defined as fever with or without hemorrhagic or neurologic signs, jaundice, or retinitis, in a person who had a history of contact with infected animals (meat, body fluid, aborted fetus). For the 2007 outbreak, the case definition was restricted to illness in persons seeking care during October–December 2007; for the 2010 outbreak, the definition was restricted to persons from the El Gezira State. A confirmed RVF case was defined as laboratory-confirmed acute or recent RVFV infection by positive RVFV IgM detection and/or positive reverse transcription PCR (RT-PCR).

Specimen Collection and Preparation

Serum was collected from a total of 256 suspected RVF case-patients. During October–December 2007, a total of 156 samples were collected from different Sudanese states: Nile, Khartoum, Kassala, El Gezira, White Nile, Sennar, and Upper Nile (now in the Republic of South Sudan; Figure 1). The remaining 100 samples were collected in October 2010 from the El Gezira State in central Sudan (Figure 1). After informed consent was obtained, a blood specimen was collected, and each participant was interviewed. A standardized questionnaire was used to collect information about demographic characteristics, such as age, sex, state of residence, and sample collection date for the 2007 suspected case-patients.

Blood samples were allowed to clot, and serum was separated and sent to the National Medical Health Laboratory (Khartoum, Sudan) for serologic diagnostic screening. RNA was extracted from serum by using a QiaAmp viral RNA Mini Kit (QIAGEN, Hilden, Germany) and sent to the Molecular Biology Laboratory, University of Khartoum, for conventional RT-PCR amplification. Aliquots of selected serum samples in Trizol reagent buffer at a 1:5 ratio were shipped to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) for whole-genome sequence analysis and subsequent phylogenetic studies.

Serologic Diagnostic Tests

Virus isolation was not attempted because of a lack of a biosafety level 3 facility at the University of Khartoum and Ministry of Health. Therefore, virus identification depended solely on serologic testing and conventional RT-PCR amplification results. In Sudan, serum was screened for RVFV IgM by using the ELISA kit from Biologic Diagnostic Supplies Ltd (Johannesburg, South Africa) following the manufacturer's instructions.

RVFV Molecular Diagnostic Tests

Selection of RVFV primers was based on a highly conserved fragment of the small (S) RNA segment of

Figure 1. Sudan and South Sudan. States with confirmed Rift Valley fever cases are in **boldface**. Light gray indicates Sudan; dark gray indicates South Sudan. The Nile, White Nile, and Blue Nile Rivers are depicted in white, and other bodies of water were removed for clarity.

the Smithburn RVFV strain (GenBank accession no. GQ862371) and multiple published RVFV sequences and by using BioEdit software (www.mbio.ncsu.edu/bioedit/bioedit.html). A forward primer RVF1 (5′-AAG CCA TAT CCT GGC CTC TT-3′) and a reverse primer RVF2 (5′-TCC AGT TGT TTC TCC CCA TC-3′) were used to amplify a 390-bp primary PCR product.

RVFV RNA was amplified with conventional RT-PCR by using a SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA), as described (*32*). Crimean-Congo hemorrhagic fever virus and dengue virus RNA were used as negative-control templates. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ, USA); reactions were incubated for 30 min at 50°C, followed by 40 cycles of 95°C for 1 min, 56°C for 30 sec, and 72°C for 45 sec, and a final incubation at 72°C for 10 min.

After amplification, the 390-bp PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and shipped to a commercial company (Seqlab, Göttingen, Germany) for partial sequencing. Sequences were edited by using BioEdit software, and BLAST (http://blast.ncbi.nlm.nih.gov) was used to confirm the identity of the generated sequences.

In addition to the RT-PCR described above, the conventional RT-PCR described by Aradaib et al. (*33*) and TaqMan-based real-time RT-PCR described by Drosten et al. (*34*) also were used. Samples were negative for dengue virus, Crimean-Congo hemorrhagic fever virus, and flaviviruses.

RVFV Whole-Genome Sequencing

Twelve serum samples from the 2007 outbreak and 18 serum samples from the 2010 RVF outbreak, all positive for RVFV by conventional RT-PCR, were sent to CDC for complete S, medium (M), and large (L) segment amplification and sequencing as described (*25*,*26*,*31*). Serum samples were sent in Trizol reagent; after a chloroform extraction, RNA was extracted by using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) was used in accordance with the manufacturer's instructions by using segment-specific primers (*31*). The samples were cycled as follows: 51°C (S, M) or 56°C (L) for 30 min; 94°C for 2 min; 40 cycles at 94°C for 15 sec, 56°C (S) or 46°C (M, L) for 30 sec, 68°C for 2 min (S) or 5 min (M, L); and a final extension at 68°C for 5 min.

RT-PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) before sequencing by using BigDye Terminator version 3.1 (Applied Biosystems, Carlsbad, CA, USA) and the ABI 3730XL automated DNA sequencer (Applied Biosystems). For sequence gaps, specific internal primers were used to amplify smaller products for additional sequencing. Thirteen complete S segments (GenBank accession nos. JQ820472–82, JQ840745–6), 5 complete M segments (GenBank accession nos. JQ820487–91), and 4 complete L segments (GenBank accession nos. JQ820483–6) were generated for phylogenetic analysis.

Phylogenetic Analysis

SeaView software (http://pbil.univ-lyon1.fr/software/seaview3.html) was used to align each complete S, M, and L RVFV segment sequence obtained from this study with those available in GenBank as of December 2011. Bayesian coalescent analyses were performed by using the BEAST and Tracer software packages (*35*). The relaxed uncorrelated exponential clock (*36*) and Bayesian Skyline population size models were chosen for the S, M, and L segments on the basis of recent analyses conducted by Carroll et al. (*25*). Runs consisted of 100 million to 240 million generations to ensure effective sample sizes of at least 200. Maximum clade credibility trees were summarized with TreeAnnotator and were depicted by using FigTree (*35*).

Statistical Analysis

Data were analyzed by using Statistical Package for Social Sciences (SPSS; IBM, Armonk, NY, USA) version 17 for Windows (Microsoft, Redmond, WA, USA). Chi-square tests were used to compare ELISA and RT-PCR data, association between RVFV infection and time period, and sex. According to data distribution, mean age was compared with RVFV infection by using the Mann-Whitney U test.

Results

Suspected RVF Case-patients, 2007

The outbreak occurred during October 9–December 4, 2007; most (16) cases were reported during the fourth week of the outbreak (Figure 2). Serum samples from 156 persons whose illness fit the clinical case definition were tested for RVFV infection by IgM ELISA, which detects an early antibody response to infection, and/or RT-PCR, which detects viral RNA during acute infection. Seventy-eight (50%) of these suspected case-patients were positive for RVFV infection by either test; 23 were positive by IgM ELISA only, 52 by RT-PCR only, and 3 by both tests. Ninety-four (60%) case-patients were male, of whom 50 had positive RVFV results; similarly, 28 of the 62 female suspected case-patients had positive RVFV results. When using the χ^2 test, we found no statistically significant differences between sex and RVF infection. The age distribution of suspected case-patients was 1.5–85 years (mean 28 years; 19.7 years ± SD). Most (46) suspected case-patients were from Khartoum State, 37 from El Gezira, 34 from

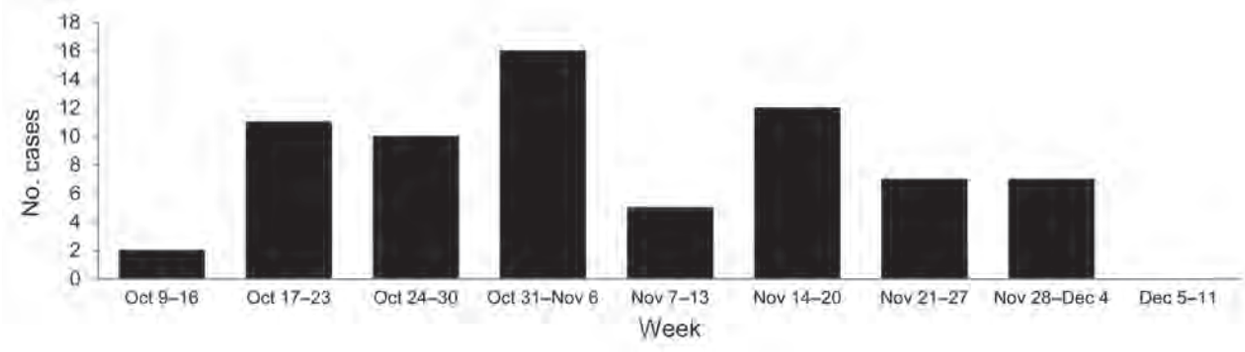

Figure 2. Laboratory-confirmed Rift Valley fever cases, Sudan, 2007.

Kassala, 24 from White Nile, 6 from Sennar, 6 from Upper Nile, and 3 from Nile State (Figure 1).

Suspected RVF Case-patients, 2010

The 2010 outbreak was restricted to a rural area in El Gezira State in central Sudan (Figure 1). One hundred serum samples were collected from suspected case-patients. Suspected RVFV samples were tested solely by RT-PCR, and 18 samples were positive.

Whole-Genome Sequencing

Serum from patients with positive test results for RVFV by conventional RT-PCR was subjected to whole-genome sequencing. Twelve samples from 2007 and 18 samples from 2010 were used to attempt generation of complete S, M, and L segment sequences (Table). From the 2007 samples, 6 complete S segments, 1 complete M segment, and 1 complete L segment were amplified and sequenced; 7 S, 4 M, and 3 L segments were obtained from the 2010 samples. Of these sequences, S segments from samples 2V, 1, and SP from 2007 were identical. S segments from samples 7 and 85 from 2010 also were identical, and the M segment of these samples contained a single base change. Sample 4 from 2010 and sample 77 from 2007 also shared an identical S segment sequence. The generated sequences represent Khartoum, El Gezira, and White Nile States (Table, Figure 1).

Phylogenetic Analysis

When conducting the phylogenetic analysis to compare the complete RVFV segment sequences available from GenBank to sequences generated in this study, the S, M, and L RNA segment datasets were analyzed separately. Previously, 2006–2007 outbreak samples from Kenya, Tanzania, and Madagascar were grouped into a genetic lineage comprising 2 sublineages referred to as Kenya-1 and Kenya-2. The analyses placed all 3 segments of the Sudan strains into either of the 2 Kenya sublineages with no evidence of reassortment (Figures 3–5; online Technical Appendix Figures 1–3, wwwnc.cdc.gov/EID/article/12-0834-Techapp1.pdf). We found no link relative to the genetically more distant Egypt RVFV strains detected during the 1970s and 1994. All Sudan RVFV strains from the 2007 outbreak, and several strains from the 2010 RVF outbreak,

Table. Full-genome Rift Valley fever virus sequences and GenBank accession numbers for Sudan Rift Valley fever virus strains, Sudan

Year collected, strain	State	Date	GenBank accession no. Full-length S	Full-length M†	Full-length L†
2007					
1	Khartoum	Oct	JQ840745		
2V	White Nile	2007*	JQ820472	JQ820490	JQ820483
77	Khartoum	Nov	JQ820482		
133	Khartoum	Nov	JQ820478		
SP	White Nile	2007*	JQ820479		
B	Unknown	2007*	JQ840746		
2010					
4	El Gezira	Oct	JQ820473		
7	El Gezira	Oct	JQ820480	JQ820487	
28	El Gezira	Oct	JQ820474	JQ820491	JQ820486
30	El Gezira	Oct	JQ820481		
34	El Gezira	Oct	JQ820475		
85	El Gezira	Oct	JQ820476	JQ820488	JQ820485
86	El Gezira	Oct	JQ820477	JQ820489	JQ820484

*Samples collected at some point during October–December but exact month of collection not known.
†Blank cells indicate no sequences.

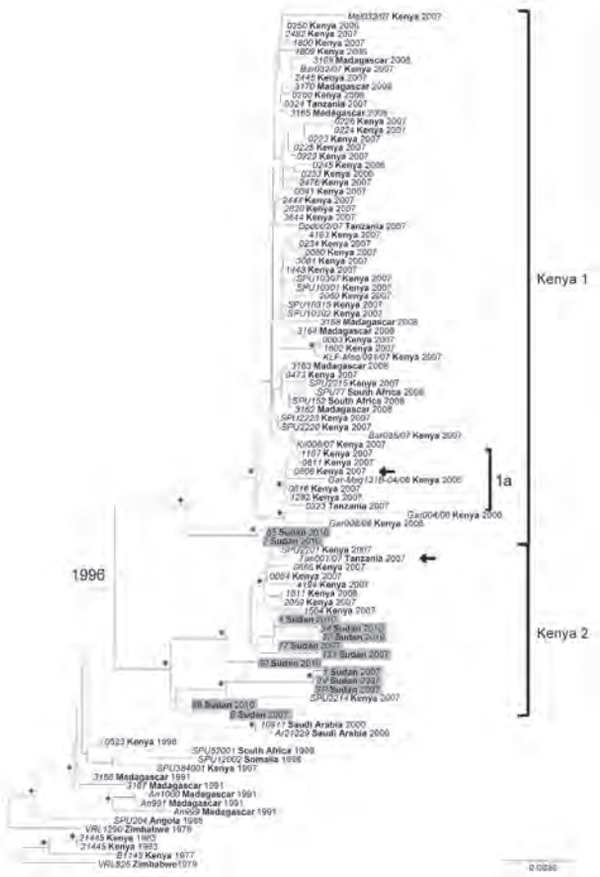

Figure 3. Phylogenetic analysis of complete Rift Valley fever virus S (small) segment sequences represented as an abbreviated maximum clade credibility tree. Asterisk indicates nodes with highest posterior density >0.95. Sudan sequences are shaded. Arrow indicates reassortant viruses. Scale bar represents substitutions per site per year. The complete tree is presented in online Technical Appendix Figure 1 (wwwnc.cdc.gov/EID/article/19/2/12-0834-Techapp.pdf). Country names appear in **boldface**, and strain names appear in *italics*.

were embedded into the Kenya-2 sublineage; however, 2 strains from 2010 were included in the Kenya-1 sublineage. Of the 7 states where suspected RVF cases were distributed, whole-genome sequences were generated from case-patients from Khartoum, White Nile, and El Gezira. The Khartoum strains grouped with strains from El Gezira and White Nile States (Figures 3–5).

When we included additional Tanzania RVFV sequences from GenBank in the analyses, 1 strain, TAN/tan-001/07, fell in the Kenya-2 sublineage for the S and L segments and in the Kenya-1 sublineage for the M segment. These findings added another reassortant to the example (Kenya strain, #0608) (Figures 3–5) (*26*,*37*).

The mean molecular evolutionary rates with 95% highest posterior density intervals were calculated for each segment and were similar to those previously reported (*25*,*26*): 4.20×10^{-4} (3.09×10^{-4} to 5.38×10^{-4}) nt substitutions per site per year for the S segment; 5.06×10^{-4} (3.27×10^{-4} to 6.76×10^{-4}) nt substitutions per site per year for the M segment; and 4.29×10^{-4} (2.65×10^{-4} to 6.03×10^{-4}) nt substitutions per site per year for the L segment. The calculations for the time to most recent common ancestor (MRCA) for all included strains were also similar to previous analyses (*25*,*26*): 91.98, 114.17, and 108.95 years for the S, M, and L segments, respectively. When the 2007 and 2010 outbreak lineages were examined more closely, the MRCA dated to 1996, 1995, and 1997 for the S, M, and L segments, respectively.

Discussion

In October 2007, RVF was detected in the several states of Sudan that border the White Nile River (*20*). Virus

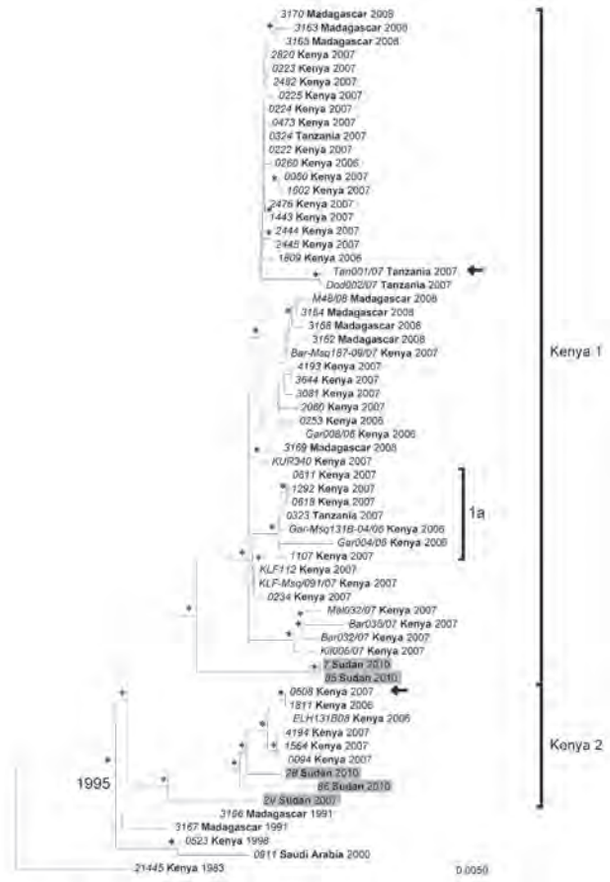

Figure 4. Phylogenetic analysis of complete Rift Valley fever virus M (medium) segment sequences represented as an abbreviated maximum clade credibility tree. Asterisk indicates nodes with highest posterior density >0.95. Sudan sequences are shaded. Arrow indicates reassortant viruses. Scale bar represents substitutions per site per year. The complete tree is presented in online Technical Appendix Figure 2 (wwwnc.cdc.gov/EID/article/19/2/12-0834-Techapp.pdf). Country names appear in **boldface**, and strain names appear in *italics*.

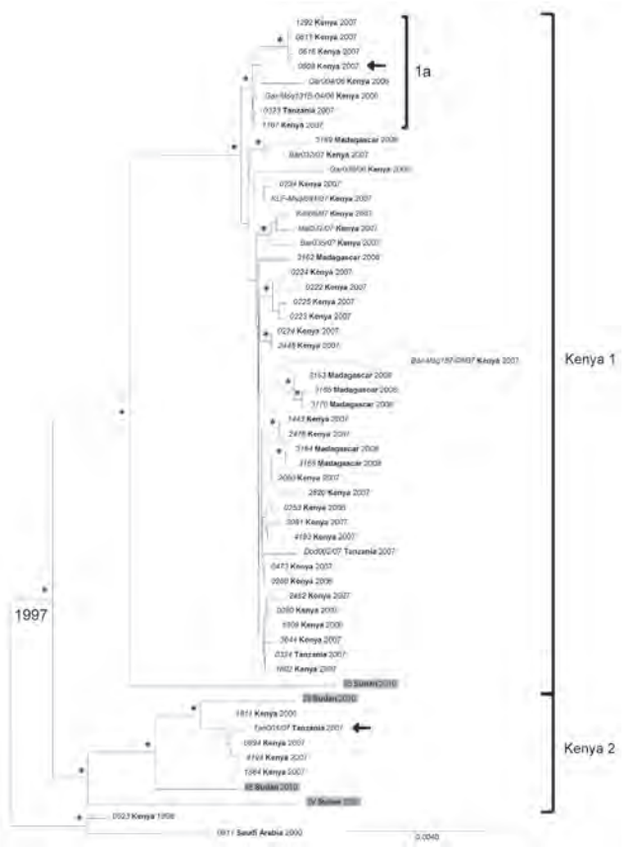

Figure 5. Phylogenetic analysis of complete Rift Valley fever virus L (large) segment sequences represented as an abbreviated maximum clade credibility tree. Asterisk indicates nodes with highest posterior density >0.95. Sudan sequences are shaded. Arrow indicates reassortant viruses. Scale bar represents substitutions per site per year. The complete tree is presented in online Technical Appendix Figure 3 (wwwnc.cdc.gov/EID/article/19/2/12-0834-Techapp.pdf). Country names appear in **boldface**, and strain names appear in *italics*.

activity was detected in 7 states: Nile, Khartoum, Kassala, El Gezira, White Nile, Sennar, and Upper Nile; large numbers of human infections occurred during the relatively short period of 2 months (Figure 1). The substantial RVF outbreak was first detected in Kenya and Tanzania in late 2006 to early 2007 after a season of heavy rainfall (*21*). RVFV activity continued for several years and covered a large geographic area, including Sudan in 2007, and South Africa and Madagascar in 2008 (*1*,*20*,*25*,*38*,*39*).

Although substantial rainfall events were most likely the major cause of spread and maintenance of the RVF outbreak (*21*,*40*), the contribution of irrigation projects is less well understood. Several recent studies have examined the effects of irrigation and agricultural practices on mosquito populations (*41–44* in online Technical Appendix). However, the effect of these agricultural processes on RVF outbreaks and maintenance during interepidemic periods has not been directly studied. The large RVF outbreak that occurred in the regions surrounding the Senegal River during 1987–1988 was thought to be linked to completion of the Maka-Diama dam built in 1986 and the extensive irrigation system developed at this time (*45* in online Technical Appendix). The potential effect of irrigation on RVF should be considered, since new industries in the Sudan are changing the landscape in several RVF-affected states. El Gezira and, to a lesser degree, Sennar, White Nile, and Khartoum States, have vast tracts of irrigated land. Khartoum State has a growing agricultural industry along the Blue Nile River, particularly in Hilat Kuku, Khartoum North, which was the focus of the 1977 Sudan RVF outbreak (*15*). During the end of November 2007, the El Gezira authorities instituted an extensive insecticide spraying program and the federal government restricted trade of livestock and associated products in the state, which may have contributed to the subsequent decline in suspected cases. Future studies would be useful for determining appropriate vector control strategies in irrigated areas.

The epidemiologic data for the 156 suspected RVF cases of 2007 indicated that more male than female case-patients fit the case definition for RVF; however, the percentage of confirmed cases was equivalent for both sexes. The mean age of suspected case-patients was 28 years, consistent with reports from Kenya and Tanzania, where persons 20–30 years old were most affected (*27*,*29*). Young adult men in these affected regions are generally more exposed than women to potentially infected mosquitoes during agricultural work or direct contact with viremic livestock and potentially infected livestock by-products, such as aborted fetal material and raw milk products.

Suspected case-patients were from the 7 states listed previously; most were from Khartoum, Kassala, El Gezira, and White Nile (Figure 1). Rainfall or the new irrigation schemes mentioned previously might have influenced case distribution. Although the overall percentage of confirmed cases was 50%, the percentage of confirmed cases in each state ranged from 15% to 84% (data not shown). The variation also could result from different interpretations of the case definition or increases in other febrile illnesses affected by weather conditions similar to those affecting RVF (e.g., an increase in disease vectors). The possibility of reporting bias also exists because patients can be referred to medical centers in neighboring states.

In 2010, RVF cases were again detected in El Gezira State. The outbreak was first characterized by abortions in ewes and does and followed by infections in persons with histories of contact with aborted fetal material (I.E. Aradaib, pers. comm.). Unfortunately, detailed information about the 100 suspected case-patients tested was not available for analysis.

RVFV-positive samples from several states of Sudan were selected for full-genome analysis to determine the relationship of the strains circulating in Sudan to other known strains identified globally, especially those from the 2006–2007 outbreaks in Kenya and Tanzania and from Egypt during the 1970s and in 1994. A total of 13 complete S segment, 5 complete M segment, and 4 complete L segment sequences were obtained. Phylogenetic analysis of these sequences identified several RVFV variants circulating during the Sudan outbreaks and placed them all in the large lineage containing the Kenya-1 and Kenya-2 sublineages, which defined the eastern Africa outbreak in 2006–2008 (*26*). No genetic relationship was found relative to the earlier Egypt strains. Previously, only RVFV strains identified in Kenya were embedded in the Kenya-2 sublineage; however, as more sequences become available, this sublineage clearly has also become widely geographically distributed (*25*,*26*). Bayesian analysis was used to help elucidate whether RVFV diversity in Sudan resulted from multiple introductions or from acquired changes over time from a single introduction event. Several observations indicate that multiple introductions of RVFV occurred as part of its spread from eastern Africa since the 1996–1997 RVF outbreak (*46* in online Technical Appendix). The date to the MRCA for the overall Kenya lineage is circa 1996, and the MRCA for the 2007 and 2010 Sudan sequences also dates to 1996 instead of 2007. The closest MRCA between the 2007 and 2010 sequences is 5 years (2005). Dating of the MRCA of the overall Kenya lineage concurs with the MRCA of previous studies, which supports the robustness of the models chosen for analysis, even considering the limitations of sample size, collection methods, and environmental factors (*25*,*26*). Identical or nearly identical sequences were identified for different states and years, Khartoum in 2007 and El Gezira in 2010, as well as Khartoum and West Nile in 2007. These sequences indicate recent movement of the virus in this region and support the necessity and utility of surveillance systems for recognizing when and where a large epidemic is imminent. Understanding where the virus is circulating during interepidemic periods can make it easier to interpret data from prediction tools (*21*) and focus preventive measures, such as vector control, livestock vaccination, and education campaigns, on high-risk areas.

The significance of detecting an additional M segment reassortant remains unclear. Reassortment seems to be a relatively rare event because only 2 reassortants were detected for the 54 complete genome sequences (S, M, and L) from the 2006–2007 RVF outbreak. However, it does support cocirculation of both Kenya sublineages in the same geographic location.

The addition of RVFV sequences from Sudan enhances our understanding of the expansion and, to some degree, maintenance of the virus during a large epidemic and the interepidemic period that follows. The ability to sequence entire viral genomes relatively quickly should lead to rapid progress in understanding the detailed ecology of RVFV. Ongoing surveillance and RVFV characterization also should help determine the pattern of virus maintenance between epizootic events. As prediction tools become more accurate and available, these data will provide public health authorities an opportunity to anticipate and prepare for RVF outbreaks.

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Lessons from the History of Quarantine, from Plague to Influenza A

Eugenia Tognotti

In the new millennium, the centuries-old strategy of quarantine is becoming a powerful component of the public health response to emerging and reemerging infectious diseases. During the 2003 pandemic of severe acute respiratory syndrome, the use of quarantine, border controls, contact tracing, and surveillance proved effective in containing the global threat in just over 3 months. For centuries, these practices have been the cornerstone of organized responses to infectious disease outbreaks. However, the use of quarantine and other measures for controlling epidemic diseases has always been controversial because such strategies raise political, ethical, and socioeconomic issues and require a careful balance between public interest and individual rights. In a globalized world that is becoming ever more vulnerable to communicable diseases, a historical perspective can help clarify the use and implications of a still-valid public health strategy.

The risk for deadly infectious diseases with pandemic potential (e.g., severe acute respiratory syndrome [SARS]) is increasing worldwide, as is the risk for resurgence of long-standing infectious diseases (e.g., tuberculosis) and for acts of biological terrorism. To lessen the risk from these new and resurging threats to public health, authorities are again using quarantine as a strategy for limiting the spread of communicable diseases (*1*). The history of quarantine—not in its narrower sense, but in the larger sense of restraining the movement of persons or goods on land or sea because of a contagious disease—has not been given much attention by historians of public health. Yet, a historical perspective of quarantine can contribute to a better understanding of its applications and can help trace the long roots of stigma and prejudice from the time of the Black Death and early outbreaks of cholera to the 1918 influenza pandemic (*2*) and to the first influenza pandemic of the twenty-first century, the 2009 influenza A(H1N1) pdm09 outbreak (*3*).

Quarantine (from the Italian "quaranta," meaning 40) was adopted as an obligatory means of separating persons, animals, and goods that may have been exposed to a contagious disease. Since the fourteenth century, quarantine has been the cornerstone of a coordinated disease-control strategy, including isolation, sanitary cordons, bills of health issued to ships, fumigation, disinfection, and regulation of groups of persons who were believed to be responsible for spreading the infection (*4*,*5*).

Plague

Organized institutional responses to disease control began during the plague epidemic of 1347–1352 (*6*). The plague was initially spread by sailors, rats, and cargo arriving in Sicily from the eastern Mediterranean (*6*,*7*); it quickly spread throughout Italy, decimating the populations of powerful city-states like Florence, Venice, and Genoa (*8*). The pestilence then moved from ports in Italy to ports in France and Spain (*9*). From northeastern Italy, the plague crossed the Alps and affected populations in Austria and central Europe. Toward the end of the fourteenth century, the epidemic had abated but not disappeared; outbreaks of pneumonic and septicemic plague occurred in different cities during the next 350 years (*8*).

Medicine was impotent against plague (*8*); the only way to escape infection was to avoid contact with infected persons and contaminated objects. Thus, some city-states prevented strangers from entering their cities, particularly, merchants (*10*) and minority groups, such as Jews and persons with leprosy. A sanitary cordon—not to be broken on pain of death—was imposed by armed guards along transit routes and at access points to cities. Implementation of these measures required rapid, firm action by authorities, including prompt mobilization of repressive police forces. A rigid separation between healthy and

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infected persons was initially accomplished through the use of makeshift camps (*10*).

Quarantine was first introduced in 1377 in Dubrovnik on Croatia's Dalmatian Coast (*11*), and the first permanent plague hospital (lazaretto) was opened by the Republic of Venice in 1423 on the small island of Santa Maria di Nazareth. The lazaretto was commonly referred to as Nazarethum or Lazarethum because of the resemblance of the word lazaretto to the biblical name Lazarus (*12*). In 1467, Genoa adopted the Venetian system, and in 1476 in Marseille, France, a hospital for persons with leprosy was converted into a lazaretto. Lazarettos were located far enough away from centers of habitation to restrict the spread of disease but close enough to transport the sick. Where possible, lazarettos were located so that a natural barrier, such as the sea or a river, separated them from the city; when natural barriers were not available, separation was achieved by encircling the lazaretto with a moat or ditch. In ports, lazarettos consisted of buildings used to isolate ship passengers and crew who had or were suspected of having plague. Merchandise from ships was unloaded to designated buildings. Procedures for so-called "purgation" of the various products were prescribed minutely; wool, yarn, cloth, leather, wigs, and blankets were considered the products most likely to transmit disease. Treatment of the goods consisted of continuous ventilation; wax and sponge were immersed in running water for 48 hours.

It is not known why 40 days was chosen as the length of isolation time needed to avoid contamination, but it may have derived from Hippocrates theories regarding acute illnesses. Another theory is that the number of days was connected to the Pythagorean theory of numbers. The number 4 had particular significance. Forty days was the period of the biblical travail of Jesus in the desert. Forty days were believed to represent the time necessary for dissipating the pestilential miasma from bodies and goods through the system of isolation, fumigation, and disinfection. In the centuries that followed, the system of isolation was improved (*13*–*15*).

In connection with the Levantine trade, the next step taken to reduce the spread of disease was to establish bills of health that detailed the sanitary status of a ship's port of origin (*14*). After notification of a fresh outbreak of plague along the eastern Mediterranean Sea, port cities to the west were closed to ships arriving from plague-infected areas (*15*). The first city to perfect a system of maritime cordons was Venice, which because of its particular geographic configuration and its prominence as a commercial center, was dangerously exposed (*12*,*15*,*16*). The arrival of boats suspected of carrying plague was signaled with a flag that would be seen by lookouts on the church tower of San Marco. The captain was taken in a lifeboat to the health magistrate's office and was kept in an enclosure where he spoke through a window; thus, conversation took place at a safe distance. This precaution was based on a mistaken hypothesis (i.e., that "pestilential air" transmitted all communicable diseases), but the precaution did prevent direct person-to-person transmission through inhalation of contaminated aerosolized droplets. The captain had to show proof of the health of the sailors and passengers and provide information on the origin of merchandise on board. If there was suspicion of disease on the ship, the captain was ordered to proceed to the quarantine station, where passengers and crew were isolated and the vessel was thoroughly fumigated and retained for 40 days (*13*,*17*). This system, which was used by Italian cities, was later adopted by other European countries.

The first English quarantine regulations, drawn up in 1663, provided for the confinement (in the Thames estuary) of ships with suspected plague-infected passengers or crew. In 1683 in Marseille, new laws required that all persons suspected of having plague be quarantined and disinfected. In ports in North America, quarantine was introduced during the same decade that attempts were being made to control yellow fever, which first appeared in New York and Boston in 1688 and 1691, respectively (*18*). In some colonies, the fear of smallpox outbreaks, which coincided with the arrival of ships, induced health authorities to order mandatory home isolation of persons with smallpox (*19*), even though another controversial strategy, inoculation, was being used to protect against the disease. In the United States, quarantine legislation, which until 1796 was the responsibility of states, was implemented in port cities threatened by yellow fever from the West Indies (*18*). In 1720, quarantine measures were prescribed during an epidemic of plague that broke out in Marseille and ravaged the Mediterranean seaboard of France and caused great apprehension in England. In England, the Quarantine Act of 1710 was renewed in 1721 and 1733 and again in 1743 during the disastrous epidemic at Messina, Sicily (*19*). A system of active surveillance was established in the major Levantine cities. The network, formed by consuls of various countries, connected the great Mediterranean ports of western Europe (*15*).

Cholera

By the eighteenth century, the appearance of yellow fever in Mediterranean ports of France, Spain, and Italy forced governments to introduce rules involving the use of quarantine (*18*). But in the nineteenth century, another, even more frightening scourge, cholera, was approaching (*20*). Cholera emerged during a period of increasing globalization caused by technological changes in transportation, a drastic decrease in travel time by steamships and railways, and a rise in trade. Cholera, the "Asiatic

disease," reached Europe in 1830 and the United States in 1832, terrifying the populations (*21–24*). Despite progress regarding the cause and transmission of cholera, there was no effective medical response (*25*).

During the first wave of cholera outbreaks, the strategies adopted by health officials were essentially those that had been used against plague. New lazarettos were planned at western ports, and an extensive structure was established near Bordeaux, France (*26*). At European ports, ships were barred entry if they had "unclean licenses" (i.e., ships arriving from regions where cholera was present) (*27*). In cities, authorities adopted social interventions and the traditional health tools. For example, travelers who had contact with infected persons or who came from a place where cholera was present were quarantined, and sick persons were forced into lazarettos. In general, local authorities tried to keep marginalized members of the population away from the cities (*27*). In 1836 in Naples, health officials hindered the free movement of prostitutes and beggars, who were considered carriers of contagion and, thus, a danger to the healthy urban population (*27*,*28*). This response involved powers of intervention unknown during normal times, and the actions generated widespread fear and resentment.

In some countries, the suspension of personal liberty provided the opportunity—using special laws—to stop political opposition. However, the cultural and social context differed from that in previous centuries. For example, the increasing use of quarantine and isolation conflicted with the affirmation of citizens' rights and growing sentiments of personal freedom fostered by the French Revolution of 1789. In England, liberal reformers contested both quarantine and compulsory vaccination against smallpox. Social and political tensions created an explosive mixture, culminating in popular rebellions and uprisings, a phenomenon that affected numerous European countries (*29*). In the Italian states, in which revolutionary groups had taken the cause of unification and republicanism (*27*), cholera epidemics provided a justification (i.e., the enforcement of sanitary measures) for increasing police power.

By the middle of the nineteenth century, an increasing number of scientists and health administrators began to allege the impotence of sanitary cordons and maritime quarantine against cholera. These old measures depended on the idea that contagion was spread through the interpersonal transmission of germs or by contaminated clothing and objects (*30*). This theory justified the severity of measures used against cholera; after all, it had worked well against the plague. The length of quarantine (40 days) exceeded the incubation period for the plague bacillus, providing sufficient time for the death of the infected fleas needed to transmit the disease and of the biological agent, *Yersinia pestis*. However, quarantine was almost irrelevant as a primary method for preventing yellow fever or cholera. A rigid maritime cordon could only be effective in protecting small islands. During the terrifying cholera epidemic of 1835–1836, the island of Sardinia was the only Italian region to escape cholera, thanks to surveillance by armed men who had orders to prevent, by force, any ship that attempted to disembark persons or cargo on the coast (*27*).

Anticontagionists, who disbelieved the communicability of cholera, contested quarantine and alleged that the practice was a relic of the past, useless, and damaging to commerce. They complained that the free movement of travelers was hindered by sanitary cordons and by controls at border crossings, which included fumigation and disinfection of clothes (Figures 1– 3). In addition, quarantine inspired a false sense of security, which was dangerous to public health because it diverted persons from taking the correct precautions. International cooperation and coordination was stymied by the lack of agreement regarding the use of quarantine. The discussion among scientists, health administrators, diplomatic bureaucracies, and governments dragged on for decades, as demonstrated in the debates in the International Sanitary Conferences (*31*), particularly after the opening, in 1869, of the Suez Canal, which was perceived as a gate for the diseases of the Orient (*32*). Despite pervasive doubts regarding the effectiveness of quarantine, local authorities were reluctant to abandon the protection of the traditional strategies that provided an antidote to population panic, which, during a serious epidemic, could produce chaos and disrupt public order (*33*).

A turning point in the history of quarantine came after the pathogenic agents of the most feared epidemic diseases were identified between the nineteenth and twentieth centuries. International prophylaxis against cholera, plague, and yellow fever began to be considered separately. In light of the newer knowledge, a restructuring of the international regulations was approved in 1903 by the 11th Sanitary Conference, at which the famed convention of 184 articles was signed (*31*).

Figure 1. Disinfecting clothing. France–Italy border during the cholera epidemic of 1865–1866. (Photograph in the author's possession).

Influenza

In 1911, the eleventh edition of Encyclopedia Britannica emphasized that "the old sanitary preventive system of detention of ships and men" was "a thing of the past" (*34*). At the time, the battle against infectious diseases seemed about to be won, and the old health practices would only be remembered as an archaic scientific fallacy. No one expected that within a few years, nations would again be forced to implement emergency measures in response to a tremendous health challenge, the 1918 influenza pandemic, which struck the world in 3 waves during 1918–1919 (online Technical Appendix, wwwnc.cdc.gov/EID/article12-0312-Techapp1.pdf). At the time, the etiology of the disease was unknown. Most scientists thought that the pathogenic agent was a bacterium, *Haemophilus influenzae*, identified in 1892 by German bacteriologist Richard Pfeiffer (*35*).

During 1918–1919, in a world divided by war, the multilateral health surveillance systems, which had been laboriously built during the previous decades in Europe and the United States, were not helpful in controlling the influenza pandemic. The ancestor of the World Health Organization, the Office International d'Hygiène Publique, located in Paris (*31*), could not play any role during the outbreak. At the beginning of the pandemic, the medical officers of the army isolated soldiers with signs or symptoms, but the disease, which was extremely contagious, quickly spread, infecting persons in nearly every country. Various responses to the pandemic were tried. Health authorities in major cities of the Western world implemented a range of disease-containment strategies, including the closure of schools, churches, and theaters and the suspension of public gatherings. In Paris, a sporting event, in which 10,000 youths were to participate, was postponed (*36*). Yale University canceled all on-campus public meetings, and some churches in Italy suspended confessions and funeral ceremonies. Physicians encouraged the use of measures like respiratory hygiene and social distancing. However, the measures were implemented too late and in an uncoordinated manner, especially in war-torn areas where interventions (e.g., travel restrictions, border controls) were impractical, during a time when the movement of troops was facilitating the spread of the virus.

In Italy, which along with Portugal had the highest mortality rate in Europe, schools were closed after the first case of the unusually severe hemorrhagic pneumonia; however, the decision to close schools was not simultaneously accepted by health and scholastic authorities (*37*). Decisions made by health authorities often seemed focused more on reassuring the public about efforts being made to stop transmission of the virus rather than on actually stopping transmission of the virus (*35*). Measures adopted in many countries disproportionately affected ethnic and

Figure 2. Quarantine. The female dormitory. France–Italy border during the cholera epidemic of 1865–1866. (Photograph in the author's possession).

marginalized groups. In colonial possessions (e.g., New Caledonia), restrictions on travel affected the local populations (*3*). The role that the media would play in influencing public opinion in the future began to take shape. Newspapers took conflicting positions on health measures and contributed to the spread of panic. The largest and most influential newspaper in Italy, Corriere della Sera, was forced by civil authorities to stop reporting the number of deaths (150–180 deaths/day) in Milan because the reports caused great anxiety among the citizenry. In war-torn nations, censorship caused a lack of communication and transparency regarding the decision-making process, leading to confusion and misunderstanding of disease-control measures and devices, such as face masks (ironically named "muzzles" in Italian) (*35*).

During the second influenza pandemic of the twentieth century, the "Asian flu" pandemic of 1957–1958, some countries implemented measures to control spread of the disease. The illness was generally milder than that caused by the 1918 influenza, and the global situation differed. Understanding of influenza had advanced greatly: the pathogenic agent had been identified in 1933, vaccines for seasonal epidemics were available, and antimicrobial drugs were available to treat complications. In addition, the World Health Organization had implemented a global influenza surveillance network that provided early warning when novel influenza (H2N2) virus, began spreading in China in February 1957 and worldwide later that year. Vaccines had been developed in Western countries but were not yet available when the pandemic began to spread simultaneously with the opening of schools in several countries. Control measures (e.g., closure of asylums and nurseries, bans on public gatherings) varied from country to country but, at best, merely postponed the onset of disease for a few weeks (*38*). This scenario was repeated during the influenza A(H3N2) pandemic of 1968–1969, the third and mildest influenza pandemic of the twentieth century. The

Figure 3. The control of travelers from cholera-affected countries, who were arriving by land at the France–Italy border during the cholera epidemic of 1865–1866. (Photograph in the author's possession).

virus was first detected in Hong Kong in early 1968 and was introduced into the United States in September 1968 by US Marines returning from Vietnam. In the winter of 1968–69, the virus spread around the world; the effect was limited and there were no specific containment measures.

A new chapter in the history of quarantine opened in the early twenty-first century as traditional intervention measures were resurrected in response to the global crisis precipitated by the emergence of SARS, an especially challenging threat to public health worldwide. SARS, which originated in Guangdong Province, China, in 2003, spread along air-travel routes and quickly became a global threat because of its rapid transmission and high mortality rate and because protective immunity in the general population, effective antiviral drugs, and vaccines were lacking. However, compared with influenza, SARS had lower infectivity and a longer incubation period, providing time for instituting a series of containment measures that worked well (*39*). The strategies varied among the countries hardest hit by SARS (People's Republic of China and Hong Kong Special Administrative Region; Singapore; and Canada). In Canada, public health authorities asked persons who might have been exposed to SARS to voluntarily quarantine themselves. In China, police cordoned off buildings, organized checkpoints on roads, and even installed Web cameras in private homes. There was stronger control of persons in the lower social strata (village-level governments were empowered to isolate workers from SARS-affected areas). Public health officials in some areas resorted to repressive police measures, using laws with extremely severe punishments (including the death penalty), against those who violated quarantine. As had occurred in the past, the strategies adopted in some countries during this public health emergency contributed to the discrimination and stigmatization of persons and communities and raised protests and complaints against limitations and travel restrictions.

Conclusions

More than half a millennium since quarantine became the core of a multicomponent strategy for controlling communicable disease outbreaks, traditional public health tools are being adapted to the nature of individual diseases and to the degree of risk for transmission and are being effectively used to contain outbreaks, such as the 2003 SARS outbreak and the 2009 influenza A(H1N1)pdm09 pandemic. The history of quarantine—how it began, how it was used in the past, and how it is used in the modern era—is a fascinating topic in history of sanitation. Over the centuries, from the time of the Black Death to the first pandemics of the twenty-first century, public health control measures have been an essential way to reduce contact between persons sick with a disease and persons susceptible to the disease. In the absence of pharmaceutical interventions, such measures helped contain infection, delay the spread of disease, avert terror and death, and maintain the infrastructure of society.

Quarantine and other public health practices are effective and valuable ways to control communicable disease outbreaks and public anxiety, but these strategies have always been much debated, perceived as intrusive, and accompanied in every age and under all political regimes by an undercurrent of suspicion, distrust, and riots. These strategic measures have raised (and continue to raise) a variety of political, economic, social, and ethical issues (*39*,*40*). In the face of a dramatic health crisis, individual rights have often been trampled in the name of public good. The use of segregation or isolation to separate persons suspected of being infected has frequently violated the liberty of outwardly healthy persons, most often from lower classes, and ethnic and marginalized minority groups have been stigmatized and have faced discrimination. This feature, almost inherent in quarantine, traces a line of continuity from the time of plague to the 2009 influenza A(H1N1)pdm09 pandemic.

The historical perspective helps with understanding the extent to which panic, connected with social stigma and prejudice, frustrated public health efforts to control the spread of disease. During outbreaks of plague and cholera, the fear of discrimination and mandatory quarantine and isolation led the weakest social groups and minorities to escape affected areas and, thus, contribute to spreading the disease farther and faster, as occurred regularly in towns affected by deadly disease outbreaks. But in the globalized world, fear, alarm, and panic, augmented by global media, can spread farther and faster and, thus, play a larger role than in the past. Furthermore, in this setting, entire populations or segments of populations, not just persons or minority groups, are at risk of being stigmatized. In the face of new challenges posed in the twenty-first century by the increasing risk for the emergence and rapid spread of infectious diseases, quarantine and other public health tools remain central to public health preparedness. But these measures, by their nature, require

vigilant attention to avoid causing prejudice and intolerance. Public trust must be gained through regular, transparent, and comprehensive communications that balance the risks and benefits of public health interventions. Successful responses to public health emergencies must heed the valuable lessons of the past (*39*,*40*).

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Crimean-Congo Hemorrhagic Fever Virus in Ticks from Migratory Birds, Morocco[1]

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Crimean-Congo hemorrhagic fever virus was detected in ticks removed from migratory birds in Morocco. This finding demonstrates the circulation of this virus in northwestern Africa and supports the hypothesis that the virus can be introduced into Europe by infected ticks transported from Africa by migratory birds.

Crimean-Congo hemorrhagic fever virus (CCHFV), the causative agent of Crimean-Congo hemorrhagic fever (CCHV), is an arthropod-borne virus (arbovirus) with clinical relevance worldwide (*1*). CCHF causes sudden onset of signs and symptoms including headache, high fever, back pain, joint pain, stomach pain, and vomiting, which can progress to severe bruising, severe nosebleeds, and uncontrolled bleeding (www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/cchf.htm).

CCHFV, belonging to the genus *Nairovirus*, circulates in an enzootic tick-vertebrate-tick cycle in which ticks can act as vectors and reservoirs. CCHFV has been found in ticks of ≥30 species; *Hyalomma marginatum* ticks are considered the most common vectors. Birds are the main hosts for the immature stages of this tick species (*2*). Viremia does not develop in most passerine birds (*3*,*4*), which are not able to pass the virus to ticks. However, migratory species could carry infected ticks over long distances and thereby disseminate the virus (*2*).

Since the first descriptions of human infections with this virus in 1944–1955 in Crimea, outbreaks of CCHF have been reported in Africa, Asia, and eastern Europe (*1*). Only imported cases have been reported in western Europe, although the causal agent has been amplified in *H. lusitanicum* ticks collected from deer in Spain (southwestern Europe) (*5*). This finding could be explained by the arrival of infected ticks transported by migratory birds coming from Africa (*5*). To confirm this hypothesis, we investigated the presence of CCHFV in ticks collected from migratory birds in northern Africa.

The Study

In April 2011, bird bandings were conducted in Zouala, Morocco (31°47□N, 4°14□W) (Figure 1). A total of 546 captured birds were checked for ticks, and parasites were found on 21 birds from 5 passerine bird species (*Phoenicurus phoenicurus*, *Erythropygia galactotes*, *Iduna opaca*, *Acrocephalus scirpaceus*, and *I. pallida*). All but *I. pallida* birds are passerine trans-Saharan migrant species, coming from central and southern Africa and able to reach the Iberian Peninsula.

A total of 52 ticks (19 larvae and 33 nymphs) were processed. Genomic DNA and total RNA from ticks were individually purified by using the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA extracts were used as templates for 16S rDNA PCR assays (*6*), and all specimens were classified as *H. marginatum* ticks. RNA was retrotranscribed by using the Omniscript RT kit (QIAGEN), according to the manufacturer's instructions. cDNA extracts were distributed in 6 pools used as templates for 2 nested PCRs with Eecf and Gre primer pairs (*7*) (Table). Negative controls (with template DNA but without primers and with primers and containing water instead of template DNA) were included in all assays.

Nested PCR assays using Eecf primers were positive for 4/6 pools; all samples were negative by Gre PCR primers (*7*). Three of 4 amplicons (associated with *P. phoenicurus*, *E. galactotes*, and *I. opaca* bird species; Figure 2) could be sequenced, and nucleotide sequences were compared with those available in GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). All nucleotide sequences were identical and showed 100% identity with the Sudan AB1-2009 and Mauritania ArD39554 CCHFV strains (GenBank accession nos. HQ378179 and DQ211641) and 98.9% identity with the sequence amplified from ticks from Spain (*5*).

Conclusions

The detection of CCHFV in ticks from migratory birds in Zouala demonstrates the circulation of this virus

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[1]Data from this study were presented in part at the XVI Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica, Bilbao, Spain, May 9–11, 2012.

Figure 1. Location of Zouala, Morocco.

in Morocco. This country has optimal conditions for the establishment of CCHF, including populations of *H. marginatum* ticks and reservoirs of the virus, such as livestock. Furthermore, autochthonous cases of the disease have been reported in neighboring Mauritania (*8*).

Our finding of 3 positive tick pools demonstrates the potential dispersion of the virus through infected ticks transported by migratory birds. Several bird species, including *P. phoenicurus*, *E. galactotes*, *I. opaca*, and *A. scirpaceus*, migrate in the spring from southern or central Africa to northern Europe. The Iberian Peninsula may be a stopover or breeding site along those routes (*9*), which suggests that migratory birds may transport *H. marginatum* ticks from Africa to Europe (*2*).

Ticks of this species are found in Africa, southern Asia, and southern Europe (*10*,*11*); some specimens have been collected in northern European regions such as Germany or England (*12*,*13*). Birds are commonly parasitized by immature stages of *H. marginatum*, a 2-host tick that has the same host for larva and nymph stages. Thus, ticks may be attached to the bird for >2–3 weeks (*11*). An average ground speed of 50 km/h for migratory birds crossing Sahara has been reported (*14*); this speed would enable birds to cover the distance between Morocco and the Iberian Peninsula in less time than it would take ticks to develop from immature to adult.

In early May 2012, our team captured *A. scirpaceus* birds parasitized by *H. marginatum* ticks in northern Spain (42°48′N; 2°39′W); some ticks were fully engorged nymphs (A.M. Palomar, unpub. data). A high probability exists that the larvae were attached to birds in Africa and molted and engorged during migration, which supports the possibility of the arrival of migratory birds with CCHFV-infected ticks.

A study conducted in England of CCHFV in ticks collected from migratory birds found negative real-time PCR results for CCHFV, although the parasitization rate of birds with *H. marginatum* ticks was low (*13*). In addition, a group of experts has reported that migratory birds may not be sufficient to establish new foci of CCHFV infection in Europe and may not represent a high risk for its implantation because adult ticks are necessary and immature specimens cannot find optimal climate conditions to molt in spring (*10*). However, this report stated that Spain's average spring temperatures are lower than those needed for birds to molt; in April 2011, the weather station located in northern Spain (LaRioja) (42°27′N, 2°19′W) recorded average temperatures >14°C for 20/30 days. In addition, *H. marginatum* tick populations are established on the Iberian Peninsula.

Table. Distribution in pools of *Hyalomma marginatum* ticks collected from birds in Zouala, Morocco, and PCR results for detection of Crimean-Congo hemorrhagic fever virus*

		No. *H. marginatum* ticks collected, by stage		PCR results	
Pool	Bird species (no. specimens)	Larvae	Nymphs	Eecf primers	Gre primers
A	*Erythropygia galactotes* (1)		2 FE	+	–
	Phoenicurus phoenicurus (1)		6 FE		
B	*E. galactotes* (2)	1 SE	7 FE	+	–
	Iduna opaca (1)	3 FE			
C	*I. opaca* (2)	6 FE	1 FE	+	–
	P. phoenicurus (1)		1 SE		
D	*Acrocephalus scirpaceus* (1)	4 SE	1 FE	+†	–
	I. opaca (1)		1 FE		
	I. pallida (1)		1 SE, 1 FE		
E	*A. scirpaceus* (1)	2 FE		–	–
	E. galactotes (1)		2 FE		
	I. opaca (4)	1 FE	2 SE, 1 FE		
	P. phoenicurus (1)		1 FE		
F	*E. galactotes* (1)		2 FE	–	–
	I. opaca (2)	2 SE	1 SE, 1 FE		
	P. phoenicurus (1)		1 SE, 1 FE		

*FE, fully engorged; +, positive; –, negative; SE, semi-engorged.
†No sequence was obtained.

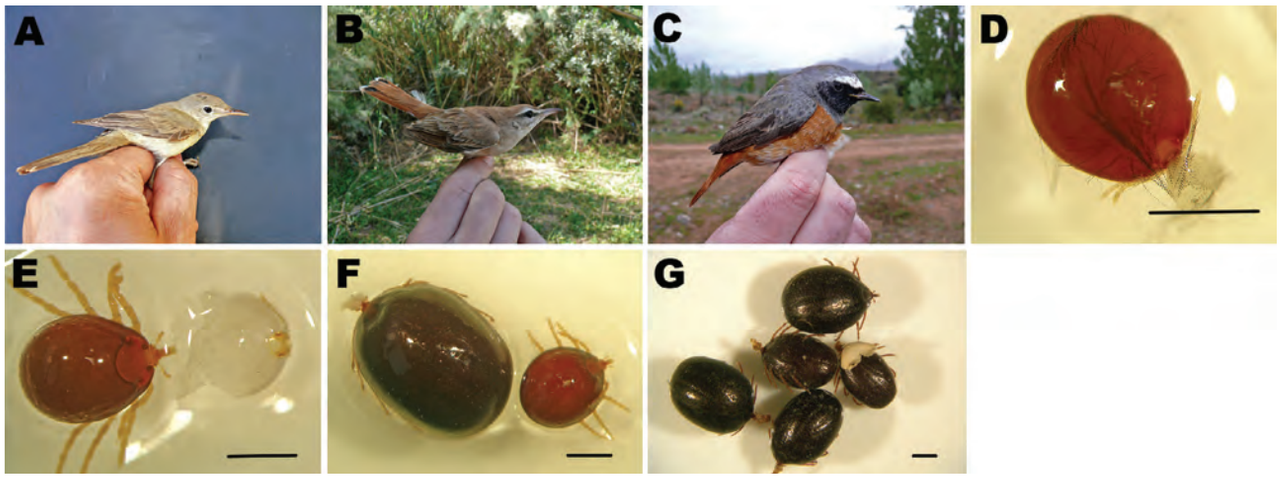

Figure 2. Bird species and tick specimens collected in Zouala, Morocco, April 2011. A) *Iduna opaca*, B) *Erythropygia galactotes*, and C) *Phoenicurus phoenicurus* birds. D–G) *Hyalomma marginatum* tick specimens removed from birds and preserved in alcohol: D) semi-engorged larva, E) semi-engorged nymph, F) semi-engorged and fully engorged nymphs, and G) fully engorged nymphs. Scale bars indicate 1 mm.

The finding of fragments of the small segment of CCHFV identical to fragments of the Mauritania and Sudan strains and closely related to the sequence previously amplified by our team (*5*), in pools of immature specimens of different engorged states (Figure 2), may explain 2 hypotheses. First, infected ticks from CCHF-endemic areas, such as Mauritania or Sudan, could have attached to birds and transported to Morocco over them. Second, these ticks could have been carried from Africa to the Iberian Peninsula, thus explaining the circulation of CCHFV in southwestern Europe.

Autochthonous cases of CCHF have not been reported in southwestern Europe, and CCHFV has not been detected in ticks from migratory birds in Europe (*13*). However, some authors support the limited role of migratory birds harboring CCHFV-infected ticks for the establishment of the virus (*10*,*15*). Nevertheless, the finding of CCHFV from migratory birds in Morocco, along with the previous detection of the virus in Spain (*5*), where *H. marginatum* tick populations and CCHFV reservoirs occur, call for further study of the distribution of CCHFV in southwestern Europe. Health care workers should be informed about the possibility of CCHF and the clinical picture in the potential disease-endemic areas, and groups potentially at risk for infection (e.g., health care workers, hunters, farmers) and reservoirs for the virus (livestock) should be investigated further.

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etymologia

Quarantine
[kwor′ən-tēn]

From the Italian quaranta (forty), "quarantine" refers to the practice established in European port cities during the Black Death requiring vessels to lie at anchor for 40 days before landing. Isolation (from the Latin insula or island), the practice of separating sick persons from those who are healthy to prevent spread of disease, goes back a long time. "As long as they have the disease they remain unclean. They must live alone; they must live outside the camp" (Leviticus 13:46).

Quarantine, on the other hand, is the practice of separating persons who appear to be healthy but may have been exposed to a disease. In 1377, the Great Council of Ragusa (modern-day Dubrovnik) established a 30-day separation period (trentino) for visitors from plague-endemic areas. In the following decades, the practice spread to other cities and was extended from 30 to 40 days (quarantino). The longer period may have been more effective at preventing disease or just a nod to the 40-day duration of Biblical events–the Great Flood or Jesus' fast in the wilderness.

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Hepatitis E Virus in Pork Liver Sausage, France

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We investigated viability of hepatitis E virus (HEV) identified in contaminated pork liver sausages obtained from France. HEV replication was demonstrated in 1 of 4 samples by using a 3-dimensional cell culture system. The risk for human infection with HEV by consumption of these sausages should be considered to be high.

Foodborne transmission of hepatitis E virus (HEV) to humans from consumption of undercooked pig liver and deer meat has been reported in Japan (*1*–*3*). In addition, commercial pig livers purchased from local grocery stores in Japan, the United States, and Europe were contaminated with HEV (*4*–*6*). Epidemiologic and PCR results linked a cluster of autochthonous acute hepatitis E cases to ingestion of raw figatelli, which is a dried, cold-smoked sausage containing ≈30% pig liver (*7*). We investigated the viability of HEV in pork liver sausages produced in France.

The Study

Four samples of pork liver sausage (designated sausages A–D) collected at the final production stage from 4 independent manufacturers in 3 locations in southern France were found to be HEV positive by using real-time reverse transcription PCR (RT-PCR). Samples were tested in 2 institutes, the Animal Health and Veterinary Laboratories Agency, United Kingdom, and Wageningen University and Research Centre, the Netherlands. Three-dimensional (3D) cell culture propagation was performed to investigate the presence of infectious HEV particles. PLC/PRF/5 hepatocarcinoma cells (American Type Culture Collection 8024) were cultured as a monolayer (2D) at 37°C in GTSF-2 medium (*8*) in a 5% CO_2 environment. Cells were trypsinized at 95% confluence and resuspended in fresh medium to a density of 2×10^5 cells/mL. Fifty milliliters of cell suspension was then introduced into a rotating wall vessel with 10 mg/mL of porous Cytodex-3 microcarrier beads (collagen type I–coated porous microspheres, average diameter 175 µm (Sigma, Dorset, UK and Zwijndrecht, the Netherlands) and incubated at 37°C in 5% CO_2. The cells were incubated for ≥28 d before inoculation to enable complete differentiation.

The inoculum was prepared by homogenizing a 2.5-mg fragment of each sample with mortar and pestle in 5 mL of culture medium. The homogenate was centrifuged at 8,000 × *g* for 3 min, and the supernatant was filtered sequentially through 1.2-µm, 0.45-µm, and 0.2-µm filters to reduce the risk for bacterial contamination. The medium was removed from the rotating wall vessel, and 2.5 mL of inoculum was incubated with the cells for 2 h at 35.5°C; at that point, 47.5 mL of fresh medium was added. Subsamples of medium (140 µL) were collected in duplicate, added to 560 µL of lysis buffer (QIAamp Viral RNA Mini Kit; QIAGEN, Crawley, UK, and Venlo, the Netherlands), and stored at −20°C until RNA extraction was performed.

Both institutes performed real-time RT-PCR by using primers and probe as described (*9*), using the Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Paisley, UK, and Bleiswijk, the Netherlands).

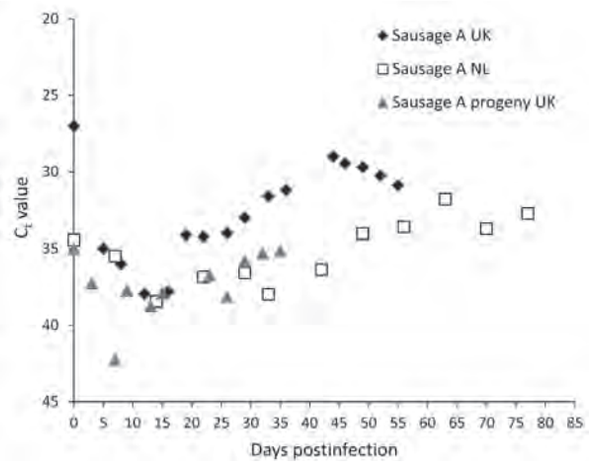

Figure 1. Cycle threshold (C_t) values detected by real-time reverse transcription PCR for hepatitis E virus–positive supernatant of 3D cells infected with homogenate of pork sausages from France. Diamonds indicate testing of sausage A in the United Kingdom; squares indicate testing of sausage A in the Netherlands; triangles indicate testing of progeny of sausage A in the United Kingdom.

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Negative (water) and positive (extract from positive fecal sample, genotype 3) controls were included.

HEV RNA was detected in the 3D cell culture supernatants of all 4 sausage samples up to 8 d postinfection (dpi). Thereafter, HEV RNA was detected only in the cells inoculated with the sample A homogenate (Figure 1); it was assumed that the signals from the other 3 samples represented residual inoculum or an abortive infection. The sample A culture showed a cycle threshold (C_t) value of 27 on the day of inoculation (dpi 0) that increased to 35 on dpi 5, continued to increase until dpi 11, when it peaked at 38 and then began to decrease. A low C_t value of 29 was observed on dpi 44. Similar results were obtained in the laboratory in the Netherlands (Figure 1).

To evaluate the infectivity of progeny viruses from the primary inoculation, supernatant positive for HEV by RT-PCR from dpi 16 was used to infect fresh 3D PLC/PRF/5 cultures according to the protocol described for the primary inoculation. Cells infected with the supernatant from the original sample A homogenate cultures had positive HEV RNA test results on most days after inoculation; C_t values remained relatively constant at an average of 37 from immediately after inoculation (dpi 0) to the end of the experiment at dpi 35.

To compare the cultured virus to the inoculum, a partial fragment of the open reading frame 2 of HEV extracted from culture subsamples (dpi 16, dpi 55; progeny subsample at dpi 35) was sequenced as described (*10*). HEV RNA sequences detected in the culture subsamples were characterized and demonstrated 100% identity with that of the inoculum (304 bp of open reading frame 2) but differed from the control strain used. These sequences were confirmed as HEV genotype 3.

Further confirmation of the presence of viable virus particles in cell culture was sought by using electron microscopy. Supernatants of the cell cultures were applied to Pioloform/carbon-coated, 400-mesh copper grids (Plano GmbH, Wetzlar, Germany) for 10 min, fixed with 2.5% aqueous glutaraldehyde solution for 1 min, and stained with 2% aqueous uranyl acetate solution for 1 min. The specimens

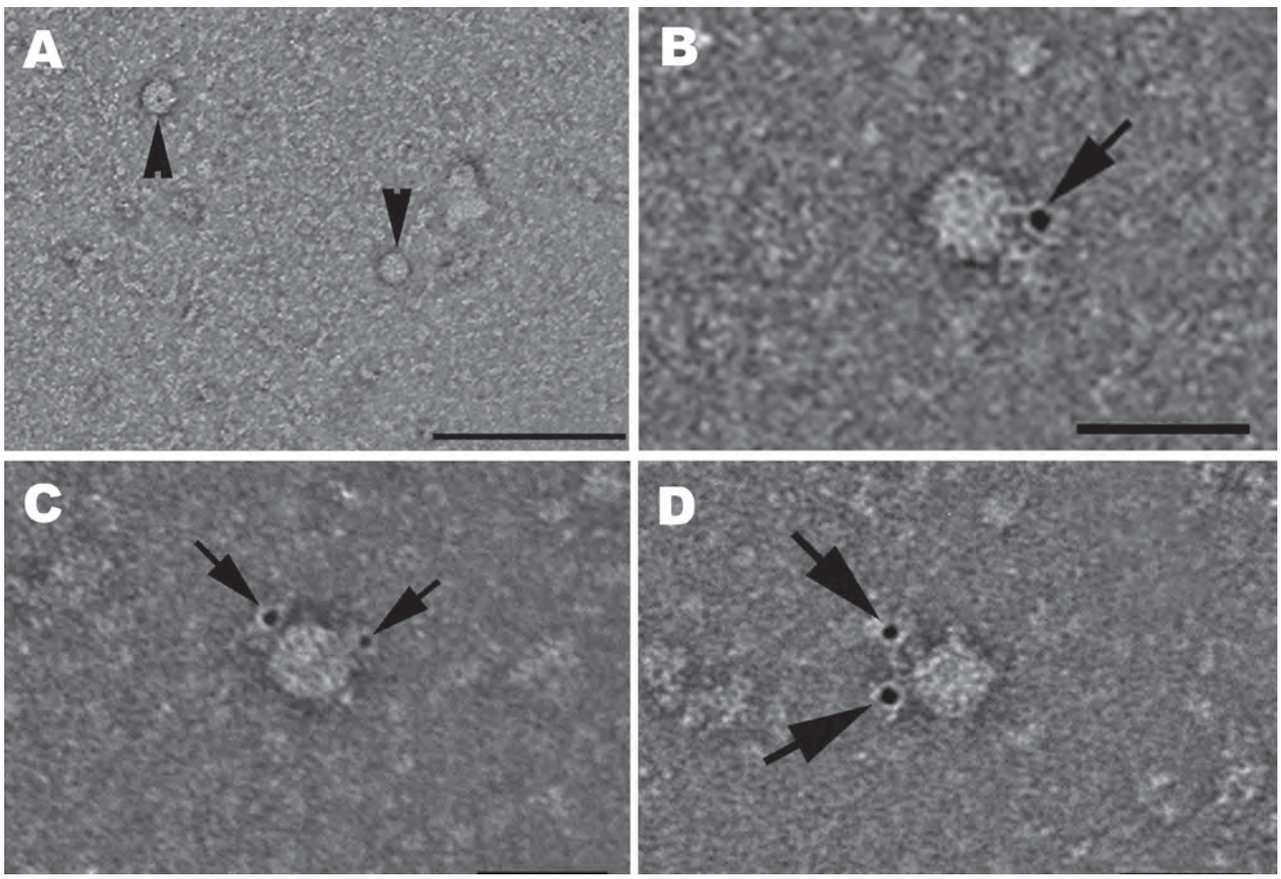

Figure 2. Hepatitis E virus (HEV) particles in the cell culture supernatant of pork liver sausage sample A, collected at 33 dpi. A) Transmission electron micrograph of negatively stained HEV particles ≈33 and 34 nm (arrowheads). Scale bar indicates 200 nm. B–D) Hepatitis E virions ≈28 (B), 33 (C), or 32 (D) nm in diameter, identified by using an HEV genotype 3–specific rabbit hyperimmune serum and a gold-labeled secondary antibody. Arrows show bound gold particles. Scale bars indicate 50 nm.

were examined through transmission electron microscopy by using a JEM-1010 microscope (JEOL, Tokyo, Japan) at an 80-kV accelerated voltage. HEV particles were observed in the supernatant of the sample A culture collected at dpi 33 (Figure 2, panel A). For additional proof by immunoelectron microscopy, an *Escherichia coli*–expressed, His-tagged HEV genotype 3 capsid protein derivative harboring amino acid residues 326–608 (*11*) was used to generate HEV-positive serum in a rabbit by 3 subcutaneous inoculations at 4-week intervals (P. Dremsek et al., unpub. data). The immunoelectron microscopy examination of this serum and goat anti-rabbit IgG linked with 5-nm gold particles (BBInternational, Oconomowoc, WI, USA) confirmed the presence of HEV particles (Figure 2, panels B–D).

Conclusions

We confirmed that 1 sample of pork liver sausage that had positive test results for HEV RNA by real-time RT-PCR contained viable HEV. We cultured 4 sausage samples in 2 different institutes by performing 3D cell-culture propagation of HEV; HEV replication was detected in the same sample independently in both institutes, and the replicated virus was shown to be infectious. We observed entire, cell-free virus particles by transmission electron microscopy at dpi 33, providing further proof of in vitro replication of the virus that contaminated the pork liver sausage.

We conclude that pork liver sausages can contain infectious HEV and that consuming these products should be regarded as a risk factor for HEV infection. Furthermore, we have shown the potential of the 3D culture system to correlate the presence of HEV RNA with the presence of infectious HEV particles.

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Human Infection with *Rickettsia sibirica mongolitimonae*, Spain, 2007–2011

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Human infection with *Rickettsia sibirica mongolitimonae* was initially reported in 1996, and reports of a total of 18 cases have been published. We describe 6 additional cases that occurred in the Mediterranean coast region of Spain during 2007–2011. Clinicians should consider this infection in patients who have traveled to this area.

The genus *Rickettsia* contains ≈25 validated species of bacteria; another 25 isolates that have not been fully characterized or have not received a species designation have also been described. Signs and symptoms of human rickettsiosis caused by spotted fever group *Rickettsia* spp. include an inoculation eschar (a necrotic area at the site of the tick bite that might not be always present), fever, local adenopathies, and rash, although some variability can be found, depending on the infecting *Rickettsia* species.

R. sibirica mongolitimonae (also spelled *mongolotimonae*) was isolated from a *Hyalomma asiaticum* tick collected in the Alashian region of Inner Mongolia in 1991 (*1*). Designated *R. mongolitimonae*, the organism was identified as a member of the *R. sibirica* species complex (*2*), but further phylogenetic analyses grouped it in a cluster separate from other strains of *R. sibirica*.

The first human case of infection with *R. sibirica mongolitimonae* was reported in France in 1996 (*3*); since then, 18 additional cases have been described in the literature (*4*–*14*). Clinical signs and symptoms of infection are fever; a discrete, maculopapular rash; and enlarged regional lymph nodes, with or without lymphangitis. Although *R. sibirica mongolitimonae* infection causes a mild, not fatal, disease, complications such as acute renal failure and retinal vasculitis have been noted (*7*,*10*). We report 6 cases of human *R. sibirica mongolitimonae* infection from the same geographic region of Spain.

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The Study

During July 2007–July 2011, six patients from the Mediterranean coast city of Elche, Spain, who had high fever and inoculation eschars received a diagnosis of infection with *R. sibirica mongolitimonae* (Table). For laboratory confirmation, DNA was extracted from eschars, lymph nodes (fine-needle aspiration), and blood samples by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For molecular detection, 200–400 ng of DNA from each sample was subjected to PCR targeting the 23S-5S rRNA intergenic spacer, followed by hybridization with specific probes by reverse line blotting, as described (*15*). When using the probe for *R. sibirica mongolitimonae*, a positive hybridization signal was obtained from eschar samples from all 6 patients; this result was confirmed by sequencing (100% similarity to a reference *R. sibirica mongolitimonae* strain [GenBank accession no. HQ710799] in all cases in the 357 bp sequenced). To further confirm this result, nested PCR targeting the gene for outer membrane protein A was performed as described (*15*); these sequences (514 bp) also showed 100% similarity to a reference *R. sibirica mongolitimonae* strain (GenBank accession no. HQ728350).

Serologic response was analyzed by using an in-house microimmunofluorescence assay for IgG and IgM, performed as described (*4*); *R. conorii* and *R. sibirica mongolitimonae* were used as antigens, and cutoff values were 1:40 for IgG and 1:20 for IgM. Acute- and convalescent-phase serum samples were obtained from 3 case-patients and single serum samples from the other 3 case-patients. Results for samples from 2 case-patients were negative, but results for the remaining 4 samples showed low to medium titers. Two samples were positive for IgM and 4 positive for IgG (Table). These results are consistent with previous reports (*6*), in which ≈30% of cases had a positive IgM result and ≈50% had negative or near-cutoff IgG results.

All 6 case-patients lived in Elche and its surroundings (230,112 inhabitants). Three of the cases occurred during the spring, which is when 10/18 cases reported in the literature occurred (*4*–*14*). All 6 case-patients had fever (38.5°C–39.5°C), myalgia, and headache; in the cases from the literature, 18/18 patients had fever, 13/18 myalgia, and 11/18 headache. In our study, 1 case-patient was confused and drowsy on arrival at the emergency department.

All 6 case-patients had a single inoculation eschar develop: 2 on the neck, 2 on a lower limb (Figure), 1 on the scalp, and 1 on an upper limb. Five (83%) case-patients had enlarged lymph nodes in the region from which the eschar drained, as reported for 10/18 (55%) cases from

Table. Epidemiologic, clinical, and microbiologic characteristics associated with 6 case-patients infected with *Rickettsia sibirica mongolitimonae*, Spain, 2007–2011*

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Patient age, y/sex	67/F	32/M	33/M	42/F	40/F	75/F
Date of illness onset	2007 Jul	2009 Sep	2010 Apr	2011 Mar	2011 Apr	2011 Jul
Type of residence	Rural	Urban	Urban	Rural	Rural	Rural
At-risk activity	Gardening	Working at golf courses	Walking in rural area	Walking in rural area	Excursion by horse	Walking in rural area
Report of tick bite	No	No	No	No	Yes	No
Duration of fever, d	10	4	5	5	6	4
Temperature, °C	38.5	39.5	39.4	39.0	39.0	39.2
Headache	Yes	Yes	Yes	Yes	Yes	Yes
Myalgia	Yes	Yes	Yes	Yes	Yes	Yes
Location of eschar	Scalp	Thigh	Leg	Shoulder	Neck	Leg
Location of enlarged regional lymph nodes	Retroauricular	Inguinal	Inguinal	Supraclavicular	Retroauricular	None
Lymphangitis	No	Yes	Yes	No	No	Yes
Rash	Yes	Yes	No	No	Yes	Yes
Leukocytes, × 10^3 cells/μL	11.1	2.93	6.40	6.05	NA	NA
Platelets, × 10^3/μL	540	126	198	217	NA	NA
AST, IU	79	50	48	NA	NA	NA
ALT, IU	65	53	27	NA	NA	NA
C-reactive protein, mg/dL	101	44	46	15.4	NA	NA
Lactate dehydrogenase, IU	642	567	NA	NA	NA	NA
Treatment	Doxycycline	Azithromycin	Doxycycline	Doxycycline	Doxycycline	Doxycycline
Complications	Hyponatremia, lethargy	NA	NA	NA	NA	NA
PCR results						
Eschar	Positive	Positive	Positive	Positive	Positive	Positive
Lymph nodes	NA	Negative	Negative	NA	NA	NA
Whole blood	Negative	NA	NA	NA	NA	NA
IgM/IgG against *R. sibirica mongolitimonae*†						
Acute-phase sample	40/160	<20/<40	<20/<40	NA	40/160	<20/80
Convalescent-phase sample	NA	NA	<20/<40	<20/40	<20/80	<20/80
IgM/IgG against *R. conorii*†						
Acute-phase sample	40/160	<20/<40	<20/<40	NA	40/160	<20/40
Convalescent-phase sample	NA	NA	<20/<40	<20/40	<20/80	<20/40

*NA, not available; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
†Determined by using in-house microimmnunofluorescence assay.

the literature. Three case-patients (50%) had lymphangitis extending from the eschar to the draining lymph nodes (Figure), compared with 6/18 (33%) in cases from the literature.

For 4/6 (67%) case-patients, a generalized maculopapular rash developed on the palms and soles but not the face; for 2 case-patients, a discrete maculopapular rash appeared after 1 day of treatment. These findings are consistent with our review of the literature, which indicated rash occurring in 13/18 (72%) cases. All 6 case-patients recovered without sequelae after antimicrobial drug treatment using doxycycline or azithromycin.

Conclusions

Fournier et al. (*4*), who reported 7 cases of *R. sibirica mongolitimonae* infection in 2005, proposed the name lymphangitis-associated rickettsiosis for the disease, on the basis of associated clinical features. However, for the case-patients reported here, the most common clinical signs and symptoms were fever and skin eschar, similar to those from previously reported case series; 5 of the case-patients reported here showed regional lymph node enlargement, 4 rash, and 3 lymphangitis. Because only 24 total cases have been reported and other rickettsioses produce lymphadenopathy and lymphangitis, the term lympangitis-associated rickettsiosis may be unwarranted for this disease.

In our case series, 1 case-patient had mental confusion after 10 days of a febrile disease before hospitalization and was found to be hyponatremic. In this patient, the eschar was located on the scalp, and neither rash nor other clinical clues were suggestive of rickettsiosis. The patient had increased C-reactive protein plasma levels and the highest serologic antibody titers for *R. sibirica mongolitimonae* of the 6 case-patients (Table, patient 1).

Since *R. sibirica mongolitimonae* was isolated from *H. asiaticum* ticks in 1991 (*1*), it has been recovered from *H. truncatum* ticks in sub-Saharan Africa (*6*) and *H. anatolicum excavatum* ticks in Greece (*7*). These findings suggest a possible association between *R. sibirica mongolitimonae* and *Hyalomma* spp. ticks. However, in Spain, *R. sibirica mongolitimonae* has been detected in 2/8 tick species tested (*Rhipicephalus pusillus* and *Rh. bursa*) at similar percentages (3.7% and 3.6%, respectively)

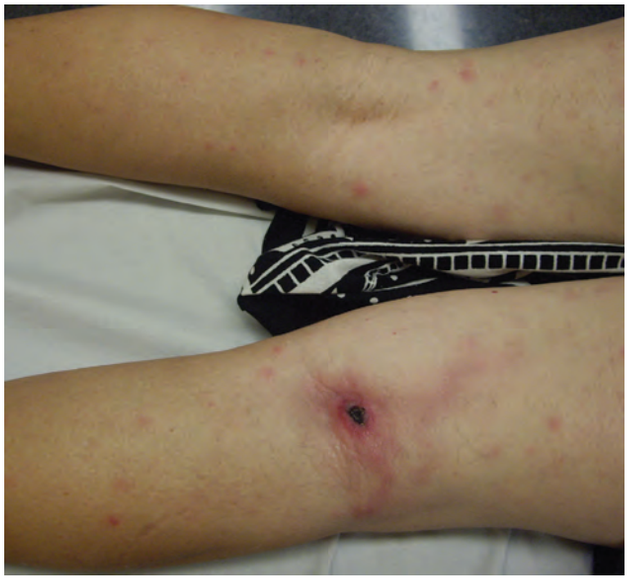

Figure. Inoculation eschar on popliteal area and discrete maculopapular elements in patient with lymphangitis infected with *Rickettsia sibirica mongolitimonae*, Spain, 2011.

but not from *Hyalomma* spp. ticks (*15*). Similarly, 1/20 samples of *Rh. pusillus* ticks from Portugal was positive for *R. sibirica mongolitimonae* (*8*), while testing of other tick species, including *H. lusitanicum*, *Rh. sanguineus*, and *Rh. bursa*, yielded negative results. That *Hyalomma* spp. (*H. lusitanicum* and *H. marginatum*) ticks have not been found to be infected in Spain does not mean that these ticks are not vectors for *R. sibirica mongolitimonae*. However, data from the literature support the hypothesis that *Rhipicephalus* spp. ticks are a vector for this rickettsia on the Iberian Peninsula, and our findings confirm that this rickettsia species is circulating in Spain, where specific vectors have yet to be described.

In summary, we report 6 cases of human infection with *R. sibirica mongolitimonae* that occurred in the same geographic area of Spain. Our results indicate that PCR of eschar samples is the most useful diagnostic procedure for this pathogen; samples from all 6 case-patients had positive results, while test results for 1 whole blood sample and 2 lymph node samples were negative. However, the limited number of samples does not make it possible to infer specific diagnostic sensitivities.

The epidemiology and pathogenicity of illness caused by *R. sibirica mongolitimonae* infection require further investigation. An active search for the vector of *R. sibirica mongolitimonae* in countries of the Mediterranean region is necessary to complete the epidemiology of this rickettsiosis, which is likely to be more widespread than originally assumed. In particular, clinicians caring for patients who have traveled to the Mediterranean coast of Spain should consider this rickettsiosis in the differential diagnosis.

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Ebola Virus Antibodies in Fruit Bats, Bangladesh

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To determine geographic range for Ebola virus, we tested 276 bats in Bangladesh. Five (3.5%) bats were positive for antibodies against Ebola Zaire and Reston viruses; no virus was detected by PCR. These bats might be a reservoir for Ebola or Ebola-like viruses, and extend the range of filoviruses to mainland Asia.

Filoviruses are zoonotic pathogens that cause episodic, lethal, hemorrhagic outbreaks among humans and nonhuman primates and case-fatality rates up to 80% (*1*). The family *Filoviridae* contains 2 genera: *Marburgvirus*, which contains Marburg virus (MARV), and *Ebolavirus*, which contains 4 viruses: Zaire Ebola virus (ZEBOV), Sudan Ebola virus, Reston Ebola virus (REBOV), and Côte d'Ivoire Ebola virus, and 2 tentative species (Bundibugyo Ebola virus and Lloviu Ebola virus) (*2*,*3*). Pathogenicity varies among Ebola viruses, from ZEBOV, which is highly lethal in humans, to REBOV, which causes disease in pigs and macaques but asymptomatically infects humans.

Despite their role in human disease, natural reservoirs of filoviruses have remained elusive for decades. Reports suggest that bats (Order Chiroptera) are the primary natural hosts, including Old World insectivorous bats (genera *Rhinolophus* and *Miniopterus*) and frugivorous bats (family *Pteropodidae*). Fruit bats of the genus *Rousettus* have been implicated as a reservoir of filoviruses in Africa (*4*–*7*) and REBOV in the Philippines (*8*). Lloviu Ebola virus was detected in *Miniopterus schreibersii* insectivorous bats from Spain and appears to cause pathologic changes in this species but is not known to infect humans (*2*). These studies point to a wide, and still poorly described, geographic distribution for viruses of the family *Filoviridae* in chiropteran hosts. We screened bats of several species from Bangladesh for Ebola virus infection to determine whether the geographic range of this virus extends to southern Asia.

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The Study

We captured and sampled 276 bats (141 *Rousettus leschenaultii* bats, 75 *Cynopterus* spp. bats, 59 *Megaderma lyra* bats, and 1 *Macroglossus sobrinus* bat) during April 2010–March 2011 from the Faridpur, Rajbari, Lalmonirhat, and Comilla Districts in Bangladesh. All bats were identified to species in the field, except *Cynopterus* spp. bats, because of cryptic diversity in this group; we are awaiting genetic species confirmation. Bats were captured in mist nets near roosts or at feeding sites and were handled in accordance with the Tufts University (Medford, MA, USA) Institutional Animal Care and Use Committee protocol (no. G2011-106).

We collected 50–800 μL of blood from brachial or cephalic veins of each bat, and diluted it 1:4 with phosphate-buffered saline in the field before serum was separated, as described (*9*). We also collected throat, urine/urogenital, and fecal swab specimens, which were placed in 750 μL of NucliSENS lysis buffer (bioMérieux, Marcy l'Etoile, France). All samples were collected in cryovials, placed in liquid nitrogen in the field, and maintained at –80°C until testing. We recorded morphologic measurements, weight, sex, age, and body condition and collected a wing biopsy specimen before releasing animals at capture sites.

We screened serum samples for IgG against REBOV and ZEBOV by using ELISA and Western blotting at the Commonwealth Scientific and Industrial Research Organisation Australian Animal Health Laboratory Biocontainment Facility (Geelong, Victoria, Australia). To inactivate potentially infectious agents, serum samples were heated at 56°C for 20 min before shipment. All samples were screened by using a 1:1 mixture of purified recombinant nucleoproteins (0.2 mg/mL) of REBOV and ZEBOV (R + Z ELISA), which were expressed in an *Escherichia coli* vector that contained a histidine tag (*10*,*11*).

Potentially positive serum cutoff values were determined to be >0.454 for the R + Z ELISA by using maximum-likelihood estimation, gamma distribution, and 95% risk for error (*7*). Potentially positive serum samples were tested by ELISA against each nucleoprotein independently to confirm reactivity and by Western blotting against nucleoproteins of Reston and Zaire virus strains as described (*10*). Serum samples were tested at a dilution of 1:50. Endpoint titrations with an optical density >3× the background reading were determined for serum samples positive against REBOV and ZEBOV antigens individually.

Total nucleic acids were extracted from samples (urine/urogenital, fecal, and throat swab specimens) by using the easyMAG NucliSENS platform (bioMérieux) at Columbia University (New York, NY, USA). Samples were tested for filovirus RNA (RNA polymerase gene) by using a consensus PCR protocol validated to amplify 19 diverse filovirus strains. This PCR has a sensitivity of 50–500 RNA copies with synthetic transcripts and has been further validated with blood samples (*12*).

Fifteen (11%) of 141 *R. lescehnaulti*, 6 (8%) of 75 *Cynopterus* spp., and 4 (7%) of 56 *M. lyra* bats were potentially positive after initial screening. Five (3.5%) of 141 (95% CI 1.5%–8.0%) *R. leschenaultii* bats were confirmed as seropositive after testing by ELISAs and Western blotting (Table 1). Bats were sampled during the breeding season; 21 (62%) of 34 sampled female *R. leschenaultii* bats were pregnant and 8 (23%) of 34 carried pups. We sampled 3× as many males as females; all 5 confirmed virus-positive animals were healthy adult males (Table 2). All 698 throat, urine/urogenital, and fecal samples were virus negative by PCR (Table 2). All confirmed seropositive samples except 1 (April 2010–042) reacted more strongly to Zaire virus antigens than Reston virus antigens (Table 1). Similarly, 2 samples (April 2010–057 and SB0311–059) showed higher reactivity to ZEBOV by Western blotting, and other samples were equally reactive to REBOV.

Conclusions

Our study provides evidence of Ebola virus infection in wildlife from mainland Asia and corroborates the observation that filoviruses are harbored across a much larger geographic range then assumed (*2*). Preferential reactivity to ZEBOV suggests exposure to an Ebola virus that is distinct from REBOV, the only filovirus currently found in Asia. We consider the likelihood of cross-reactivity with MARV as low because there is only a 35% aa identity between nucleoprotein genes of REBOV/ZEBOV and MARV. However, we cannot rule out co-infection with multiple filoviruses.

Seroprevalence found in this study is consistent with that found in another study (*4*). However, other studies of *Rousettus* spp. bats have reported higher values (e.g., 7%–20% and 8% of *R. aegyptiacus* bats seropositive for MARV and ZEBOV, respectively) (*6*,*7*), and 5 (31%) of 16 *R. amplexicaudatus* bats seropositive for REBOV (*8*). These differences might have been caused by poor specificity of the assay if this virus is novel, an artifact of low volume of blood collected, the potential that other species may have greater roles as reservoirs than *Rousettus* spp. in Bangladesh, or timing of sampling. *R. leschenaultii* bats have a large range (China to India) (*13*); and more detailed studies of virus ecology and diversity are warranted to better understand their role as a potential reservoir of zoonotic disease agents.

Table 1. Ebola virus serologic assay results for bats, Bangladesh, 2010–2011*

Year, specimen no.	Age of bat	Sex of bat	Species or control	ELISA OD (endpoint titration)			Western blot	
				R + Z	R	Z	R	Z
2010								
Rab691/d0	ND	ND	Negative control	0.138	0.116	0.097	–	–
April 2010–001	A	F	Negative control (*Rousettus leschenaultii*)	0.215	0.117 (50)	0.058 (50)	–	–
April 2010–002	A	F	Negative control (*R. leschenaultii*)	0.092	0.096	0.059	–	–
Rab691/EboV-N	ND	ND	Positive control	**2.303**	**1.72**	**1.23**	**++**	**++**
Monkey/EboV	ND	ND	Positive control	**1.753**	**0.676**	**0.445**	NT	NT
April 2010–042	A	M	*R. leschenaultii*	**1.512**	**0.511 (400)**	0.07 (50)	+	+
April 2010–057	A	M	*R. leschenaultii*	**0.684**	0.072 (50)	**0.477 (800)**	+	++
66 additional negative	ND	ND	*R. leschenaultii*	<0.60	–	–	NT	NT
2011								
Rab691/d0	ND	ND	Negative control	0.165	0.116	0.145	–	–
SB0311–115	A	F	Negative control (*Megaderma lyra*)	0.515	0.074	0.083	–	–
SB0311–117	A	F	Negative control (*M. lyra*)	0.775	0.075	0.072	–	–
Rab691/ REboV-N	ND	ND	Positive control	**1.598**	**1.123**	**1.106**	**++**	**++**
SB0311–001	A	M	*R. leschenaultii*	**0.494**	0.213 (50)	**0.538 (100)**	**+**	**+**
SB0311–004	A	M	*R. leschenaultii*	**0.557**	0.152 (50)	**0.497 (100)**	+	+
SB0311–059	A	M	*R. leschenaultii*	**0.757**	0.079 (50)	**0.816 (400)**	–	++
SB0311–016	A	F	*R. leschenaultii*	**0.542**	0.182 (100)	**0.367 (400)**	NT	NT
67 additional negative	ND	ND	*R. leschenaultii*	<0.60	NT	NT	NT	NT
55 additional negative	ND	ND	*M. lyra*	<0.775	NT	NT	NT	NT
75 negative	ND	ND	*Cynopterus* sp.	<0.595	NT	NT	NT	NT
1 negative	A	M	*Macroglossus sobrinus*	<0.256	NT	NT	NT	NT

*Values in **boldface** are positive results. OD, optical density; R + Z, ELISA using a 1:1 mixture of recombinant nucleoproteins of Reston and Zaire Ebola viruses; R, Reston Ebola virus ELISA; Z, Zaire Ebola virus ELISA; ND, not determined; A, adult; –, negative; ++, strongly positive; NT, not tested; +, positive.

Table 2. Bat specimen results for filovirus by PCR and Ebola virus by serologic analysis, Bangladesh, 2010–2011*

Bat species, sex, and sample type	No. positive/ no. tested
Cynopterus spp., n = 75, 43 M, 32 F	
Feces swab	0/74
Throat swab	0/75
Serum	0/75
Urine/urogenital swab	0/39
Macroglossus sobrinus, n = 1, 1 M	
Feces swab	0/1
Throat swab	0/1
Serum	0/1
Urine/urogenital swab	0/1
Megaderma lyra, n = 56, 23 M, 33 F	
Feces swab	0/56
Throat swab	0/56
Serum	0/56
Urine/urogenital swab	0/50
Rousettus leschenaultii, n = 141, 106 M, 34 F, 1 ND	
Feces swab	0/141
Throat swab	0/140
Serum	5/141
Urine/urogenital swab	0/58
Total	5/971

*ND, sex not determined.

We demonstrated that serologic and virus surveys of bats can be informative for identifying potential virus hosts. Previous studies amplified ZEBOV nucleic acid from bat feces (*14*). We also screened bat feces to identify potential routes of virus excretion, which is useful when the route of exposure from bats to humans is known. A short interval for Ebola virus shedding by reservoir hosts and an inverse relationship between viremia and antivirus titer probably explain our negative PCR results for seropositive bats. Failure to detect filovirus nucleic acid might reflect our relatively small sample size, low virus prevalence, or use of a PCR that has low sensitivity for filoviruses circulating in Bangladesh.

In Bangladesh, human outbreaks of Nipah virus have been linked to drinking date palm sap contaminated with bat excreta, presumably from *Pteropus giganteus* bats (*15*). *R. leschenaultii* bats and other small fruit bat species visit date palm trees 10× more frequently than *Pteropus* spp. bats (*15*). This finding could indicate potential transmission of filoviruses or any other novel viruses that *R. leschenaultii* bats carry. It also highlights the need for more research to understand this ecologic system and for better implementation of low-cost barriers to reduce bat–human contact during periods of date palm harvesting (*15*).

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Usutu Virus, Italy, 1996

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Retrospective analysis of archived tissue samples from bird deaths in the Tuscany region of Italy in 1996 identified Usutu virus. Partial sequencing confirmed identity with the 2001 Vienna strain and provided evidence for a much earlier introduction of this virus into Europe than previously assumed.

In early fall 1996, an episode of wild bird deaths occurred in the provinces of Florence and Pistoia (Tuscany region), Italy. Several bird species were affected; however, most observed bird carcasses were Eurasian blackbirds (*Turdus merula*). Several animals were subjected to necropsy, which predominantly showed swollen livers and spleens, necrotizing pericloacal dermatitis, and a variety of endoparasites. Bacteriologic, virologic, and toxicologic investigations produced no conclusive results. Formalin-fixed and paraffin wax–embedded tissue samples were archived. This event was reported in an Italian veterinary journal with local distribution and thus did not receive broad attention (*1*)

Five years later in late summer 2001, similar seasonal deaths of wild birds, again predominantly blackbirds, were observed in neighboring Austria (*2*). A particular strain of Usutu virus (USUV) was determined as the causative agent of this fatal bird disease outbreak, which recurred in Austria in subsequent years (*3*), and was later identified in Hungary (*4*), Switzerland (*5*), Italy (*6*), and Germany (*7*).

There were similarities between 1996 bird deaths in Tuscany and subsequent USUV-associated bird deaths in other areas of Europe. Thus, we retrospectively analyzed archived paraffin wax–embedded material from the Tuscany cases for USUV.

The Study

Thirty-three paraffin blocks generally containing multiple tissue samples (such as brain, liver, spleen, kidney, lung, heart, proventriculus, gizzard, intestine, pancreas, and skeletal muscle) were used for detection of USUV. Most organ samples were from blackbirds, but some were from other bird species (Table). Tissue blocks were assigned to 4 groups: group 1 (7 blocks, blackbirds found dead or severely ill during August 28–September 25, 1996); group 2 (6 blocks, blackbirds found during October 1–November 19, 1996); group 3 (10 blocks, blackbirds, starlings [*Sturnus vulgaris*], and redwings [*Turdus iliacus*] found during August 3–September 18, 1997); and group 4 (10 blocks, blackbirds and fieldfares [*T. pilaris*] found during October 5–December 20, 1997).

Recut samples from these blocks were placed on positively charged slides (Superfrost plus; Menzel Gläser, Braunschweig, Germany) and processed for immunohistochemical staining by using a rabbit USUV-specific antibody at a dilution of 1:4,000. Immunohistochemical analysis was performed by using an automated immunostainer (Autostainer 360–2D; Thermo-Fisher, Kalamazoo, MI, USA). From the same paraffin blocks, three 10 μm–thick samples were cut and used for RNA extraction.

Viral RNA was purified from paraffin-embedded tissue samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) after deparaffinization with xylene. Because of the formaldehyde fixation, the paraffin wax embedding procedure, and the long storage time, a high degree of RNA fragmentation was expected. Therefore, the PCR-based nucleic acid detection methods were specific for short (<300 nt) sequences.

In a TaqMan-based real-time reverse transcription PCR (RT-PCR), genomic (5′-GCCAATGCCCTGCACTTT-3′) and reverse (5′-TCCCGAGGAGGGTTTCCA-3′) primers amplify part of the nonstructural protein 5 (NS5) gene region of USUV between nt positions 9721 and 9795 (according to the USUV complete genome sequence, GenBank accession no. NC_006551). The TaqMan probe (FAM-5′-CGATGTCCAAGGTCAGAAAAGACGTGC-3′-TAMRA) hybridizes the amplification product between nt positions 9746 and 9773. The SuperScript III Platinum One-Step qRT-PCR System (Invitrogen, Carlsbad, CA, USA) was used for amplifications according to the manufacturer's instructions. Primers and probe were used at concentrations of 0.2 μmol/L. Reactions were performed in an Applied Biosystems (Foster City, CA, USA) 7300 Real Time PCR System with a thermal profile of 48°C for 15 min, 95°C for 2 min, and 45 cycles at 95°C for 15 sec and 60°C, for 30 sec. Selected real-time RT-PCR virus-positive samples were also tested by using conventional RT-PCRs that generated short amplification products. Two primer pairs (Usu9247f-Usu9445r and Usu10626f-Usu10828) (*6*) amplified specific products.

Nucleotide sequences of real-time and conventional RT-PCR amplification products were determined and identified by using a BLAST search (http://blast.ncbi.nlm.nih.gov/). Sequences were aligned with USUV sequences

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Table. Results of IHC analysis and real-time RT-PCR for Usutu virus in birds, Italy, 1996*

Group, protocol no.	Tissue	Bird species	IHC result	RT-PCR result
1				
6439-A1	Lung, kidney, spleen	*Turdus merula*	+ (kidney)	+
6439-C2	Brain	*T. merula*	+	+
6439-C3	Brain	*T. merula*	+	+
6439-C4	Brain	*T. merula*	+	+
6439-D5	Proventriculus, gizzard	*T. merula*	–	+
6439-D6	Kidney	*T. merula*	–	+
6439-D7	Liver	*T. merula*	I	+
2				
6484-A	Skeletal muscle	*T. merula*	–	–
6484-B	Spleen	*T. merula*	–	–
6484-C	Intestine	*T. merula*	–	–
6484-D	Liver	*T. merula*	–	–
6484-E	Heart	*T. merula*	–	–
6484-F	Lung	*T. merula*	I	–
3				
7665-A1	Brain	*T. merula, T. iliacus, Sturnus vulgaris*†	–	–
7665-A2	Brain	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-B1	Brain	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-B2	Brain, intestine	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-B3	Liver, lung, kidney	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-C1	Brain	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-C2	Heart	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-C3	Liver, intestine, skeletal muscle	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-D1	Brain	*T. merula, T. iliacus, S. vulgaris*†	–	–
7665-D2	Liver, kidney	*T. merula, T. iliacus, S. vulgaris*†	–	–
4				
7714–1	Heart	*T. merula, T. pilaris*†	–	–
7714–2	Liver, kidney	*T. merula, T. pilaris*†	–	–
7714–3	Spleen, bursa	*T. merula, T. pilaris*†	–	–
7714–4	Brain	*T. merula, T. pilaris*†	–	–
7714–5	Brain	*T. merula, T. pilaris*†	–	–
7714–6	Intestine, pancreas	*T. merula, T. pilaris*†	–	–
7714–7	Heart	*T. merula, T. pilaris*†	–	–
7714–8	Heart	*T. merula, T. pilaris*†	–	–
7714–9	Brain	*T. merula, T. pilaris*†	–	–
7714–10	Kidney	*T. merula, T. pilaris*†	–	–

*IHC, immunohistochemical; RT-PCR, reverse transcription PCR; +, positive; –, negative; I, inconclusive.
†Blocks not assignable to individual bird species.

available in GenBank. Phlyogenetic analysis with the neighbor-joining algorithm was performed to infer genetic relatedness between sequences.

Immunohistochemical analysis showed positive results for USUV in several brain samples (Figure 1) and 1 kidney sample from only group 1 blocks. Liver, lung, spleen, and proventriculus were negative for USUV. In all blocks from the other 3 groups, there was no specific staining.

Results of real-time RT-PCR were positive for all samples from group 1; samples from other groups were negative. Nucleotide sequences of amplification products were 100% identical with available USUV sequences in GenBank. Selected samples (brains for group 1) were also positive by conventional RT-PCRs.

Nucleotide sequences of the amplification products in the partial NS5 gene region (between nt positions 9267 and 9425, excluding primer sequences) were 100% identical with the corresponding sequences of USUV detected in Austria in 2001 and in Italy and in Switzerland in 2006. Sequences were 98.7%–99.3% identical with other USUV sequences from Austria, Italy, Hungary, and Germany. However, these sequences were only 95.6% identical with USUV detected in Spain in 2009 and 96.2% identical with the reference strain isolated in South Africa in 1959.

Phylogenetic relatedness of sequences is shown in Figure 2. Nucleotide sequences of the 3′ untranslated region (between nt positions 10646 and 10808) were 100% identical with USUV sequences from central Europe and 98.1% identical with the reference strain from South Africa.

Conclusions

These investigations provide evidence that USUV emerged in a pathogenic form in Europe in 1996 or even earlier. This date is ≥5 years before USUV-associated bird deaths in Vienna, Austria (*2*), which has been generally assumed to have been the starting point of the spread of the virus to other countries in Europe. Partial sequencing of the 1996 strain confirmed its identity with the 2001 Vienna strain and all its descendants. However, the assumed epicenter of virus spread being Austria must be reconsidered

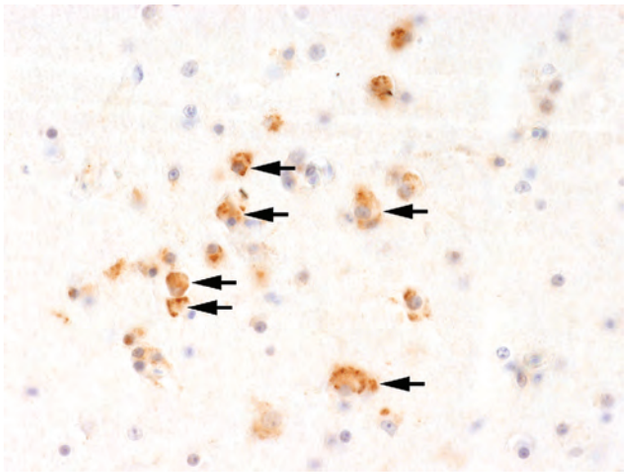

Figure 1. Immunohistochemical staining with Usutu virus–specific antibody showing virus antigen in the brain of a blackbird that died during an Usutu virus outbreak in Italy, 1996. Numerous neurons show characteristic, frequently coarsely granular cytoplasmic labeling (arrows). Original magnification ×390.

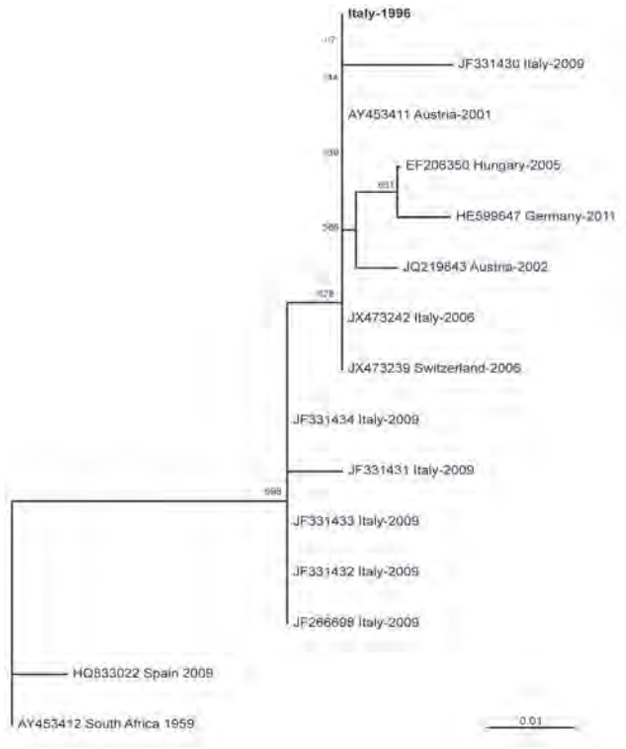

Figure 2. Genetic relationships of partial nonstructural protein 5 nucleotide sequences of Usutu virus, Italy, 1996. Sequences are indicated by codes containing GenBank accession number, country of origin, and year of sample collection. Virus reported in this study is indicated in **boldface**. Bootstrap values ≥500 (50%) are displayed. Scale bar indicates genetic distance.

because the source has been in Italy much longer and may have given rise to subsequent local episodes of bird deaths in Italy and other countries.

USUV has an established stable mosquito-to-bird transmission cycle in Europe, which can remain silent for many seasons. There are no reports of bird deaths during 1996–2001, which might have been caused by unfavorable climatic conditions or lack of larger numbers of susceptible birds. Local herd immunity (*8*) prevented further bird deaths and supported silent spread of the virus. Large-scale wild bird deaths, as later reported in Austria, Switzerland, and Germany (*2*,*3*,*5*,*7*), had not been observed in Italy, despite widespread viral activity (*6*,*9*). Introduction of a potentially pathogenic vector-borne virus into a new area does not necessarily lead to immediate deaths, which has been repeatedly shown by seropositivity of sentinel birds or virus detection in vectors before epidemics (*10*,*11*). Episodes of bird deaths tend to occur when virus spreads to areas without prior exposure, thus affecting virus-naive birds. Also, specific climatic conditions, such as longer periods of hot and dry weather, seem to affect vector abundance and competence and efficient virus transmission to susceptible hosts (*12*).

There is evidence for introduction of other USUV strains into Europe. Direct evidence exists for a strain so far found only in mosquitoes in Spain, which is genetically different from the strain from central Europe (*13*,*14*). This strain has never been associated with bird deaths, which might have resulted from its lower virulence, but low levels of bird deaths might have occurred unnoticed. Thus, results of studies in the United Kingdom that reported several USUV seroreactive resident birds without obvious bird deaths may be explained accordingly (*15*).

Acknowledgments

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Kyasanur Forest Disease, India, 2011–2012

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To determine the cause of the recent upsurge in Kyasanur Forest disease, we investigated the outbreak that occurred during December 2011–March 2012 in India. Male patients >14 years of age were most commonly affected. Although vaccination is the key strategy for preventing disease, vaccine for boosters was unavailable during 2011, which might be a reason for the increased cases.

Kyasanur Forest disease (KFD), a tick-borne viral disease, was first recognized in 1957 in Shimoga District, India, when an outbreak in monkeys in Kyasanur Forest was followed by an outbreak of hemorrhagic febrile illness in humans (*1*). KFD is unique to 5 districts (Shimoga, Chikkamagalore, Uttara Kannada, Dakshina Kannada, and Udupi) of Karnataka State and occurs as seasonal outbreaks during January–June (*2*–*4*).

Since 1990, vaccination campaigns using formalin-inactivated tissue-culture vaccine have been conducted in the districts to which KFD is endemic (Directorate of Health and Family Welfare Services, Government of Karnataka, Manual on Kyasanur Forest disease. 2005, unpub. data). Earlier studies showed vaccine efficacy of 79.3% with 1 dose and 93.5% with 2 doses (*5*,*6*). The vaccination program identifies villages reporting KFD activity (laboratory-confirmed cases in monkeys and/or humans, or infected ticks), and all villages within 5 km of the affected location are targeted for vaccination. Two doses are administered to persons 7–65 years of age at 1-month intervals. Because the immunity conferred by vaccination is short-lived, booster doses are administered at 6–9-month intervals consecutively for 5 years after the last reported KFD activity in the area (Directorate of Health and Family Welfare Services, Government of Karnataka, Manual on Kyasanur Forest disease. 2005, unpub. data). If KFD activity is reported where vaccination has been administered during pretransmission seasons, additional vaccination campaigns are conducted.

Thirthahalli Taluka in the Shimoga District (Figure 1), where vaccination campaigns were ongoing, reported 0 cases of KFD during 2007–2010. A vaccination campaign was conducted in the area during October 2010. Because 11 cases were reported from the Thirthahalli Taluka in March 2011, vaccination campaigns were conducted during April–May 2011; however, no booster doses were administered in the affected areas during October–November 2011 because the vaccine was not available. Suspected KFD cases were reported in the area again in December 2011. We investigated this cluster to 1) confirm the etiology, 2) identify risk factors, and 3) propose recommendations for control.

The Study

We defined a suspected KFD case as sudden onset of fever, headache, and myalgia among residents of Shimoga during December 2011–March 2012 (Directorate of Health and Family Welfare Services, Government of Karnataka, Manual on Kyasanur Forest disease, 2005, unpub. data). Health workers conducted door-to-door searches to identify suspected case-patients within 5 km of villages that reported monkey deaths or laboratory-confirmed KFD cases in humans since December 2011. We established stimulated passive surveillance in health facilities in the district to identify suspected case-patients. Health workers collected information about sociodemographic profile, date of onset, and place of residence from all suspected case-patients. We recorded clinical history and vaccination details of laboratory-

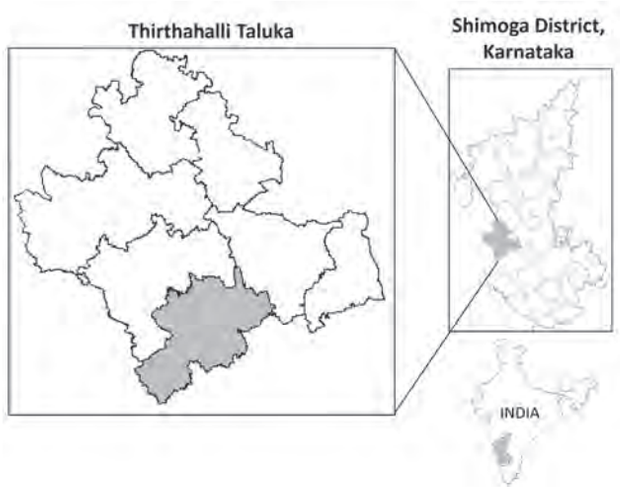

Figure 1. Location of Thirthahalli Taluka, Shimoga District, Karnataka State, India.

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confirmed case-patients. We analyzed the data to describe the disease by time, place, and person. The investigation was exempted from ethical committee clearance because it was part of the state-level public health response to the outbreak.

Blood specimens were collected from all suspected case-patients. We tested for KFD virus by using nested reverse transcription PCR (RT-PCR) and Taqman-based RT-PCR at the National Institute of Virology (Pune, India) (*7*) and/or intracerebral injection of the serum into suckling mice at the Virus Diagnostic Laboratory, Shimoga (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/2/12-0544-Techapp1.pdf).

We conducted a matched case–control study to identify risk factors for the illness. Persons with laboratory-confirmed infection who were admitted to health facilities were considered case-patients, and healthy persons were used as controls. We recruited 2 controls per case-patient (total 51 cases, 102 controls). Case-patients and controls were matched with age group (±5 years), sex, and locality. We interviewed participants to collect information about any recent exposure to the forest and number of doses of KFD vaccine received in 2011. We conducted conditional logistic regression analysis by using Epi Info software (Centers for Disease Control and Prevention, Atlanta, GA, USA) to identify risk factors. All risk factors evaluated were included in the logistic regression model.

During December 2011–March 2012, we identified 215 suspected case-patients from 80 villages (total population 22,201) in Shimoga (attack rate 9.7 cases/1,000 persons). Of these, 61 (28%) cases were laboratory confirmed (57 by RT-PCR; 4 by suckling mice intracerebral inoculation). Most (92%) laboratory-confirmed case-patients were >14 years of age, and 70% were male (Table 1). The cases began occuring in the last week of December 2011, peaked during the first 2 weeks of February, and then declined gradually (Figure 2). Of the 215 suspected cases, 166 (77%) occurred in 4 primary health center areas of Thirthahalli Taluka.

Besides fever and myalgia, common clinical manifestations among the 61 laboratory-confirmed case-patients included bleeding (38 [63%] persons), vomiting (28 [46%]), and abdominal pain (26 [42%]). The hemorrhagic manifestations included conjunctival congestion (30 [49%]), hematemesis (5 [8%]), epistaxis (1 [2%]), hematuria (1 [2%]), and rectal bleeding (1 [2%]). One patient died (case-fatality rate 0.5%). Of the 61 laboratory-confirmed case-patients, 20 (33%) had received 2 doses of KFD vaccine, and 2 (3%) received 1 dose; 39 (64%) did not receive any vaccination during April–May 2011. Twelve case-patients were housewives or students; the rest reported multiple occupations requiring frequent visits to the forest, such as cultivator, dry leaf gatherer, agriculture laborer, and cattle grazer.

Behavioral factors, such as handling cattle (adjusted odds ratio [aOR] 5.1, 95% CI 1.3–20.4) and frequent visits to forest for livelihood (aOR 4.8, 95% CI 1.2–20.3) and piles of dry leaves within the compounds of the house (aOR 4.1, 95% CI 1.3–12.3) were independently associated with illness. Of the 51 case-patients, 20 had received 2 doses of vaccine and 2 had received 1 dose. The odds of developing illness did not differ significantly for nonvaccinated case-patients and case-patients who received 2 doses (Table 2).

Conclusions

Vaccination is the key strategy for preventing KFD in Karnataka. However, during 2011, a booster vaccination campaign was not conducted in the district because of vaccine unavailability, which might be a reason for the upsurge of KFD cases during 2012. Two doses of the vaccine given during April–May 2011 did not confer adequate protection against the disease during December 2011–March 2012, suggesting the possibility of short-lived immunity conferred by 2 doses of vaccine and the need for periodic boosters.

In the affected areas, local villagers stay in and around the forest area, frequently visit the forest for their livelihood, and get infected through tick bites. We identified certain risk factors for the illness, including frequent

Table 1. Age and sex distribution of suspected and laboratory-confirmed case-patients during Kyasanur Forest disease outbreak, Shimoga District, Karnataka State, India, December 2011–March 2012

Characteristic	Population	Suspected		Laboratory-confirmed	
		No. case-patients	Attack rate*	No. case-patients	Attack rate*
Age group, y					
≤14	7,193	32	4	5	1
15–29	6,349	51	8	16	3
30–44	4,440	70	16	22	5
45–59	2,642	48	18	13	5
≥60	1,577	14	9	5	3
Sex					
M	11,194	136	12	43	4
F	11,007	79	7	18	2
Total	22,201	215	10	61	3

*Per 1,000 population.

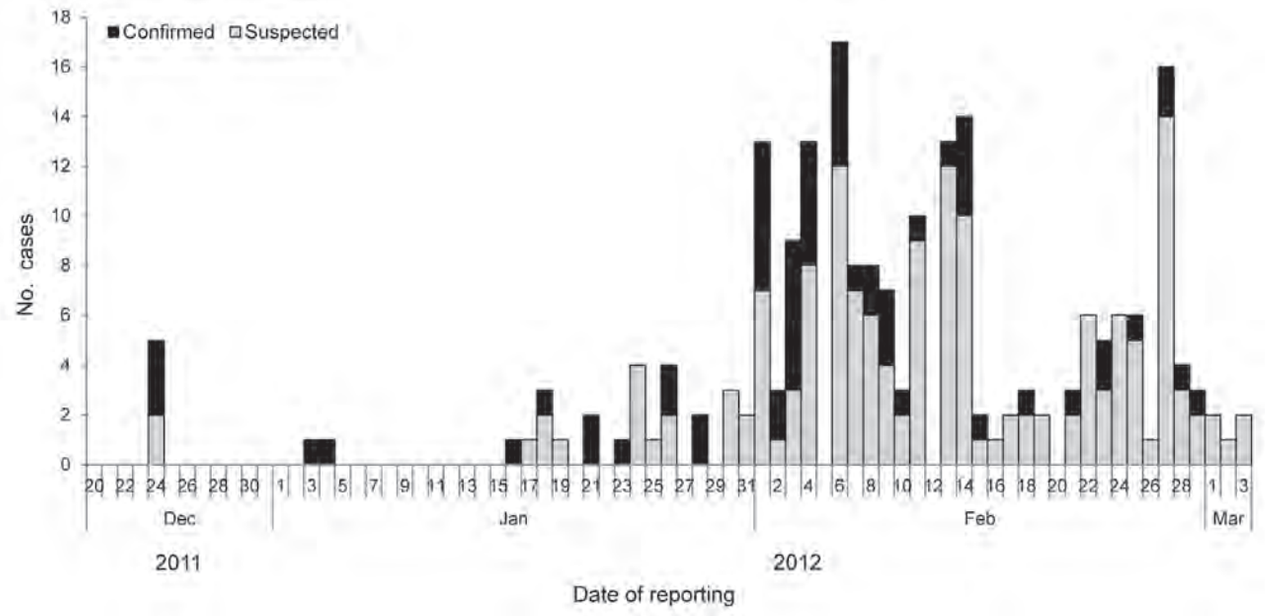

Figure 2. Distribution of suspected and confirmed Kyasanur Forest disease cases, Shimoga District, Karnataka State, India, December 2011–March 2012.

visits to the forest, handling of cattle, and piles of dry leaves within the compounds. The higher attack rates for male case-patients aged >14 years during this outbreak are consistent with their frequent exposure to the forest. Health authorities advise use of tick repellent; however, it was infrequently used in the area. Educating the community to wear long-sleeved clothing might help reduce exposure to ticks.

Although the transmission cycle of KFD virus is well documented, its control remains challenging. Measures to minimize the human–tick interface are less likely to succeed considering the forest ecosystem and the dependence of local villagers on it. Control of ticks in the forest is far from easy, but health authorities need to continue educating villagers about using tick repellent before visiting the forest, especially during spring and summer, and ensure distribution of tick repellents to them. Health authorities must ensure that vaccination campaigns are initiated on time and completed before November every year. More epidemiologic studies are needed to evaluate the long-term protection offered by booster doses of vaccine. Molecular studies also are needed to understand the phylogenetic relationships of the past and contemporary strains of the virus and to identify possible sources and origins of outbreak strains.

Acknowledgments

We thank M. Sulochana for her valuable inputs and guidance during the investigation. We gratefully acknowledge the medical officers and staff of Konandur, Humchadakatte, Yogimalali, Mandagadde primary health centers and Taluk Health office, Thirthahalli and Jayachamrajendra Taluk Hospital, Thirthahalli, for their help during the investigation.

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Dr Kasabi is a senior medical officer with Department of Health and Family Welfare, Government of Karnataka, in Shimoga District. He conducted this outbreak investigation as a part of a Master of Public Health (Epidemiology and Health Systems) course at the National Institute of Epidemiology, Chennai. His research interests include health system research and reemerging infectious diseases.

Table 2. Univariate and multivariate analysis of risk factors associated with Kyasanur Forest disease outbreak, Shimoga District, Karnataka State, India, December 2011–March 2012*

Variable	No. (%) case-patients, n = 51	No. (%) controls, n = 102	Odds ratio (95% CI) Matched	Odds ratio (95% CI) Adjusted
Handled cattle in last week	47 (92)	72 (71)	5.4 (1.6–18.2)	**5.1 (1.3–20.4)**
Went to forest in last week	44 (86)	72 (71)	4.2 (1.2–14.3)	**4.7 (1.1–20.3)**
Had pile of leaves within the compound	39 (76)	58 (57)	3.2 (1.3–7.9)	**4.0 (1.3–12.3)**
Had cattle shed in household	48 (94)	89 (87)	3.3 (0.7–16.3)	3.7 (0.5–25.9)
Received 2 doses of vaccine in 2011	20 (39)	42 (41)	0.7 (0.2–2.9)	2.4 (0.4–15)
Used tick repellent before going to forest	8 (16)	18 (18)	0.8 (0.3–2.4)	1.1 (0.3–3.8)

*__Boldface__ indicates significance.

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Macrolide- and Rifampin-Resistant *Rhodococcus equi* on a Horse Breeding Farm, Kentucky, USA

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Macrolide and rifampin resistance developed on a horse breeding farm after widespread use was instituted for treatment of subclinical pulmonary lesions in foals. Resistance occurred in 6 (24%) of 25 pretreatment and 8 (62%) of 13 (62%) posttreatment isolates from affected foals. Drug-resistant isolates formed 2 distinct genotypic clusters.

Rhodococcus equi is a major cause of pneumonia in young horses and a common opportunistic pathogen of immunocompromised humans (*1*). Over the past decade, control of *R. equi* infections at many horse farms to which the disease is endemic has relied on early detection of subclinical pulmonary disease by use of thoracic ultrasonography and initiation of treatment with antimicrobial drugs before development of clinical signs (*2*). This approach appears to have decreased deaths caused by *R. equi* pneumonia at some farms, although controlled studies are lacking (*2*). However, the temporal association between widespread use of macrolides and rifampin and a perceived increase in the frequency of detection of drug-resistant isolates in the past decade (*3*) suggest that this practice may not be innocuous.

We describe emergence of resistance to macrolides and rifampin among *R. equi* isolates obtained from a horse breeding farm. We conducted this study after initiation of an ultrasonographic screening program on the farm and resulting widespread use of these drugs in foals with subclinical pulmonary lesions.

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The Study

This study was conducted at a Thoroughbred horse breeding farm in Kentucky, USA. The farm initiated an ultrasonographic screening program in 2001 in an attempt to decrease deaths associated with pneumonia caused

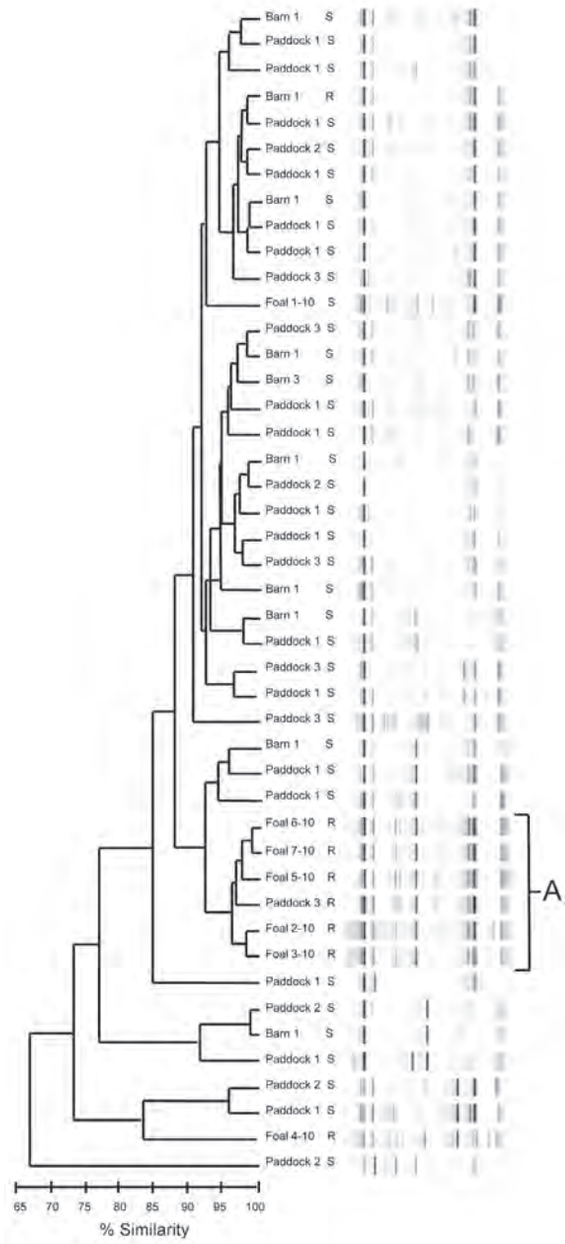

Figure 1. Dendrogram and virtual gel repetitive sequence–based PCR fingerprint patterns of foal and air (barn and paddock)–derived isolates of *Rhodococcus equi* on horse breeding farm, Kentucky, USA, 2010. Macrolide and rifampin susceptibility (S) or resistance (R) are indicated. A indicates the main cluster of drug-resistant isolates (5 foal and 1 air).

[1]Current affiliation: Kalon Biotherapeutics LLC, College Station, Texas, USA.

Table 1. Macrolide- and rifampin-resistant *Rhodococcus equi* on horse breeding farm, Kentucky, USA*

Year	No. foals born	No. (%) foals with lesions treated	No. foals tested	No. foals with positive *R. equi* culture	No. (%) foals with macrolide- and rifampin-resistant *R. equi*
2001	95	30 (32)	30	30	0
2002	117	53 (45)	0	NA	NA
2003	148	58 (32)	2	?	?
2004	181	88 (49)	28	19	0
2005	168	70 (42)	30	?	?
2006	170	42 (41)	5	2	0
2007	181	93 (51)	4	?	?
2008	171	52 (30)	21	16	4 (25)†
2009	162	50 (31)	30	22	5 (23)†
2010	138	45 (33)	28	22	9 (41)†
2011	132	24 (18)	27	25	9 (36)†

*NA, not applicable; ?, data missing from farm records.
†Includes pretreatment and posttreatment isolates.

by *R. equi* through early diagnosis and treatment of foals with subclinical lesions. During March–July 2010, the farm reported 9 foals infected with macrolide- and rifampin-resistant *R. equi* isolates. This finding led to a disease investigation consisting of retrospective data collection (Table 1), collection of air samples in September 2010 to determine the prevalence of drug-resistant *R. equi* in the environment, and prospective culture of pulmonary lesions from all foals in 2011 before initiation of antimicrobial drug therapy.

A total of 124 air samples were collected from the 4 barns at the farm and from each of their respective surrounding paddocks by using a portable air-sampling device as described (*4*). For the 2011 breeding season, a tracheobronchial aspirate was collected transendoscopically from each foal that had ultrasonographically detected pulmonary lesions before initiation of therapy by using a triple-guarded microbiological aspiration catheter. A second tracheobronchial aspirate was collected by using the same method 2 weeks after initiation of therapy. Decisions regarding the need for therapy and selection of antimicrobial agents were made by the farm veterinarian and manager.

After standard microbiological culture of air and tracheobronchial aspirate samples, confirmation of *R. equi* was accomplished by amplification of the *choE* gene and detection of the virulence plasmid by amplification of the *vapA* gene by using multiplex PCR (*5*). For each isolate, the MICs of erythromycin, azithromycin, clarithromycin, and rifampin were determined from 3–5 isolated colonies by broth dilution in accordance with Clinical and Laboratory Standards Institute guidelines (*6*). *R. equi* isolates with MIC values ≤2 μg/mL for azithromycin and clarithromycin, ≤0.5 μg/mL for erythromycin, and ≤1 μg/mL for rifampin were considered susceptible, and isolates with MIC values ≥8 μg/mL were considered resistant.

The similarity of *R. equi* isolates from air and tracheobronchial aspirate samples was determined by using a repetitive sequence–based PCR (DiversiLab; bioMérieux Inc., Durham, NC, USA) previously validated for *R. equi* (*7*). Isolates were clustered as the same strain on the basis of ≥95% similarity and a difference of ≤1 band (*8*).

The ultrasonographic screening program was introduced in 2001, and the first isolates of *R. equi* resistant to macrolides and rifampin were identified in 2008 (Table 1). There were no *R. equi* isolates that were resistant to either a macrolide or rifampin alone. Air sampling yielded 82 isolates of *R. equi*. Of these isolates, 15 (18%) contained the plasmid required for virulence in foals and 67 (82%) were avirulent.

All 15 virulent isolates and 23 randomly selected avirulent isolates were used for in vitro antimicrobial drug susceptibility testing and genotyping. Two (5%) of 38 isolates tested were resistant to azithromycin, clarithromycin, erythromycin, and rifampin. One resistant isolate was virulent and came from an outdoor paddock location that had been used to house foals given a diagnosis of *R. equi* pneumonia caused by drug-resistant isolates. The other resistant isolate was avirulent and was isolated from an air sample obtained from an indoor barn location.

During the 2011 season, 132 foals were born on the farm. Thoracic ultrasonography showed evidence of pulmonary disease in 27 (20%) foals. Culture of a tracheobronchial aspirate before initiation of therapy yielded *R. equi* in 25 (93%) of the 27 foals sampled (Table 1). Of the 25 pretreatment *R. equi* isolates, 6 (24%) were resistant to macrolides and rifampin. Twenty-four foals were treated with clarithromycin and rifampin. Tracheobronchial aspirates were collected 2 weeks after initiation of therapy in 19 foals, and *R. equi* was cultured from 13 (68%) of the 19 samples. Of these 13 posttreatment isolates, 8 (62%) were resistant to macrolides and rifampin. After identification of resistant isolates, 3 foals had a third antimicrobial agent added to their therapy (gentamicin, n = 2; doxycycline, n = 1). All foals with a diagnosis of pneumonia caused by *R. equi* in 2011 survived.

Genotypes by repetitive sequence–based PCR from all 2010 isolates grouped into 1 main cluster consisting of resistant isolates from 5 foals and 1 air sample (Figure 1, cluster A). Isolates resistant to macrolides and rifampin

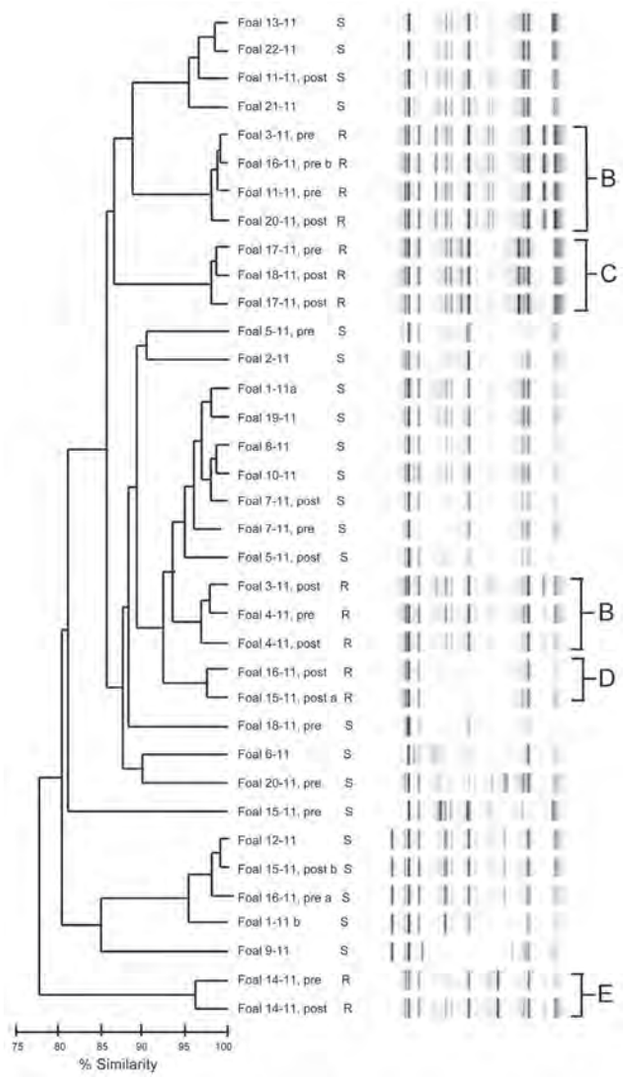

Figure 2. Dendrogram and virtual gel repetitive sequence–based PCR fingerprint patterns of 36 *Rhodococcus equi* isolates obtained from foals on horse breeding farm, Kentucky, USA, 2011. Macrolide and rifampin susceptibility (S) and resistance (R) are indicated. B–E indicates clusters of drug-resistant isolates. Foals from which pretreatment (pre) and posttreatment (post) samples were obtained are indicated. a and b indicate samples from which 2 isolates were obtained.

from the 2011 foals grouped closely, forming 4 clusters (B–E); the largest cluster (B) contained 7 isolates (Figure 2). Of the 11 foals for which pretreatment and posttreatment samples were collected in 2011, a total of 5 had similar pretreatment and posttreatment isolates and 6 had different pretreatment and posttreatment isolates (Table 2).

Conclusions

Recently, development of macrolide resistance in clinically relevant pathogens has been recognized in human and animal populations that received intensive macrolide treatment (*9*,*10*). We documented that macrolide- and rifampin-resistant isolates of *R. equi* occurred 7 years after initiation of an ultrasonographic screening program, which resulted in treatment of all foals with subclinical pulmonary lesions.

Compared with macrolide-susceptible *Campylobacter jejuni*, acquisition of macrolide resistance impairs the fitness and transmission of the pathogen in chickens, suggesting that the prevalence of macrolide-resistant *C. jejuni* would probably decrease in the absence of antimicrobial drug selection pressure (*11*). Similarly, studies in humans have shown that macrolide resistance in *Streptococcus pneumoniae* decreased 2–5 years after use of azithromycin was stopped and selection pressure was abolished (*10*). Although the fitness cost of macrolide resistance among *R. equi* isolates might be sufficient to ensure its eventual elimination, this elimination will take time and elimination of resistance will only occur in the absence of antimicrobial drug selection pressure (*10*).

A recent study on a large horse farm indicated that the proportion of foals with ultrasonographic pulmonary lesions associated with *R. equi* infection that recovered was not different between foals given azithromycin and rifampin and foals given a placebo (*12*). This surprising finding, combined with the apparent increase in macrolide- and rifampin-resistance demonstrated in the present study, support the need to stop the practice of mass macrolide treatment for subclinical infection with *R. equi* in foals on horse breeding farms. The goal should be to more accurately identify, of the many subclinically infected foals, which few are likely to show development of disease and thus require treatment for it.

Table 2. Characteristics of pretreatment and posttreatment *Rhodococcus equi* isolates obtained from foals on a horse breeding farm, Kentucky, USA, 2011*

Foal ID no.	Pretreatment isolates (cluster)	% Similarity†	Posttreatment isolates (cluster)
7-11	S	97.5	S
5-11	S	84.3	S
14-11	R (E)	96.4	R (E)
3-11	R (B)	96.5	R (B)
4-11	R (B)	97.4	R (B)
17-11	R (C)	98.5	R (C)
20-11	S	83.1	R (B)
18-11	S	78	R (C)
11-11	R (B)	87.2	S
15-11‡	S	83.1	S
	NA	73.2	R (D)
16-11‡	S	78.8	R (D)
	R (B)	80.8	NA

*ID, identification; S, susceptible; R, resistant; NA, not applicable.
†Determined by using Pearson correlation coefficient and unweighted pair-group method with arithmetic means.
‡Two isolates were recovered from the pretreatment (foal ID 16-11) or posttreatment (foal ID 15-11) sampling.

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Campylobacter Infection in Poultry-Processing Workers, Virginia, USA, 2008–2011

Marie A. de Perio, R. Todd Niemeier, Seth J. Levine, Karen Gruszynski, and John D. Gibbins

During a health hazard evaluation, we investigated 29 cases of laboratory-diagnosed *Campylobacter* infection among workers at a poultry-processing plant. Most infected employees worked at the plant <1 month, worked as live hangers, and lived at a state-operated center. To lessen the infection risk, we recommended improvements to engineering and administrative controls at the plant.

Campylobacter spp., commensal organisms of poultry, are several common bacterial causes of gastrointestinal infection in the United States (*1*). *Campylobacter* infection, or campylobacteriosis, affects an estimated 2.4 million persons each year (*2*) and is most often associated with sporadic illness rather than outbreaks. Transmission typically occurs through consumption of undercooked poultry or handling of raw poultry (*3*,*4*). As part of a health hazard evaluation requested by plant management (*5*,*6*), we report a case series of laboratory-diagnosed *Campylobacter* infections among employees at a poultry-processing plant in Virginia during 2008–2011. As a public health response, according to Title 45 Code of Federal Regulations Part 46, this evaluation was determined not to require review by an institutional review board.

The Study

During the period studied, the poultry plant processed 300,000–350,000 birds per day and employed ≈1,000 persons who worked in 2 main processing areas: first processing and second processing. In first processing, birds were unloaded, shackled (in an area called live hang), stunned, killed, scalded, defeathered, eviscerated, and chilled. In second processing, carcasses were rehung, washed, cooled, and packaged. The plant was under the regulatory authority of the Food Safety and Inspection Service of the US Department of Agriculture. At any given time, the plant employed 24–35 persons who were residents of 1 of 2 local diversion centers (i.e., participants of a 16- to 20-week residential work assignment program operated by the Virginia Department of Corrections). The plant had a medical office, with limited diagnostic capabilities, staffed by licensed practical nurses. Employees requiring additional medical evaluation were referred to outside providers.

Using occupation data in Virginia Department of Health case reports and Virginia Department of Corrections records, we identified persons who had laboratory-diagnosed *Campylobacter* infection while employed at the plant during January 2008–May 2011. To capture all possible cases, we defined a case-patient as a plant employee with *Campylobacter* infection diagnosed by culture or enzyme immunoassay. We reviewed case-patient records from the Virginia Department of Health, Virginia Department of Corrections, and local medical providers and obtained additional work history information from the plant.

To determine the background incidence of reported gastrointestinal illness among plant employees, we reviewed encounter records (for January 2010–September 2011) from the plant's medical office. We categorized an encounter as gastrointestinal illness–related if the employee reported diarrhea, abdominal cramps, nausea, or vomiting without another reason listed, such as nausea related to pregnancy or migraine headaches. We then tabulated gastrointestinal illness–related encounters by month.

We identified 29 cases of laboratory-diagnosed *Campylobacter* infection during January 2008–May 2011 in persons employed at the poultry-processing plant. Of the 29 persons, 23 were infected with *C. jejuni*, 1 was infected with *C. coli*, and 5 were infected with an unspecified *Campylobacter* species. Twenty-seven cases were diagnosed by stool culture; 2 were diagnosed by stool enzyme immunoassay.

The median age of case-patients was 29 years (range 19–52 years); 28 (97%) were men. Twenty-six (90%) case-patients were residents of a diversion center, and 3 lived at a private residence. Of the 29 case-patients, 27 (93%) worked in first-processing areas, including the live-hang (n = 18), evisceration (n = 8), and kill (n = 1) rooms, and 2 worked in second-processing areas, including the rehang (n = 1) and cut-up (n = 1) rooms. Twenty-four (83%) case-patients worked at the plant for <1 month before illness onset.

We obtained medical records for 24 case-patients, 3 of whom reported having been sought care in the plant medical office. These 24 case-patients were all reported to have had diarrhea while sick. Other signs and symptoms included abdominal cramping (n = 14), fever (n = 9), nausea and vomiting (n = 6), headache (n = 7), and muscle aches

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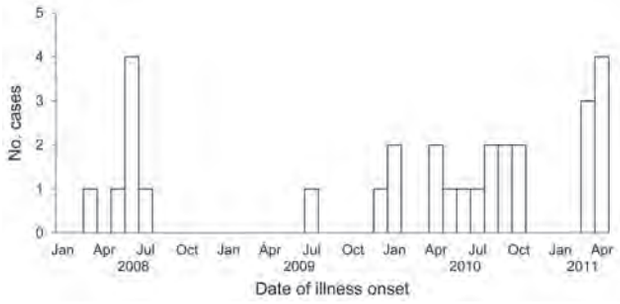

Figure 1. Number of laboratory-diagnosed *Campylobacter* infections, by month of illness onset, in employees at a poultry-processing plant, Virginia, USA, 2008–2011.

(n = 3). Of the 29 case-patients, 1 was hospitalized; there were no deaths. Figure 1 shows the number of cases by month of symptom onset.

In 2010, a total of 1,716 encounters at the plant's medical office were recorded; 273 (16%) were associated with gastrointestinal symptoms. During January 2011–September 2011, a total of 1,543 encounters at the plant's medical office were recorded, of which 221 (15%) were related to gastrointestinal symptoms (Figure 2). Multiple peaks of visits for gastrointestinal illness were seen during summer 2010 and winter 2010–11, and a smaller peak occurred in summer 2011. Most other reasons for plant medical office visits were for injury reporting, first aid, and musculoskeletal symptoms.

Conclusion

Our investigation revealed 29 cases of laboratory-diagnosed *Campylobacter* infection in employees at the poultry-processing plant during January 2008–May 2011. Most (62%) cases occurred among employees in the live-hang area who are responsible for lifting live chickens from the supply conveyer and hanging them by their feet from a shackle conveyor. This area has a known high potential for contamination with *Campylobacter* spp. because the feathers, skin, crop, cloaca, and feces of birds brought to slaughter are often highly contaminated with *Campylobacter* spp. (*7*,*8*). Only ≈50 of the ≈1,000 employees work in the live-hang area, suggesting that these employees are disproportionately infected with *Campylobacter* spp. Preharvest practices by the plant and hatcheries may not be sufficient for controlling *Campylobacter* spp. contamination of live birds. The US Department of Agriculture has noted that high bacterial loads of *Campylobacter* spp. on live birds can undermine other in-plant interventions (*9*).

All but 3 case-patients were residents of a diversion center. Many diversion center residents are assigned to work in the live-hang area. Most (83%) case-patients had worked at the plant for <1 month before illness onset. Our finding of illness in new employees is similar to findings from previous investigations of poultry workers. For example, an outbreak investigation of *Campylobacter* infection among poultry workers in Sweden revealed that infection attack rates among inexperienced teenage holiday workers were higher than those among experienced staff (*10*). Another study found that levels of antibodies to *Campylobacter* spp. in long-term workers (employed >1 month) in Sweden were significantly higher than levels in short-term workers (employed ≤1 month) and in blood donors with no special exposure to poultry (*9*). Those findings indicate that for poultry workers, the highest risk for work-related *Campylobacter* infection is during the first weeks of work, after which the workers develop immunity that may be protective against future symptomatic infection (*11*).

In our investigation, the apparent overrepresentation of diversion center residents among employees with *Campylobacter* infection may be partially attributed to their better access to health care compared with access by permanent employees. Approximately 15% of ≈3,000 encounters at the plant's medical office during January 2010–September 2011 were related to gastrointestinal disorders. The numbers of cases of *Campylobacter* infection and gastrointestinal illness that we found among plant employees are likely an underestimation of the true numbers. This may be due to an unwillingness to report illness because of the plant's lack of paid sick leave and employees' difficulty in accessing medical care.

On the basis of our findings, we recommended that plant management strengthen efforts to reduce *Campylobacter* contamination, particularly in the live-hang area. Efforts should incorporate engineering controls, such as improved sanitation, ventilation system modifications, and installation of hands-free soap dispensers and waste receptacles. We also recommended that employee training (in English and Spanish) and compliance with plant policies related to hand hygiene and the use of personal

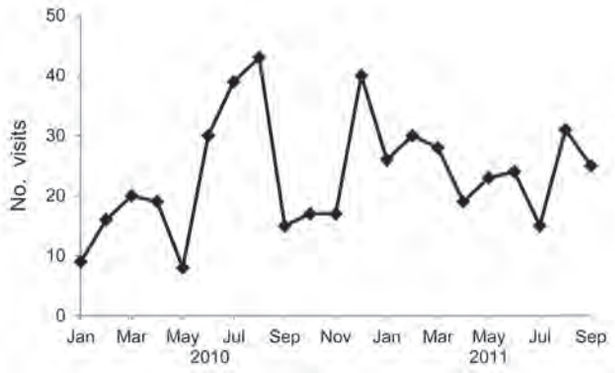

Figure 2. Number of gastrointestinal illness–related visits to the medical office in a poultry-processing plant, Virginia, USA, 2008–2011.

protective equipment be improved, especially among temporary employees. Poultry-processing plants should regularly review their illness records and work with local health departments to ensure that all cases and outbreaks of *Campylobacter* infection are reported.

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Human Granulocytic Anaplasmosis, Japan

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We retrospectively confirmed 2 cases of human *Anaplasma phagocytophilum* infection. Patient blood samples contained unique *p44/msp2* for the pathogen, and antibodies bound to *A. phagocytophilum* antigens propagated in THP-1 rather than HL60 cells. Unless both cell lines are used for serodiagnosis of rickettsiosis-like infections, cases of human granulocytic anaplasmosis could go undetected.

Japanese spotted fever (JSF) and scrub typhus, which are caused by infection with *Rickettsia japonica* and *Orientia tsutsugamushi*, respectively, are common rickettsioses in Japan (*1*). National surveillance (http://idsc.nih.go.jp/idwr/CDROM/Main.html [in Japanese]) indicates that JSF occurs frequently in central and western Japan and that scrub typhus is present throughout Japan, except in Hokkaido. In JSF- and scrub typhus–endemic areas, cases of non-JSP and non–scrub typhus disease with rickettsiosis-like fever have often been reported. And, human infection with *R. heilongjiangensis*, a spotted fever group (SFG) rickettsia, has been identified in Japan (*2*). Furthermore, *Anaplasma phagocytophilum* has been detected in *Ixodes persulcatus* and *I. ovatus* ticks, and *Ehrlichia chaffeensis* has been detected in deer (*3*–*6*). More recently, we identified *A. phagocytophilum* infection in ticks (*Haemaphysalis formosensis*, *H. longicornis*, *H. megaspinosa*, and *Amblyomma testudinarium*) from central and western Japan, the JSF-endemic areas of the country (*7*,*8*). We conducted this retrospective study to determine the cause of non-JSP and non–scrub typhus disease in 2 men in western Japan who had rickettsiosis-like fever.

The Study

In 2002–2003 in Kochi Prefecture, western Japan, 2 men sought medical care for rickettsiosis-like signs and symptoms. Case-patient 1 (61 years old) sought care for fever (39.2°C), chills, and malaise 10 days after traveling to the mountains (day 0, the day of symptom onset). His physician prescribed cefdinir (300 mg/day). By day 3, signs and symptoms had not improved and an erythematous rash on his trunk had spread; the physician suspected infection with *R. japonica* or *O. tsutsugamushi*. The patient was hospitalized and intravenously administered minocycline (200 mg/day). Results (and reference values) for laboratory tests (day 3) follow: leukocytes, 5.8×10^9 cells/L ($3.5–9.2 \times 10^9$ cells/L); thrombocytes, 225×10^9 cells/L (155–365 × 109 cells/L); aspartate aminotransferase, 59 U/L (<38 U/L); alanine aminotransferase, 61 U/L (<36 U/L); and C-reactive protein, 12.1 mg/dL (<0.3 mg/dL).

Case-patient 2, a 73-year-old lumberjack, sought medical care for fever (39.2°C), headache, and malaise (day 0, the day of symptom onset). On day 4, a disseminated maculopapular rash was noticed, especially on the trunk and lower limbs; JSF or scrub typhus infection was suspected. The patient was hospitalized and intravenously administered minocycline (200 mg/day). Results for laboratory tests (day 4) follow: leukocytes, 6.4×10^9 cells/L; aspartate aminotransferase, 100 U/L, alanine aminotransferase, 45 U/L; and C-reactive protein, 17.2 mg/dL.

In 2003, blood clots and serum samples from the 2 patients were transferred from Kochi Institute of Health to the University of Shizuoka, where they were stored at -20°C until a retrospective analysis could be performed. DNA was extracted from the blood clots, and nested PCR was performed, as described (*3*,*9*), to detect SFG rickettsiae 16S rDNA, *O. tsutsugamushi* 16S rDNA, *A. phagocytophilum p44/msp2*, and *Ehrlichia* spp. *p28/omp-1* (Table 1). To avoid DNA contamination, we performed PCR, electrophoresis, and cloning were performed in separate laboratories. As a negative control, nested PCR without DNA template samples was performed for each sample. PCR detected *A. phagocytophilum p44/msp2* multigenes in acute-phase blood clots from both case-patients, and SFG

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Table 1. Results of PCR for select rickettsial organisms for 2 men with human granulocytic anaplasmosis, Kochi Prefecture, Japan*

Days after symptom onset†	Nested PCR result‡			
	SFG rickettsia 16S rDNA	*Orientia tsutsugamushi* 16S rDNA	*Anaplasma phagocytophilum p44/msp2*	*Ehrlichia* sp. *p28/omp-1*
Case-patient 1				
3	Negative	Negative	Positive	Negative
19	Negative	Negative	Negative	Negative
Case-patient 2				
4	Positive	Negative	Positive	Negative
11	NA	NA	NA	NA

*SFG, spotted fever group; NA, not available.
†After in-hospital treatment with minocycline (200 mg/d), both case-patients improved clinically and were discharged on days 20 and 12, respectively, after symptom onset.
‡Before being used in PCR, blood clots from the patients were homogenized by using BioMasher (Nippi Inc., Tokyo, Japan) and treated overnight with 100 U of streptokinase (WAKO Pure Chemical Industries Ltd, Osaka, Japan). DNA then was extracted by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). Multiplex nested first-step PCR for SFG rickettsiae and *O. tsutsugamushi* was performed by using the following primers: RO-1F (5'-CCGTAAACGATGAGTGCTAGA-3') and RO-R1 (5'-CCGAGAACGTATTCACCGC-3'). Multiplex nested second-step PCR for SFG rickettsiae 16S rDNA was performed by using the following primers: R-2F (5'-GAAGATTCTCTTTCGGTTTCGC-3') and R-2R (5'-GTCTTGCTTCCCTCTGTAAAC-3'). Multiplex nested second-step PCR for *O. tsutsugamushi* 16S rDNA was performed by using the following primers: O-2F (5'-GACATGGTAGTCGCGAAAAATG-3') and O-2R (5'-TGCAATCCGAACTGAGATACC-3'). *A. phagocytophilum p44/msp2* was amplified by using primers p3726, p4257, p3761, and p4183, and *Ehrlichia* spp. *p28/omp-1* was amplified by using primers conP28-F1, conP28-R1, conP28-F2, and conP28-R2, as described (*3*,*9*).

rickettsia 16S rDNA was amplified from a sample from case-patient 2 (Table 1).

Amplicons of *p44/msp2* were subjected to TA cloning (TA Cloning Kit; Life Technologies, Grand Island, NY, USA), and randomly selected recombinant clones were sequenced and analyzed phylogenetically (Figure 1). A total of 28 *p44/msp2* clones from case-patient 1 shared 27.5%–100% similarity with each other and were widely dispersed in the tree. The 40 clone sequences from case-patient 2 shared 97.5%–100% similarity with each other and grouped into a single cluster. Using Blast (http://blast.ncbi.nlm.nih.gov), we compared the sequences with those in GenBank; 27 previously identified *p44/msp2* variants from human isolates and ticks collected in Japan were identified as the closest relatives to *p44/msp2* cloned from the 2 patients. We included the 27 variants in the tree; however, some were widely separated from the related clones (Figure 1). For case-patient 2, the 389-bp sequence of the 16S rDNA amplicon (determined by direct sequencing) was 100% identical to that of *R. japonica* YH (GenBank accession no. AP011533).

Serologic evidence of infection was demonstrated by using indirect immunofluorescence assay (IFA) and Western blot analysis as described (*10*,*11*). In IFAs, IgM and/or IgG from serum samples from the case-patients reacted with *A. phagocytophilum* cultured in THP-1 rather than HL60 cells, and seroconversion was stronger in convalescent-phase serum samples (Table 2). IgG titers against *R. japonica* were also higher in convalescent-phase samples from case-patient 2. Western blot analysis further confirmed the specific reaction to the 44-kDa outer membrane proteins (P44s) of *A. phagocytophilum* cultured in THP-1 cells and/or to the recombinant P44-1 (rP44-1) in serum samples (Figure 2, Table 2). However, using the same serum samples, we could not detect P44 antigens of *A. phagocytophilum* propagated in HL60 cells (data not shown), supporting the IFA result.

In central and western Japan, most cases of tickborne infectious and febrile disease have been reported as JSF (*1*,*13*), and *R. japonica* has been frequently detected in ixodid ticks in these areas. We found *A. phagocytophilum* infection in

Figure 1. Phylogenetic analysis of *Anaplasma phagocytophilum p44/msp2* multigenes detected in blood from 2 men in Kochi Prefecture, Japan. Each *p44/msp2* PCR product was cloned (TA Cloning Kit; Life Technologies, Grand Island, NY, USA) into the PCR2.1 vector, after which recombinant clones were randomly selected and the DNA inserts were sequenced. The tree was constructed on the basis of the 117–133 aa sequences of the *p44/msp2* genes by using the neighbor-joining method. The closest relatives to sequences for the 2 case-patients are included in the tree. Those sequences have been published in GenBank: patient2-day27 (obtained from a US patient); P44-2, P44-8, P44-10, P44-11, P44-13, P44-18E, P44-28, P44-35, P44-39, P44-40, P44-41, P44-48, varHH2, and WMSP5 are from human isolates; and 44-kDa outer membrane proteins are from ticks collected in Japan. **Boldface** font indicates the 28 *p44/msp2* genes from case-patient 1 and the 40 from case-patient 2. Numbers on the tree indicate bootstrap values for branch points. Scale bar indicates the percent of sequence divergence. Data in parentheses indicate the number of *p44/msp2* clones with identical sequences and the sequence accession numbers.

Table 2. Detection of IgM and IgG in serum samples from 2 men with human granulocytic anaplasmosis, Kochi Prefecture, Japan*

Days after symptom onset	Antibody titers, IgM/IgG†			
	R. japonica, cultured in L929 cells‡	*O. tsutsugamushi*, cultured in L929 cells§	*Anaplasma phagocytophilum*, propagated in HL60 cells	*Anaplasma phagocytophilum*, propagated in THP-1 cells
Case-patient 1				
3	<20/<20	<20/<20	<20/<20	80/<20
19	<20/<20	<20/<20	<20/<20	320/80
Case-patient 2				
4	<20/<20	<20/<20	20/<20	40/40
11	<20/320	<20/<20	<20/<20	160/80

*All Western blot testing using recombinant P44-1 antigen detected IgM and IgG; the antigen reacted with all sera tested, as shown in Figure 2.
†Determined by using indirect immunofluorescence assay.
‡*Rickettsia japonica* strain YH.
§*Orientia tsutsugamushi* strains Gilliam, Karp, Kato, and Kawasaki.

several species of ticks, and at least 3 species (*H. formosensis*, *H. longicornis*, and *I. ovatus*) seem to be associated with *R. japonica* and *A. phagocytophilum* (*7*,*8*). National surveillance during 1999–2010, showed that JSF was endemic in Kochi Prefecture during 1999–2004. More recently, JSF-endemic areas are Mie, Kagoshima, Wakayama, and Kumamoto Prefectures rather than Kochi Prefecture. Our survey demonstrating the presence of *A. phagocytophilum*–infected ticks in Mie and Kagoshima Prefectures (*8*) indicates that there is a risk for dual infection with *R. japonica* and *A. phagocytophilum* in JSF-endemic areas of Japan.

A. phagocytophilum cultured in HL60 cells is generally used as a source of antigen for serodiagnosis of human anaplasmosis. Our findings show, however, that titers of antibody against *A. phagocytophilum* propagated in THP-1 cells were higher than those propagated in HL60 cells. We further analyzed the transcription of *p44/msp2* multigenes encoding P44 repertoires (major antigens of *A. phagocytophilum*) in infected HL60 and THP-1 cells by using reverse transcription PCR followed by TA cloning as described (*7*). The analyses showed that a transcript from the *p44-60* gene and another from the *p44-47* gene (75% and 25% of transcripts tested, respectively) were dominantly expressed in *A. phagocytophilum* propagated in THP-1 cells but not in HL60 cells; several transcript species other than *p44-60* and *p44-47* of *p44/msp2* multigenes were expressed in *A. phagocytophilum* propagated in HL60 cells (data not shown). A previous proteomic study supported the variety of P44 repertoires produced by *A. phagocytophilum* in HL60 cells (*14*). The difference of *p44/msp2* expression between HL60 and THP-1 cell cultures may reflect the discrepancy of antibody titers obtained by IFAs. Furthermore, in IFAs using infected THP-1 antigens, IgM titers tended to be higher than IgG titers, even in convalescent-phase serum samples. These patients probably produced IgG reactive with P44 species other than P44-60 and P44-47 that were dominantly expressed in *A. phagocytophilum* propagated in THP-1 cells; Western blot analysis showed that IgG in patients strongly bound to recombinant P44-1 rather than P44s (probably including P44-60 and P44-47) of *A. phagocytophilum* propagated in THP-1 cells. Thus, cases of human anaplasmosis could go undiagnosed if only infected HL60 cells, and not THP-1 cells, are used as antigen for serodiagnosis of rickettsiosis-like infections, as is currently done when using IFAs.

Conclusions

We documented 2 cases of human granulocytic anaplasmosis in Japan, 1 with and 1 without JSF coinfection.

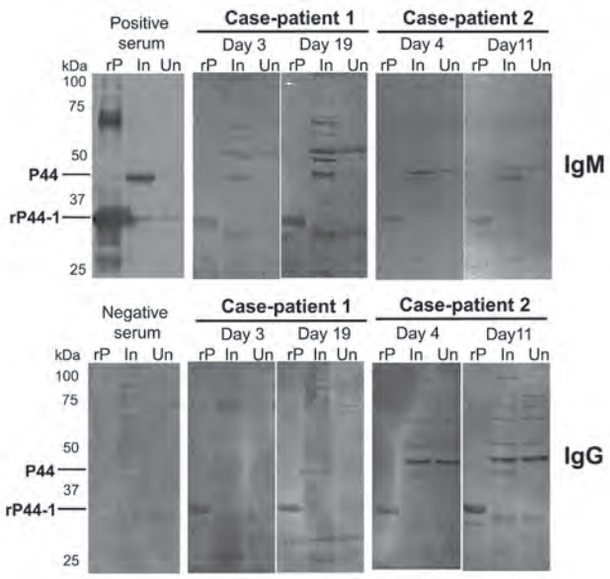

Figure 2. Western blot analyses, using recombinant P44-1 protein (rP44-1) and *Anaplasma phagocytophilum*–infected THP-1 cells as antigens, of serum samples from 2 men, case-patients 1 and 2, who had *A. phagocytophilum* infection, Kochi Prefecture, Japan. The *Escherichia coli*, which produced rP44-1, was kindly provided by Yasuko Rikihisa (Ohio State University, Columbus, OH, USA). The rP44-1 and the rabbit hyperimmune serum (positive control serum) were prepared as described (*11*,*12*). Results for a negative control (human serum sample) are included. The primary human serum samples tested were diluted 200- to 400-fold; rabbit serum sample (positive control) was diluted 2,000-fold. The goat antihuman IgG and IgM alkaline phosphatase conjugates (Life Technologies, Grand Island, NY, USA) were used as secondary antibodies. Days represent days after symptom onset. rP, rP44-1 antigen; In, infected THP-1; Un, uninfected THP-1 cells.

To avoid misdiagnosing cases of human anaplasmosis, we recommend that *A. phagocytophilum* propagated in THP-1 and in HL60 cells be used as antigens for the serodiagnosis of rickettsiosis-like infections.

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Genetic Variants of Echovirus 13, Northern India, 2010

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Nonpolio acute flaccid paralysis is increasing in India. To determine viral causes, we conducted cell culture and molecular analysis identification of nonpolio human enteroviruses associated with acute flaccid paralysis during March–August 2010 in northern India. The predominant nonpolio enterovirus found was echovirus 13, a serotype rarely isolated in India.

Although polio has not been reported from India since January 2011, spurts in the rate of nonpolio acute flaccid paralysis (AFP) are of concern. Therefore, other viral agents associated with AFP need to be identified, especially nonpolio human enteroviruses (HEVs), which are a major cause of neurologic illness.

The classic method for HEV serotypic identification requires virus isolation in susceptible cell cultures, followed by virus neutralization tests. However, the procedure is laborious, slow, and incapable of identifying new serotypes for which no reference antiserum is available (*1*). Nonpolio HEV typing based on viral protein (VP) 1 sequences has been developed and correlates well with antigenically defined serotypes (*2*).

Since 1998, we have been actively involved in polio surveillance and passively reporting nonpolio HEV activity in Uttar Pradesh State, northern India. Most nonpolio HEVs were isolated during the summer–autumn season and identified by using methods based on antigenic serotyping (*3*). We used a method combining a single cell culture passage with VP1 reverse transcription PCR (RT-PCR) and sequencing for rapid identification of nonpolio HEV serotypes. The predominant nonpolio HEV found was echovirus (E) 13, a serotype rarely isolated in India.

The Study

Nonpolio AFP case-patients showing signs of acute onset of focal weakness or paralysis characterized as flaccid (reduced muscle tone) and preceded by fever were considered for NPEV analysis. A total of 347 fecal specimens collected in Uttar Pradesh during March–August 2010 from children <15 years of age who had AFP symptoms were analyzed by using a modified World Health Organization (WHO) method (*4*,*5*).

Fecal specimens were processed according to the WHO protocol (*4*). Human rhabdomyosarcoma and L20B (mouse fibroblast cells expressing the poliovirus receptor) cell lines used for virus isolation were observed for cytopathic effect (CPE). The positive isolates with enterovirus (EV)-like CPE were tested by using pan-poliovirus and pan-EV RT-PCR (*4*). All poliovirus isolates characterized according to WHO algorithm were excluded from the analysis presented here. Viral RNA was extracted from the first-pass rhabdomyosarcoma cell suspension with the Viral RNA Mini Kit (QIAamp, QIAGEN, Hilden, Germany). Genotyping was done by RT-PCR of a portion of the VP1 gene and sequencing as described (*2*,*6*).

MEGA version 5.05 software (*7*) was used for phylogenetic analysis. Distance matrices of the nucleotide and amino acid sequences were analyzed with the MegAlign program in the Lasergene software package (DNASTAR, Madison, WI, USA).

Of 347 fecal samples analyzed, 73 (21%) cell cultures tested positive by pan-EV 5′ nontranslated region RT-PCR. Nineteen (5%) were pan-poliovirus positive, leaving 54 (16%) nonpolio HEVs for analysis. Genotyping of 45 (83%) EVs by partial VP1 sequencing followed the criteria of Oberste et al. (*2*). CPE was observed in rhabdomyosarcoma cells in the first passage for 33 samples, of which 27 were pan-EV positive, identified as E13 (21 [64%]), E7 (2 [6%]), E4 (1 [3%]), E33 (1 [3%]), and EV75 (2 [6%]); 6 isolates remained untypeable. Of the 21 samples that did not yield CPE in rhabdomyosarcoma cells in the first passage, 18 were pan-EV positive, identified as E25 (4 [19%]), E13 (9 [43%]), coxsackievirus B3 (CVB3; 3 [14%]), CVB6 (2 [10%]). Three isolates could not be identified because of poor quality sequence data.

E13 was the most common nonpolio HEV identified. Because E13 has rarely been isolated in India, we analyzed the Uttar Pradesh E13 isolates for March–August 2010 from 5 adjoining districts of western Uttar Pradesh (Lakhimpur-Kheri, Sitapur, Lucknow, Raebareli, and Hardoi). Clinical findings (Table 1) showed that 20 (67%) of the 30 patients had asymmetric paralysis at onset, including 3 AFP case-patients with fever at onset of paralysis and residual paralysis mimicking poliolike illness; however, when compared with other etiologies, no conclusion was inferred (Table 2).

The 244-nt partial VP1 gene sequence of all the Uttar Pradesh E13 isolates shared 75.4%–79.9% nt identity and 87.7%–93.8% aa identity with the prototype EV13 strain, Del Carmen (GenBank accession no. AY302539).

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Table 1. Clinical and virologic findings for AFP case-patients from whom echovirus 13 was isolated, Uttar Pradesh, India, 2010*

Clade,† isolate name‡	Month§	District¶	Patient age, y/sex	Disease onset		Paralysis status after 60-day follow-up (residual paralysis)
				Fever	Asymmetric paralysis	
A						
Har01/IND/UP/Echo13/2010	Mar	LNO	3.10/M	–	–	+
Har05/IND/UP/Echo13/2010	Mar	STP	2.9/F	–	–	+
IND-29-2010	Mar	LNO	4.4/F	–	–	–
IND-30-2010	Mar	LNO	6.0/M	–	–	–
IND-34-2010	Mar	KRI	8.0/M	–	+	–
IND-35-2010	Mar	STP	1.5/M	+	+	–
IND-36-2010	Mar	STP	4.0/M	+	–	–
IND-43-2010	May	LNO	2.5/M	+	+	–
IND-37-2010	May	STP	2.3/F	–	+	–
IND-38-2010	May	STP	2.8/M	+	+	–
IND-42-2010	May	STP	4.7/M	–	–	+
IND-45-2010	May	STP	9.0/M	–	+	–
IND-20-2010	Aug	KRI	4.0/M	+	+	–
IND-21-2010	Aug	KRI	8.0/M	–	–	–
IND-12-2010	Apr	KRI	0.9/F	+	+	–
IND-17-2010	Apr	KRI	2.0/M	–	+	–
B						
IND-23-2010	Jul	HDO	2.0/F	+	+	+
IND-24-2010	Jul	HDO	2.5/M	+	+	–
C						
IND-14-2010	Jul	KRI	1.4/M	+	+	–
IND-16-2010	Jul	KRI	2.0/M	+	+	–
IND-19-2010	Jul	KRI	2.5/F	+	+	–
IND-18-2010	Jul	KRI	3.0/F	–	+	–
D						
IND-15-2010	Jul	KRI	6.5/M	+	+	+
IND-22-2010	Aug	HDO	4.5/M	–	–	–
IND-40-2010	Aug	RBL	2.0/M	–	–	–
IND-39-2010	Aug	LNO	4.0/F	–	+	–
IND-41-2010	Aug	LNO	4.5/M	+	–	+
IND-44-2010	Aug	LNO	3.0/M	+	+	–
IND-33-2010	Aug	HDO	2.0/F	+	+	+
IND-32-2010	Aug	HDO	2.0/M	–	+	–

*AFP, acute flacid paralysis; LNO, Lucknow; STP, Sitapur; KRI, Lakhimpur-Kheri; HDO, Hardoi; RBL, Raebareli; –,absence of clinical feature; + presence of clinical feature.
†Different clades (Figure) of echovirus 13 identified in the study.
‡Designation of investigated isolates of all Uttar Pradesh 2010 echovirus 13 isolates.
§Month of isolation.
¶District of AFP patient.

Phylogenetic analysis indicated the existence of 2 different Uttar Pradesh E13 clades in the neighbor-joining tree (Figure). Uttar Pradesh 2010 E13 viruses in the largest clade segregated into 3 groups (labeled A–C, Figure), whereas the second large 2010 Uttar Pradesh E13 clade was monophyletic (labeled D, Figure). Partial VP1 sequences of Uttar Pradesh E13 clade A shared 96.5%–100% nt identity (100% aa identity) with one another. Clade B shared 89.4%–90.7% nt identity (95.5%–98.8% aa identity) with clade A and 84.2%–84.5% nt identity (97.5% aa identity) with clade C. Clade C shared 88.9%–88.1% nt identity (95.5%–98.8% aa identity) with clade A. Uttar Pradesh E13 clade D shared 73%–76% nt identity (90.1%–93.5% aa identity) with clades A, B, and C. Although variation exists among the partial VP1 sequences of E13 strains from India, the E13 clades A, B, and C appear to be closely related to earlier India isolates (2007 and 2008) followed by the isolates from Pakistan (2009), Bangladesh (2000), and the Republic of Georgia (2004). The E13 D clade was genetically more distant and grouped together with year 2000 strains from India and Bangladesh (isolated in 1999), and 1 isolate from Georgia (isolated in 2004).

Conclusions

We demonstrated the utility of a modified WHO laboratory protocol by combining classical and molecular methods for rapid detection and identification of nonpolio HEV (*2*,*4*–*6*). Identifying the EV in mixed EV infections is challenging when this protocol is used, but on balance, pan-EV testing followed by VP1 RT-PCR and sequencing delivers rapid results and more efficient use of labor and resources than does the traditional method and enables identification of newer EV types.

Numerous reports have described AFP patients infected with EVs, coxsackieviruses, and newer numbered

Table 2. Clinical characteristics of 45 AFP case-patients with nonpolio enterovirus serotypes, Uttar Pradesh, India, 2010*

Sign/symptom	AFP case-patients with serotype, no. (%)							
	E13, n = 30	E25, n = 4	E7, n = 2	E4, n = 1	E33, n = 1	EV75, n = 2	CVB6, n = 2	CVB3, n = 3
Fever at onset of paralysis	15 (50)	3 (75)	–	–	1 (100)	2 (100)	–	3 (100)
Asymmetric paralysis	20 (67)	2 (50)	1 (50)	1 (100)	–	2 (100)	2 (100)	2 (67)
Residual paralysis	7 (23)	–	–	1 (100)	–	1 (50)	–	–

*AFP, acute flacid paralysis, E, echovirus; EV, enterovirus; CVB, coxsackievrius B; –, absence of clinical feature.

EVs (*3*,*8*). Nonpolio HEVs also have been detected in epidemics of paralytic disease (*9*). Although nonpolio HEVs can be isolated from asymptomatic children, few studies have compared the nonpolio HEV serotypes found in AFP case-patients and in healthy children. Identifying nonpolio HEV serotypes associated with AFP in India provides useful EV surveillance data and should be explored further in conjunction with virologic evaluation of appropriate community controls and additional clinical specimens from AFP case-patients (cerebrospinal fluid, throat swab samples, serum), which could indicate the nonpolio HEV type causing paralytic disease.

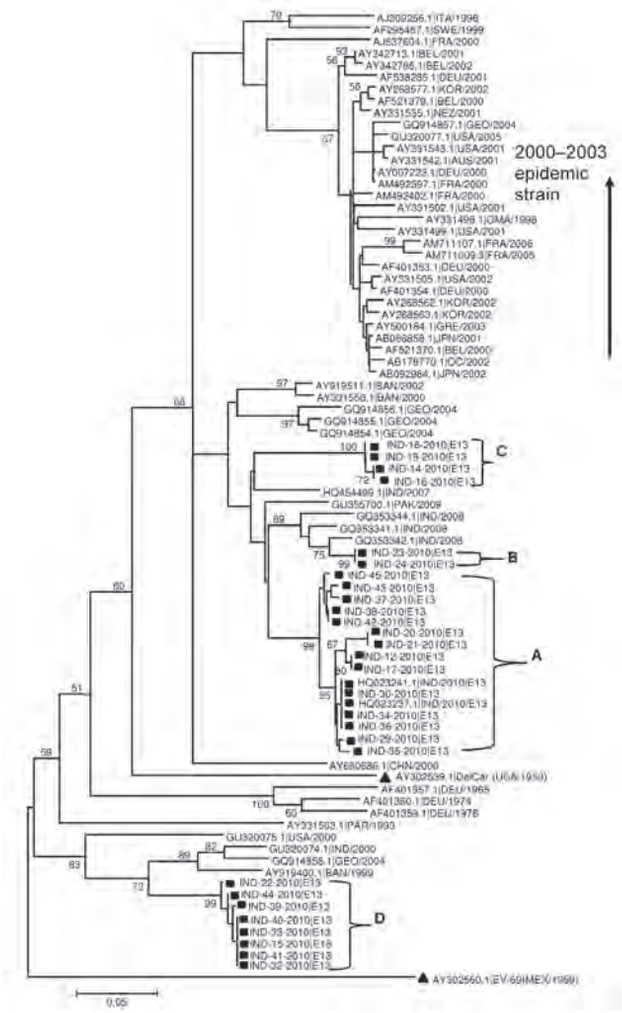

Figure. Phylogenetic tree based on alignments of partial viral protein 1 gene sequences of echovirus 13 (E13) constructed by the neighbor-joining method implemented in MEGA version 5.05 software (*7*) by using the Kimura-2 parameter nucleotide substitution model. Bootstrap analysis included 1,000 pseudoreplicate datasets. Clusters are labeled A, B, C, and D. Square indicates Uttar Pradesh E13 from fecal samples. All Uttar Pradesh E13 isolates on the tree are identified by using the same numbers listed in Table 1. Triangle indicates E13 prototype Del Carmen and the out-group enterovirus 69. Scale bar indicates nucleotide substitutions per site.

We identified 8 nonpolio HEV serotypes (E4, E7, E13, E25, E33, EV75, CVB3, and CVB6). E13, the predominant serotype, has been isolated rarely (*3*,*10*); however, nonpolio HEVs have been reported from India in connection with sporadic and epidemic meningitis/encephalitis cases (*11*,*12*). A wide spectrum of illnesses have been reported with E13 (*13*), so finding E13-associated AFP cases mimicking poliomyelitis (*14*) is not surprising.

The global aseptic meningitis epidemic caused by E13 during 2000–2003 was associated with a single E13 genotype (*15*); however, our findings indicate that several different and genetically diverse E13s have been cocirculating in India. The Uttar Pradesh E13 partial VP1 gene sequences are most closely related to recent E13 strains from central and southern Asia. The high genetic diversity among the Uttar Pradesh E13 isolated from patients in a local area over 6 months implies a continuous pattern of circulation. High-density human populations, immunologically susceptible cohorts, and nonhygienic environmental conditions probably facilitated the E13 genetic heterogeneity in our study.

Although we report a high prevalence of E13 infection in AFP case-patients, our study is restricted by the modest number of cases from a small geographic area sampled over a short period. The epidemiologic and nonpolio HEV genetic information gathered warrants further studies in larger groups of children to gain better insight into AFP caused by nonpolio HEV and to identify the etiologic nonpolio HEV type(s) associated with sporadic or epidemic AFP as India nears the goal of polio elimination.

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Transmission and Maintenance Cycle of *Bartonella quintana* among Rhesus Macaques, China

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We detected Bartonella quintana in 48.6% of captive rhesus macaques from an animal facility in Beijing, China. Prevalence of infection increased over the period of observation. Our findings suggest that macaques may serve as reservoir hosts for B. quintana and that Pedicinus obtusus lice might act as efficient vectors.

Bartonella quintana is a vector-transmitted, hemotropic, and extremely fastidious gram-negative bacterium. Infection with *B. quintana* has been recognized to cause a broad spectrum of disease, including trench fever, chronic bacteremia, endocarditis, and bacillary angiomatosis (*1–4*). Humans are the primary reservoir host for *B. quintana*, which, unlike most other *Bartonella* species, lacks an identified animal reservoir, although some recent reports have found *B. quintana* in dogs and in cynomolgus and rhesus macaques (*5–7*). Almost 60 years ago, rhesus macaques were able to be experimentally infected with *B. quintana* (*8*). However, nonhuman primates have not been shown to support long-term maintenance, multiplication, and transmission of this pathogen, all of which would be expected if these animals were to act as reservoir species. Observations of monkey ectoparasites transmitting *B. quintana* between nonhuman primates or infecting humans have also not been reported.

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The Study

A laboratory animal surveillance program intended to screen for the presence of adventitious pathogens was performed at the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China. Four blood samples from 10 captive-bred rhesus macaques (*Macaca mulatta*) were presumed to be infected with *Bartonella* spp. according to Giemsa-stained smears and transmission electron microscopy (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/2/12-0816-Techapp1.pdf). Further PCR and sequence analysis of 3 gene targets (internal transcribed spacer [ITS], *gltA*, and *rnp*B) confirmed the existence of *B. quintana* in the 4 parasite-positive macaques (online Technical Appendix Table 1). In addition, *B. quintana* was successfully isolated from the 4 monkeys by blood plating.

During the 36-day period of observation, 3 screening tests of the 10 macaques showed an increasing prevalence of *B. quintana*: 4 were found positive at day 1, 7 positive at day 15, and all 10 positive at day 35 (Figure 1, panel A). Close examination of the monkeys revealed no skin scratch or wound indicative of direct contact between them.

Examination for ectoparasites at the last day of observation (day 36) revealed that all 10 monkeys were infested with lice (mean 10.3 lice/monkey, range 4–28 lice). Lice from each infested monkey were combined in 2 pools. *B. quintana* was identified in all pools of lice by PCR selective for ITS, *glt*A, and *rnp*B. Partial Cytb sequence (660-bp) of the louse was obtained (GenBank accession no. JX070558) (online Technical Appendix Table 1); phylogenetic analysis of Cytb identified the louse as a relative of lice of the genus *Pedicinus* (Figure 2, panel A). By means of stereomicroscopy, the louse was then identified as *Pedicinus obtusus* (Figure 1, panel B), a macaque-specific ectoparasite, according to morphologic criteria (*9,10*).

Of the 60 rhesus macaques (27 male, 33 female) housed in 5 other rooms in individual cages in the same facility, an additional 30 were found to be positive for *B. quintana* by PCR. *B. quintana* prevalence among sexually immature macaques was higher than that among sexually mature macaques, but this difference was not significant (29/54 [53.7%] vs. 1/6 [16.7%], respectively; p = 0.195). *B. quintana* prevalence among male macaques was similar to that among females (15/27 [55.6%] vs. 15/33 [45.5%], respectively).

Nucleotide sequences of ITS (123-bp), *gltA* (539-bp), and *rnpB* (336-bp) from all macaques and pools of lice were identical; they differed from from those of *B. quintana* strain Toulouse by1–3 bp . Phylogenetic markers *rnpB*, 16S rRNA, and 23S rRNA (*11*) were amplified and sequenced from the strain identified in this study

[1]These authors contributed equally to this article.

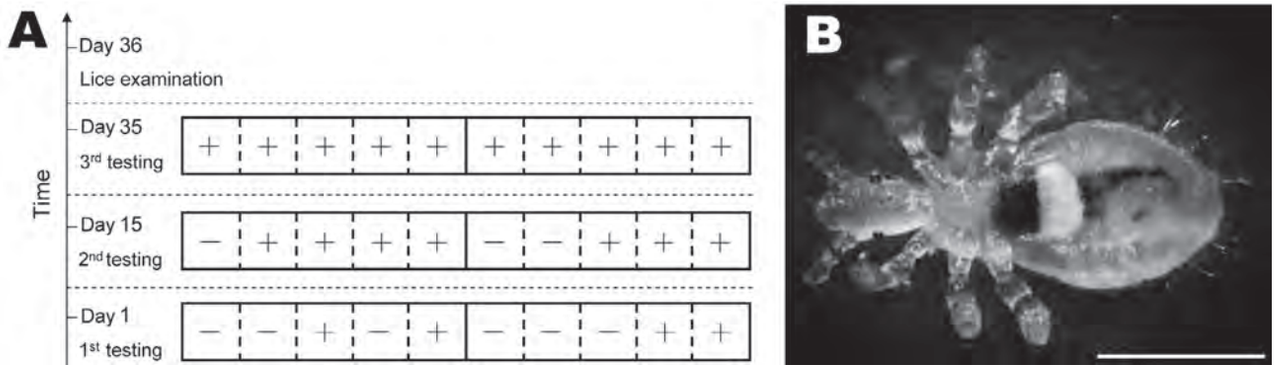

Figure 1. Monitoring surveillance of *Bartonella quintana* infection in macaques and identification of lice. A) Macaques were housed in linked cages (squares); dashed lines indicate wired net enabling direct contact between macaques, and solid line indicates wall that does not enable contact between macaques. +, positive result for PCR; –, negative result for PCR. B) Stereomicroscope image of a *Pedicinus obtusus* louse, a macaque-specific ectoparasite characterized by a slender body ≈1.0–3.0 × 0.5–1.0 mm; long, sharp claws in distal end of 6 legs of the same length; and a plurality of rows of small hairs on both sides of the abdomen. Scale bar indicates 500 μm.

(RM-11) (online Technical Appendix Table 1). Phylogenetic analysis of their combined sequence alignment placed the RM-11 strain on a separate branch along with the strain of *B. quintana* from a cynomolgus macaque and in the same clade as strains from patients in Europe who had trench fever (strains Toulouse and Fuller) (Figure 2, panel B).

To evaluate the ability of the isolate to cause disease, we intravenously inoculated 4 *Bartonella* spp.–negative rhesus macaques with *B. quintana* isolated from a blood sample of a macaque from this study and twice passaged on agar (detailed methods described in the online Technical Appendix). Bacteremia reached a peak in 1 monkey on day 7 postinoculation (160 CFU/mL), in 2 monkeys on day 14 postinoculation (290 and 240 CFU/mL), and in 1 monkey on day 42 postinoculation (240 CFU/mL). Bacteremia then dropped to below a detectable level after 15 weeks postinoculation for all monkeys (online Technical Appendix Figure 2). A relapsing pattern of bacteremia was observed during the experiment. Rectal temperature, hemogram, and blood biochemistry results for the 4 monkeys remained within normal limits.

The animal facility employees who had direct contact with monkeys during cage cleaning and feeding activities were tested for *B. quintana* infection. Paired serum samples collected at 2 time points 3 months apart were tested for IgG against *B. quintana* by indirect immunofluorescence assay, as described (*12*). The baseline serum samples were all negative at a dilution of 1:64. Among the serum samples collected 3 months later, 3 had IgG titers of 256, 1 had a titer of 512, and 4 were negative. For all blood samples collected at the 2 time points, PCR detection, blood-smear staining, and blood culture for *Bartonella* spp. were negative. Analysis of questionnaires revealed that all 4 of the workers with evidence of seroconversion reported lice exposure; 2 of them were scratched or bitten by monkeys.

Conclusions

We demonstrated high prevalence of *B. quintana* in a colony of rhesus macaques and postulated the transmission among macaques by *P. obtusus* lice. Our findings suggest that macaques are susceptible to *B. quintana* infection and can sustain vector infection and subsequent transmission. In addition, rhesus macaques showed long-lasting chronic bacteremia without apparent clinical abnormalities after experimental inoculation, suggesting a high level of adaptation of the pathogen to macaques.

It is unknown how the macaques were initially exposed to infected lice or how the lice became infected with *B. quintana.* We postulate that the lice became infected with *B. quintana* from an infected macaque and thereafter acted as efficient vectors among the rest of the macaques in the colony. We cannot completely exclude the possibility that transmission occurred through direct contact between macaques within the colony; however, we did not observe any skin scratches or wounds on the animals.

Four workers involved with care of the macaques showed seroconversion to antigens derived from our strain. This finding may relate to exposure to the *P. obtusus* lice found on macaques in the animal facility; however, we cannot exclude the possibility of direct contact with these animals as the mode of *Bartonella* spp. transmission because information obtained from the questionnaires indicates that 2 of the 4 seropositive workers were scratched or bitten by the colony's macaques. No clinical signs were observed from these persons, indicating an asymptomatic course of *B. quintana* infection, which has been frequently reported in other studies (*1*,*13*). However, we cannot exclude the possibility that bacteremia continued for an extended time in humans, because the animal care personnel were sampled only 2 times, so we might have missed bacteremia of short duration. Bacterial levels obtained after monkey

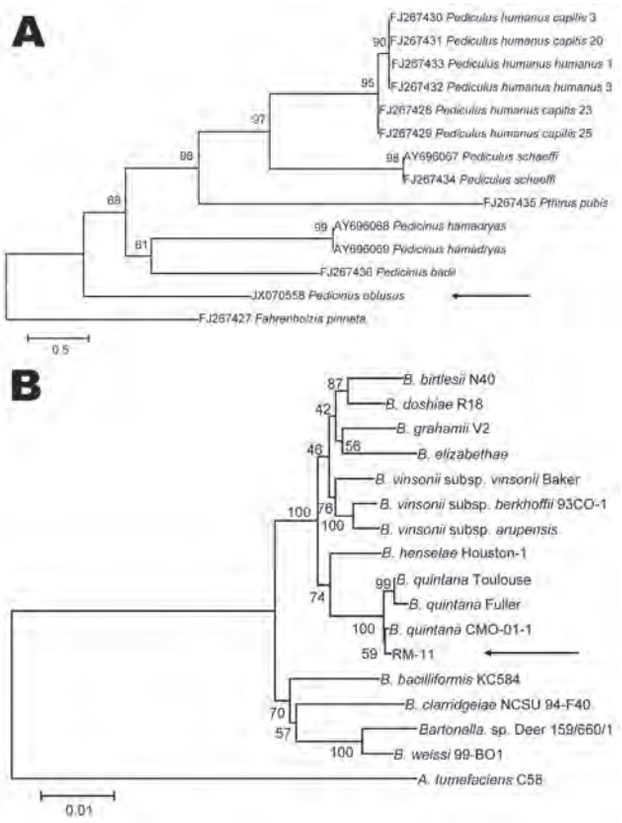

Figure 2. Phylogenetic analyses of louse species and *Bartonella* spp. A) Phylogenetic tree of louse species based on the partial Cytb sequence (364-bp), obtained by using the neighbor-joining method with maximum composite likelihood analysis and bootstrap analysis of 1,000 replicates. Arrow indicates the *Pedicinus obtusus* louse identified in this study. The tree was rooted with the louse species *Fahrenholzia pinnata*. Numbers shown at each node indicate percentage of replicates that reproduced the topology of each clade. Scale bar indicates estimated evolutionary distance of 0.5 substitutions per position. B) Phylogenetic tree of *Bartonella* spp. based on the combined RNase P RNA, 16S, and 23S rRNA sequence alignment (4131-bp), obtained by using the same analytical method as described in panel A. Arrow indicates the RM-11 isolate. The tree was rooted with the louse species *Agrobacterium tumefaciens*. The GenBank accession numbers of *Bartonella* strains used for phylogenetic analysis are shown in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/19/2/12-0816-Techapp1.pdf). Scale bar indicates estimated evolutionary distance of 0.01 substitutions per position.

inoculation was low compared with levels in the initial inoculum, a finding similar to that of a previous study (*14*).

In summary, our findings suggest that the macaques might serve as reservoir hosts for *B. quintana* and that lice might act as efficient vectors. Our data also indicate that macaques could be a source for human infection with *B. quintana*. Further research is needed to understand the underlying mechanism of *B. quintana* transmission by the *P. obtusus* louse.

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Borrelia crocidurae Meningoencephalitis, West Africa

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Borrelia crocidurae–associated relapsing fever is endemic to West Africa and is considered benign. We report 4 patients with B. crocidurae–associated neurologic symptoms; 2 of their cases had been misdiagnosed. Frequency and severity of this disease could be underestimated; molecular methods and serodiagnostic tests for Lyme disease might be helpful in its detection.

Tick-borne relapsing fever (TBRF) is caused by several *Borrelia* species that are transmitted through the bites of *Ornithodoros* ticks (*1*). TBRF is an acute febrile illness characterized by multiple recurrences of nonspecific signs and symptoms, including fever, headache, myalgia, and arthralgia. Neurologic complications might occur, particularly related to *B. hispanica*, *B. crocidurae*, *B. duttoni*, and *B. turicatae* infections (*2*–*7*). Conventional diagnosis is made by microscopic detection of spirochetes in blood samples collected during acute febrile episodes and by direct examination of the cerebral spinal fluid (CSF) of patients with neurologic manifestations. Recently, molecular methods have been shown to be more reliable for *Borrelia* spp. detection in blood and CSF (*3*,*8*). *B. crocidurae* is endemic to West Africa; in Senegal, the rising incidence of infections reported recently has been associated with climate change (*1*,*9*). We report 3 cases of meningitis and 2 cases of encephalitis in 4 persons among a total of 11 consecutive travelers who returned from West Africa to France with *B. crocidurae* infections.

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The Study

Persons included in our study had clinical signs and symptoms of meningitis or encephalitis, or both, and were selected from the 11 patients with cases of *B. crocidurae* TBRF that were reported to and confirmed by the National Reference Center for Borrelia (NRCB) in France during 2009–2011. The NRCB is the reference laboratory responsible for the epidemiologic surveillance of TBRF in France. Clinical meningitis or encephalitis was defined as previously reported (*2*). *Borrelia* species were detected in Giemsa-stained thin blood smears by microscopy and quantitative buffy coat analysis (Becton Dickinson, Le Pont de Claix, France) when available (*4*). *Borrelia* spp. were detected and identified by using 16SrRNA PCR and subsequent sequencing as described (*8*). We tested serum and CSF samples with standardized antibody assays for detection of *Borrelia* spp. that cause Lyme disease (Table).

Among the 11 TBRF cases reported to NRCB during the 3-year study, we identified 4 (36%) cases of clinical meningitis or encephalitis, or both. The epidemiologic, clinical, and laboratory findings and the treatment of the 4 patients are documented in the Table. Three of the 4 patients were adult men, 26–57 years of age, and 1 was a 7-year-old girl. None of the patients were immunocompromised. They were all given appropriate antimalarial chemoprophylaxis. Patients 1 and 4 experienced their first febrile episode in Africa and were empirically treated with antimalarial drugs without biological confirmation of *Plasmodium* infection.

At the time of admission to health care facilities, all patients had fever and headache. Patients 2, 3, and 4 had signs of meningitis, including neck stiffness; patients 2 and 4 also had phonophobia and photophobia. Patients 1 and 4 had encephalitis with drowsiness, which for patient 4 was accompanied by cerebellar syndrome (dysarthria and dysmetria). All patients except patient 3 underwent computed tomography scanning of the brain; no abnormality was detected. The 2 patients with encephalitis were examined by magnetic resonance imaging; in patient 4, a predominant positive contrast of the cerebellum leptomeninges on the right side was observed.

For all patients, 16S rRNA PCR and sequencing identified *B. crocidurae* (*8*) in blood samples. Laboratory analysis of the 4 CSF samples showed a lymphocytic pleocytosis, high protein concentrations, and a glucose value within reference range (Table). The molecular methods applied to CSF samples confirmed neurologic *B. crocidurae* infection in patients 1, 2, and 3. Serum samples collected from patients 1 and 4 at the time of diagnosis were tested by using Lyme disease serodiagnostic assays. ELISA detected substantial levels of IgM and IgG in samples from both patients; 1 was

[1]These authors contributed equally to this work.

Table. Epidemiologic, clinical, and laboratory findings and treatment for patients with *Borrelia crocidurae* meningoencephalitis*

Variables	Patient 1	Patient 2	Patient 3	Patient 4
Demographic factor				
Age, y/sex	36/M	57/M	7/F	26/M
Country of origin/of residence	Senegal/France	France/France	France/France	Senegal/France
Travel country	Senegal	Senegal	Senegal	Senegal
Travel dates	2009 Mar–May	2010 May	2011 Feb–May	2011 Aug–Sep
Travel duration, d	53	15	15	35
Travel accommodation	Family house	Hotel	Hotel	Family house
Arthropod or insect bite report	No	Yes	No	No
Individual vector protection	No	No	No	No
First suspected diagnosis†/presumptive treatment	Malaria/quinine	Sinusitis	Gastroenteritis	Malaria/piperaquine
Symptoms				
Oral temperature >38.5°C	Yes	Yes	Yes	Yes
Chills	No	No	Yes	Yes
Total no. febrile episodes/no. before diagnosis	2/1	4/2	6/5	2/1
Length of acute febrile episodes, d	2–8	2–6	2	2–6
Afebrile periods between febrile episodes, d	15	2–15	2–13	34
Asthenia/anorexia/weight loss	Yes/no/no	Yes/yes/yes‡	Yes/no/no	Yes/no/no
Headache	Yes (severe)	Yes	Yes (severe)	Yes (severe)
Myalgia	No	No	No	Yes
Photophobia, phonophobia	No	Yes	No	Yes
Neck stiffness	No	Yes	Yes	Yes
Cerebellar syndrome	No	No	No	Yes
Drowsiness	Yes	No	No	Yes
Imaging results				
Brain CT scan	Normal	Normal	ND	Normal
Brain MRI	Normal	ND	ND	Abnormal
Serologic results				
Leukocytes, g/L	4.7	15.8	13.0	8.4
Hemoglobin, g/L	134	139	115	131
Platelets, g/L	245	273	295	103
C-reactive protein, mg/L	103	4	57	150
Creatinine, μmol/L	107	76	42	99
Borrelia spp. detection, Giemsa-stained blood smear	+§	+§	+§	+¶
Quantitative buffy coat	ND	ND	ND	+
ELISA anti–*B. burgdorferi* (titer)				
Siemens Enzygnost Lyme link VlsE/IgG#	+(11.3)	ND	ND	+(19)
Siemens Enzygnost Borreliosis/IgM#	+(3.7)	ND	ND	+(1.21)
Western blot anti–*B. burgdorferi*				
Bio-Advance IgG anti-VlsE/p41/p83/p21#	+/–/–/+	ND	ND	ND
EUROIMMUN IgG anti-p17/p19/p21/p25/p30/p31/p39/p83/VlsE#	ND	ND	ND	–/–/–/–/–/–/–/–/**/**
Meridian Bioscience IgM garinii/afzelii/p41/p39/p17#	ND	ND	ND	ND
Bio-Advance IgM anti-p25/p83#	+/+	ND	ND	ND
16S rRNA PCR *Borrelia*/identification	+/*B. crocidurae*	+/*B. crocidurae*	+/*B. crocidurae*	+/*B. crocidurae*
CSF test results				
Leukocytes, cells/mm^3	405	217	258	156
% Lymphocytes	94	80	90	84
Erythrocytes, cells/mm^3	0	7	12	5
Protein, g/L	0.66	1.38	0.39	0.44
Glucose, mmol/L	3.1	2.7	2.69	2.9
Chloride, mmol/L	114	113	117	115
Lactate, mmol/L	ND	ND	1.5	2.2
Direct examination (Gram stain)	–	–	–	–
Conventional bacterial culture	–	–	–	–
16S rRNA PCR *Borrelia*/identification	+/*B. crocidurae*	+/*B. crocidurae*	+/*B. crocidurae*	–
ELISA anti–*B. burgdorferi*				
Siemens Enzygnost Lyme link VlsE/IgG#	ND	ND	ND	+††
Siemens Enzygnost Borreliosis/IgM#	ND	ND	ND	–
Treatment (daily dose/total d)	Ceftriaxone (2 g/21)	Doxycycline (100 mg 2×d/10)	Ceftriaxone (2 g/14)	Doxycycline (100 mg 2×d/21), ceftriaxone (2 g/15)

*CT, computed tomography; MRI, magnetic resonance imaging; ND, not done; +, positive; –, negative; CSF, cerebrospinal fluid.
†First suspected diagnosis was not biologically confirmed.
‡7 kg in 3 wk.
§Second sample was positive.
¶First sample was positive after review prompted by the quantitative buffy coat result.
#Siemens, Erlangen, Germany; Bio-Advance, Bussy Saint Martin, France; EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany; Meridian Bioscience, Paris, France.
**Ambiguous.
††At 12-fold dilution (low level).

confirmed by Western blot analysis. The CSF sample from patient 4 showed a low level of IgG (Table).

All cases were treated with doxycycline or ceftriaxone, or both (Table). In all patients, fever resolved within 3 days of the beginning of the appropriate treatment, and the outcomes were favorable. No Jarisch-Herxheimer reaction was observed.

B. crocidurae–associated TBRF is an emerging disease that is considered to be benign (*1*,*9*). However, the series of infections reported here suggest that severe neurologic complications, notably, meningitis and encephalitis, occur more frequently than previously thought and could be particularly common in travelers who acquired this infection in West Africa. For the patients we studied, the earliest neurologic signs occurred during the second febrile episode, confirming previous studies reporting the onset of neurologic complications after the first episode (*2*). However, facial palsy, often considered to be among the main clinical signs and symptoms of neuroborreliosis caused by TBRF-associated *Borrelia* species, was not observed in these patients (*2*). A similar clinical manifestation described in a recent case report of *B. crocidurae* encephalitis is entirely consistent with our observations (*3*).

Functional and experimental studies have focused on the capacity of TBRF-associated *Borrelia* species to cross the blood–brain barrier and to persist in the brain (*2*–*7*,*10*,*11*). These studies have established *B. crocidurae* as the most neurotropic species, an observation consistent with this and other case series and case reports. In animal models, this feature has been associated with the presence of vascular microemboli in the brain of infected animals and the particular ability of *B. crocidurae* to form and bind to erythrocyte rosettes, a phenomenon also involved in cerebral malaria pathogenesis. Erythrocyte aggregation might prevent host–pathogen interactions and thereby protect the spirochetes from the specific immune response (*10*,*12*,*13*).

The rather high frequency and severity of neurologic complications associated with *B. crocidurae* infection raise the problem of distinguishing it from cerebral malaria, because the areas of endemicity of these 2 diseases largely coincide (*1*,*9*). Indeed, relapsing fever is frequently misdiagnosed as malaria, as it was for 2 of the patients we studied, who were initially treated with antimalarial drugs (*14*). In this context, quantitative buffy coat analysis that can effectively detect each pathogen in blood might be of particular interest (*4*). In addition, our study confirms the usefulness of molecular methods applied to blood and CSF samples to confirm *Borrelia* infection (*3*,*8*). The negative result obtained by PCR of CSF from patient 4 could have been the consequence of inappropriate storage of the sample at high room temperature for 72 hours before analysis.

Lyme disease serodiagnostic testing of serum and CSF samples might be helpful. Indeed, cross-reacting IgG and IgM were detected by ELISAs and in Western blot assays. Because Lyme disease is endemic to France, our results could have been caused by the actual detection of *B. burgdorferi* sensu lato antibodies, although none of the patients had a known history of Lyme disease.

No specific recommendations have been proposed for the treatment of patients with TBRF neuroborreliosis. Erythromycin and penicillin have been reported to be ineffective (*5*,*6*). In our series, all patients were prescribed either ceftriaxone or doxycycline, or both (Table), resulting in successful treatment of the disease. Thus, from the literature and our own experience, we suggest that TBRF with neurologic involvement should be treated with ceftriaxone or doxycycline for at least 21 days.

Conclusions

Our study highlights the frequent occurrence of meningitis or encephalitis in patients with *B. crocidurae* TBRF acquired in West Africa. The clinical and radiologic manifestations suggest that this infection could be more severe than previously thought. Consequently, travelers returning from West Africa with febrile neurologic disorders should be tested immediately for biological confirmation of *Borrelia* infection through blood and CSF analyses, including molecular methods.

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Influenza A(H5N1) Virus Surveillance at Live Poultry Markets, Cambodia, 2011

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In Cambodia, influenza A(H5N1) virus surveillance at live poultry markets (LPMs) relies on virus isolation from poultry specimens; however, virus is rarely detected by this method. We tested 502 environmental LPM samples: 90 were positive by PCR, 10 by virus isolation. Virus circulation could be better monitored by environmental sampling of LPMs.

Highly pathogenic avian influenza (HPAI) A(H5N1) virus was first detected in 2004 in Cambodia. Since then, outbreaks of subtype H5N1 infection among poultry have been regularly detected, and 21 human cases associated with 19 deaths have been recorded (*1*).

In Cambodia, where poultry vaccination is not allowed, illegal poultry trade has been a repeated source of reintroduction of the virus (*2*–*4*). Surveillance for subtype H5N1 virus at live poultry markets (LPMs) has been conducted in Cambodia by inoculating cloacal or throat swab specimens from poultry into embryonated chicken eggs; however, virus has rarely been detected by this method (S. Sorn, unpub. data). After outbreaks of subtype H5N1 virus in poultry, the viral genome can be detected for >1 week in environmental samples from the outbreak area; thus, environmental surfaces are potential sources of virus transmission to humans and animals (*5*,*6*). LPMs have also been reported as sources of virus involved in human subtype H5N1 infection (*7*–*9*).

Birds sold at LPMs can originate from many regions of a country; thus, conducting surveillance for subtype H5N1 virus at these markets would probably be an effective way to monitor the circulation of virus within a country (*7*,*9*,*10*). It has been recommended that samples of drinking water shared by birds maintained within the same cage and samples of poultry feces be used to detect virus within LPMs (*10*,*11*). We performed this study to determine whether subtype H5N1 virus circulation in Cambodia could be better monitored by using environmental sampling in LPMs.

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The Study

In 2011 in Cambodia, environmental samples were collected from 4 LPMs each week for 7 weeks, including during the Khmer New Year festival (Figure 1). Two of the markets were in Phnom Penh, the capital city: Orussey market (M1) and Chamkar Doung market (M2), which also served as an overnight resting place and a place to keep unsold birds from various markets. The third market (M3) was in Takeo (Takeo Province), and the fourth (M4) was in Kampong Cham (Kampong Cham Province). Local chickens, Sampov (domesticated mallards), and Kaki Campbell ducks (domesticated Muscovy ducks) were the only live poultry observed in the markets. Other poultry species were usually available only upon customer request, or they were sold dead.

During the study, we observed that chickens and ducks were mixed together in cages or stalls. In each market, we collected environmental samples from 4–5 poultry cages or from stalls where poultry were gathered. From each sampling site, we collected the following into sterile 50-mL tubes: 50 mL of water used by poultry for drinking, 50 mL of water used to wash carcasses or found on the floor near the slaughtering area, 40–50 g of soil/mud, and 2–3 g of fresh feces present on the soil. Feathers (10–20 g) dropped by birds were gathered and placed in sterile plastic bags.

Virus in water, soil, and mud samples was concentrated, as described (*6*,*12*,*13*), in the biosafety level 3 labora-

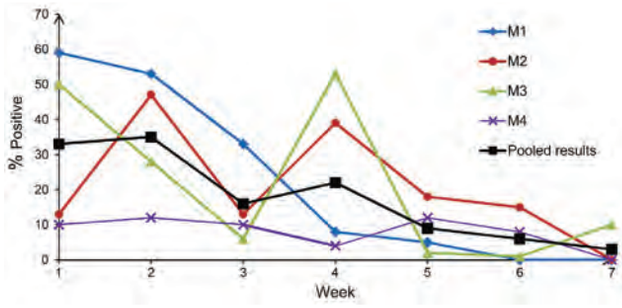

Figure 1. Prevalence of influenza A(H5N1) virus–positive environmental samples from live poultry markets, by collection week, during the Khmer New Year festival, Cambodia, 2011. The New Year festival occurred during week 4 of the study. M1, Orussey market (Phnom Penh); M2, Chamkar Doung market (Phnom Penh); M3, Takeo market (Takeo Province); M4, Kampong Cham market (Kampong Cham Province). Samples positive for the matrix, hemagglutinin 5, and neuraminidase 1 genes by quantitative real-time reverse transcription PCR were considered positive for subtype H5N1 virus. In rare instances, neuraminidase 1–negative samples that were positive for the matrix and hemagglutinin genes were considered positive for subtype H5N1 virus.

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tory at Institut Pasteur, Phnom Penh. Samples were then tested by quantitative real-time reverse transcription PCR (qRT-PCR) targeting the matrix (M), hemagglutinin 5 (H5), and neuraminidase 1 (N1) genes. Feces and feather samples were homogenized with sterile phosphate-buffered saline before nucleic acid extraction and testing. Samples were considered subtype H5N1 virus–positive if qRT-PCR was positive for the M, H5, and N1 genes. The N1 qRT-PCR we used is less sensitive than those used for detection of the M and H5 genes. Thus, in rare instances, we considered N1-negative but H5 and M gene–positive samples to be positive for subtype H5N1 virus. All positive samples were subsequently inoculated into specific pathogen–free embryonated hen eggs for virus isolation (*6*).

Of 502 samples collected, 90 (18%) were positive for subtype H5N1 virus by qRT-PCR, and 10 (2%) were positive by virus isolation (Table). We did not detect >1 positive sample at a time in each cage sampled; thus, each positive sample corresponded to 1 contaminated sampling site. No correlation was observed between viral load measured by qRT-PCR and the ability to isolate the virus in eggs. The overall positivity rate for detection of RNA was >20% for water, feather, and soil/mud samples; the rate was significantly lower for feces samples (6%; $p<0.05$) (Table). The virus was isolated from 8 (6%) water samples and 2 (2%) soil/mud samples.

Compared with the market in Kampong Cham (M4), the markets in Phnom Penh and Takeo (M1–3) had a higher percentage of samples positive for H5N1virus (8% vs. >20%; $p<0.05$). Overall, the level of environmental viral contamination in LPMs was highest at the beginning of the study (i.e., 4 weeks before the Khmer New Year, when poultry sales began for the annual festival) and corresponded with intense movement of poultry within the country and higher densities of poultry populations on farms (*2*,*4*); contamination levels tended to progressively decrease, reaching low levels 2 weeks after the event (Figure 1).

The full genomic sequence of 4 strains and the hemagglutinin sequence of 3 other isolates were generated (GenBank accession nos. JQ673600–JQ673606). Phylogenetic analyses showed that the virus strains detected during this study belong to lineage 6, a group of viruses that seems to be endemic to Cambodia (*14*) (Figure 2), and not to lineage 5, a more regional group of viruses that was also circulating in Cambodia at that time and that includes strains originating from Cambodia and Vietnam. Sequence analyses did not detect reassortment events or mutations associated with

Table. Results of laboratory testing for influenza A(H5N1) virus in environmental samples from live poultry markets, Cambodia, 2011*

	Samples tested by qRT-PCR		Samples tested by virus isolation†	
Sample type, market	No. positive/no. tested (%)	Total no. positive/total no. tested (%)	No. positive/no. tested (%)	Total no. positive/total no. tested (%)
Water		30/145 (21)‡		8/145 (6)§
M1	7/46 (15)		2/46 (4)	
M2	9/37 (24)		3/37 (8)	
M3	11/21 (52)		3/21 (14)	
M4	3/41 (7)		0/41 (0)	
Soil or mud		27/120 (23)‡		2/120 (2)
M1	7/28 (25)		0/28 (0)	
M2	8/28 (29)		1/28 (4)	
M3	9/33 (27)		0/33 (0)	
M4	3/31 (10)		1/31 (3)	
Feces		7/117 (6)‡		0/117 (0)§
M1	3/30 (10)		0/30 (0)	
M2	0/25 (0)		0/25 (0)	
M3	3/33 (9)		0/33 (0)	
M4	1/29 (3)		0/29 (0)	
Feathers		26/120 (22)‡		0/120 (0)§
M1	11/30 (37)		0/30 (0)	
M2	8/28 (29)		0/28 (0)	
M3	4/32 (13)		0/32 (0)	
M4	3/30 (10)		0/30 (0)	
Total		90/502 (18)		10/502 (2)
M1	28/134 (21)¶		2/134 (1)	
M2	25/118 (21)¶		4/118 (3)	
M3	27/119 (23)¶		3/119 (3)	
M4	10/131 (8)¶		1/131 (1)	

*qRT-PCR, quantitative real-time reverse transcription PCR; M1, Orussey market in the capital city of Phnom Penh; M2, Chamkar Doung market in Phnom Penh; M3, a market in Takeo, Takeo Province; M4, a market in Kampong Cham, Kampong Cham Province.
†Environmental samples were inoculated into specific pathogen–free embryonated hen eggs for virus isolation.
‡The percentage of samples that were positive was significantly different (by χ^2 test) for feces vs. water ($p = 0.0007$), feces vs. soil/mud ($p = 0.0003$), and feces vs. feathers ($p = 0.0005$).
§The percentage of samples that were positive was significantly different (by χ^2 test) for water vs. feces ($p = 0.001$) and water vs. feathers ($p = 0.009$).
¶The percentage of environmental samples that were positive was significantly different (by χ^2 test) for M4 vs. M1 ($p = 0.002$), M4 vs. M2 ($p = 0.002$), and M4 vs. M3 ($p = 0.0008$).

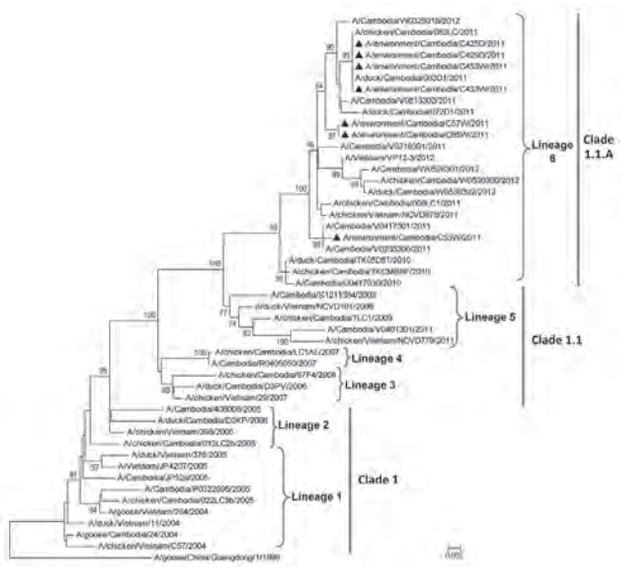

Figure 2. Phylogenetic relationship of the hemagglutinin (HA) gene among various influenza A(H5N1) strains; HA sequences for 48 strains (36 from Cambodia, 11 from Vietnam, and 1 from China) were included in the analysis. Black triangles indicate viruses detected during this study of environmental samples from live poultry markets in Cambodia. Phylogenetic trees were generated by using the distance method and applying the neighbor-joining algorithm with bootstrap analysis (1,000 replicates). Analysis was based on nt 1–1,661 of the HA gene. The trees were rooted to A/goose/China/Guangdong/1/96 (H5N1). Numbers above and below branch nodes indicate bootstrap value of ≥70%. Scale bar represents the number of nucleotide changes per site. Lineage numbers 1–6, clades, and subclades indicate strains that are grouped in closely related phylogenetic lineages, as described (*14*). All sequences included in the analysis are available in GenBank.

higher virulence or increased transmission to humans. Sequences for a virus detected in March 2011 in M3 (Takeo) clustered with sequences for strains isolated from 2 subtype H5N1 virus–infected humans in February near Phnom Penh and in April in Prey Veng Province, respectively. This finding suggests that the strain detected in Takeo was part of a phylogroup that circulated in different regions of the country for several months. Another strain was isolated from M3 on the same day in March. Sequences for that strain did not cluster with those for the isolates from humans in Phnom Penh and Prey Veng Province; however, the strain shared a high degree of homology with a strain detected a week later in Phnom Penh, suggesting the cocirculation in markets of strains with different origins.

Conclusions

HPAI A(H5N1) virus circulation in Cambodia has traditionally been monitored by using the moderately sensitive egg inoculation method to test cloacal and tracheal swab samples from birds randomly selected from LPMs or farms. Our results show that a more effective approach—especially before and during the main annual festivals, when the movement of poultry within the country is increased—would be to use highly sensitive qRT-PCR to test environmental samples from LPMs. In our study, water samples proved to be the best choice for isolation of infectious subtype H5N1 virus (Table). The lower detection rate of virus among feces samples was expected because the analysis of such samples represents viral shedding by only 1 or a few birds. Available sequence data from other surveillance efforts indicate that all strains detected in this study originated in Cambodia.

In Cambodia, birds not sold the same day they arrive at a live poultry market are transported to an overnight resting place, which is sometimes another market (*7*). Such movement of poultry could increase exposure to environmental contamination with HPAI A(H5N1) virus and thus contribute to virus spread among poultry. It is not known what effect the high levels of HPAI A(H5N1) virus contamination in LPMs in Cambodia have on human health; the effect should be evaluated by conducting clinical and serologic surveillance of vendors and poultry workers.

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Risk Factors for **Influenza A(H1N1)** pdm09 among Students, Beijing, China

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To identify risk factors associated with influenza A(H1N1)pdm09 among students in Beijing, China, we conducted a case–control study. Participants (304 case-patients and 608 controls, age range 6–19 years) were interviewed by using a standardized questionnaire. We found that in addition to vaccination, nonpharmaceutical interventions appeared to be protective.

Influenza A(H1N1)pdm09 virus first emerged in Mexico and southern California, USA, in early April 2009 and rapidly spread worldwide (*1*). The mode of transmission of this novel virus was similar to that of other influenza viruses. Notably, the virus disproportionately affected children and young adults (*2*). Therefore, further research was required to understand etiologic factors associated with spread of influenza A(H1N1)pdm09 among school-age children to limit transmission within schools and in the community. We conducted a case–control study to identify risk factors associated with influenza A(H1N1)pdm09 among students in Beijing, China.

The Study

Beijing is one of the largest cities in China and has 18 districts and a population of >20 million persons. Although there is considerable variation in district size and a greater population density in urban areas, health care is accessible for residents in all districts. During the pandemic period, the Notifiable Disease Surveillance System (NDSS) was established in Beijing. Fifty-five collaborating laboratories covering all hospitals were authorized to conduct confirmation testing for influenza A(H1N1)pdm09 virus (*3*). All confirmed cases were reported through the NDSS.

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Case-patients were students for whom diagnosis was confirmed during October 1, 2009–January 31, 2010. Stratified sampling was used to recruit case-patients through the NDSS. We randomly selected 3 urban and 3 rural districts from the 18 districts and listed all case-patients ≤18 years of age. We aimed to randomly select 50 patients from each district.

Controls were matched with case-patients at a ratio of 2:1 by sex and age (± 1 year) and were recruited from the same school and grade but from different parallel classes than case-patients. Students who reported having influenza-like symptoms since September 2009 were excluded.

The survey was conducted as a face-to-face interview by trained investigators from the Centers for Disease Control and Prevention in Beijing by using a standardized questionnaire. This interview had a 100% response rate and no data were missing. All variables were self-reported.

Data entry and statistical analysis were conducted by using EpiData software version 3.1 (www.epidata.dk/download.php) and SPSS version 16.0 (IBM, Armonk, NY, USA). Bivariate and multivariate conditional logistic regression analyses were used to determine risk factors associated with infection. All variables with $p<0.05$ in bivariate analysis were included in multivariate analysis. Collinearity was evaluated for all variables in the final model. Backward conditional logistic regression was conducted by removing variables with $p>0.10$, and statistical significance was defined as $p<0.05$ (online Technical Appendix, wwwnc.cdc.gov/EID/article/19-2/12-0628-Techapp1.pdf).

A total of 304 case-patients and 608 controls were recruited from either primary or middle schools. Age range was 6–18 years for case-patients and 6–19 years for controls (median age 13 years for both groups).

Bivariate analysis identified factors associated with having influenza A(H1N1)pdm09. These factors were vaccination history, eye rubbing, handwashing immediately after sneezing, handwashing after lessons in communal classrooms, sleep time per day, participation in outdoor activities after class, population density of classrooms, classroom ventilation, mode of transportation to and from school, and participation in clustered social activities after school (Table 1).

Multivariate analysis showed that in addition to vaccination, a series of environmental and behavioral factors were associated with reducing the risk for influenza A(H1N1)pdm09. These factors included provision of classroom space ≥1.6 m^2/student, participation in outdoor activities after school, decreased interval of classroom ventilation, immediate handwashing after sneezing, having more sleep time (≥7 h/day), and use of open modes of travel (walking, bicycle, and motorcycle) (Table 2).

[1]These authors contributed equally to this article.

Conclusions

We found several variables that determined whether students would have influenza A(H1N1)pdm09. These factors were vaccination, classroom space, outdoor activities, classroom ventilation, handwashing, sleep time, and modes of travel.

Vaccination against influenza A(H1N1)pdm09 was more common among controls than case-patients, suggesting its potential value of protection. However, the vaccination rate is low in Beijing, China. Limited knowledge and misconceptions regarding vaccination safety were contributing risk factors (*4*–*6*).

Because transmission modes for this virus appeared to be similar to those for seasonal influenza viruses, involving close, unprotected contact with respiratory droplets (*7*), we found that environmental issues appeared to be protective.

Table 1. Bivariate analysis of potential factors associated with influenza A(H1N1)pdm09 infection among students ≤18 years, Beijing, China*

Variable	No. (%) case-patients, n = 304	No. (%) controls, n = 608	p value	OR (95%CI)
Vaccination against influenza A(H1N1)pdm09				
No	276 (90.8)	264 (43.4)	Referent	
Yes	28 (9.2)	344 (56.6)	<0.001	0.08 (0.05–0.12)
Vaccination with pneumococcal vaccine				
No	279 (91.8)	542 (89.1)	Referent	
Yes	25 (8.2)	66 (10.9)	0.193	0.72 (0.43–1.18)
Use of traditional Chinese medicine				
No	103 (33.9)	175 (28.8)	Referent	
Yes	201 (66.1)	433 (71.2)	0.068	0.73 (0.51–1.02)
Eye rubbing				
No	163 (53.6)	395 (65.0)	Referent	
Yes	141 (46.4)	213 (35.0)	0.001	1.68 (1.25–2.26)
Handwashing immediately after sneezing				
No	151 (49.7)	205 (33.7)	Referent	
Yes	153 (50.3)	403 (66.3)	<0.001	0.48 (0.36–0.65)
Use of soap during handwashing				
No	37 (12.2)	63 (10.4)	Referent	
Yes	267 (87.8)	545 (89.6)	0.402	0.83 (0.53–1.29)
Handwashing after lessons in communal classrooms				
No	176 (57.9)	285 (46.9)	Referent	
Yes	128 (42.1)	323 (53.1)	0.002	0.63 (0.48–0.84)
Handwashing after participation in outdoor sports activities				
No	46 (15.1)	69 (11.3)	Referent	
Yes	258 (84.9)	539 (88.7)	0.088	0.69 (0.45–1.06)
Duration of handwashing, s				
<20	176 (57.9)	347 (57.1)	Referent	
≥20	128 (42.1)	261 (42.9)	0.800	0.96 (0.71–1.30)
Sleep time, h/day				
<7	99 (32.6)	162 (26.6)	Referent	
≥7	205 (67.4)	446 (73.4)	0.030	0.67 (0.47–0.96)
Sharing of tableware with classmates				
No	263 (86.5)	534 (87.8)	Referent	
Yes	41 (13.5)	74 (12.2)	0.556	1.14 (0.74–1.75)
Classroom space/student, m^2				
<1.6	223 (73.4)	412 (67.8)	Referent	
≥1.6	81 (26.6)	196 (32.2)	<0.001	0.17 (0.07–0.41)
Participation in outdoor activities after class				
No	232 (76.3)	411 (67.6)	Referent	
Yes	72 (23.7)	197 (32.4)	0.003	0.58 (0.40–0.83)
Frequency of classroom ventilation				
>1×/h	109 (35.9)	160 (26.3)	Referent	
1×/h	195 (64.1)	448 (73.9)	0.002	0.61 (0.44–0.83)
Having meals in small restaurants near school				
No	232 (76.3)	460 (75.7)	Referent	
Yes	72 (23.7)	148 (24.3)	0.808	0.96 (0.67–1.37)
Modes of transportation to and from school				
Closed (taxi, public transportation, school bus, car)	188 (61.8)	325 (53.5)	Referent	
Open (walking, bicycle, motorcycle)	116 (38.2)	283 (46.5)	0.009	0.66 (0.48–0.90)
Participation in clustered social activities after school closure				
No	266 (87.5)	559 (91.9)	Referent	
Yes	38 (12.5)	49 (8.1)	0.023	1.76 (1.08–2.86)

*Bivariate conditional logistic regression was used to generate p values. OR, odds ratio.

When the interval of classroom ventilation exceeded 1 h, air renewal was determined to be inadequate, increasing potential risk for infection. These findings are consistent with those of other studies, which reported that influenza can spread in a confined space with insufficient air flow and that clustering of students within classrooms or during after-school activities can facilitate transmission of infectious diseases (*8*,*9*).

Social distancing might be another protective nonpharmaceutical measure. When available classroom space per student was <1.6 m^2, there was a greater chance that students having influenza A(H1N1)pdm09 would have close contact with healthy classmates, who would be at higher risk of acquiring this disease.

We found that use of closed modes of transportation was also a risk factor. Although other studies reported that transmission rates of influenza A(H1N1)pdm09 were not increased by close and frequent contact with other persons on public transportation, we advocate use of open modes of transportation for travel to and from school, and self-protection measures when using closed modes of transportation (*10*). For instance, because wearing of face masks is easily applicable and has been shown to be protective, it tended to be a preventative measure for students who use closed transport systems (*11*).

School closure has been identified as a protective measure for controlling influenza pandemics (*12*). Some students after school closure continued to participate in clustered social activities, thereby having potentially increased their risk for contact with patients with influenza A(H1N1)pdm09 outside the school environment. Thus, after school closure, avoidance of large gatherings and clustered social activities may further reduce infection among students.

Some variables that we analyzed were not risk factors (vaccination with pneumococcal vaccine, drug prophylaxis [using traditional Chinese medicine], some handwashing habits (e.g., duration of handwashing), and sharing of tableware with classmates. Further studies might be needed to determine their effects.

There were limitations to this study. Case-patients were recruited into the study 3–8 months after receiving a confirmed diagnosis. Therefore, data collection was retrospective and had potential recall bias. Not all risk factors for influenza could be comprehensively assessed by the questionnaire. Controls were not subjected to laboratory testing, and some asymptomatic infected students may have been misclassified as controls, resulting in underestimation of odd ratios of certain risk factors and overestimation of odd ratios of certain protective factors. We did not include face mask use in the analysis because it was difficult to accurately categorize wearing face masks, given the large variety of face masks in different sizes and varying tightness in use during the pandemic in Beijing, and because we had no data for time, place, or duration of face mask use.

Table 2. Multivariate analysis of independent factors associated with influenza A(H1N1)pdm09 infection among students ≤18 years of age, Beijing, China*

Variable	p value	Matched OR (95% CI)
Vaccination against influenza A(H1N1)pdm09		
No	Referent	
Yes	<0.001	0.07 (0.04–0.11)
Handwashing immediately after sneezing		
No	Referent	
Yes	<0.001	0.49 (0.33–0.72)
Sleep time, h/day		
<7	Referent	
≥7	0.042	0.62 (0.38–0.98)
Classroom space/student, m^2		
<1.6	Referent	
≥1.6	<0.001	0.11 (0.04–0.31)
Participation in outdoor activities after class		
No	Referent	
Yes	0.029	0.60 (0.38–0.95)
Frequency of classroom ventilation		
>1×/h	Referent	
1×/h	0.023	0.60 (0.39–0.93)
Mode of transportation to and from school		
Closed (taxi, public transportation, school bus, car)	Referent	
Open (walking, bicycle, motorcycle)	0.010	0.58 (0.39–0.88)
Participation in clustered social activities after school		
No	Referent	
Yes	0.025	2.08 (1.10–3.95)

*Ten variables were included in multivariate conditional logistic regression analysis. Backward conditional logistic regression was conducted by removing variables with p>0.10, and 8 variables remained in the final regression model. All statistical tests were 2-sided, and significance was defined as p<0.05. The statistic for each variable was obtained after adjustment for other 7 variables in the final regression model.

In conclusion, administration of vaccine and nonpharmaceutical interventions were beneficial for control of influenza A(H1N1)pdm09. Thus, it is essential to increase awareness regarding severity of influenza A(H1N1) pdm09 to improve knowledge of the protective effect of influenza vaccine and to promote use of nonpharmaceutical interventions among school-age children.

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Risk Perceptions for Avian **Influenza Virus** Infection among Poultry Workers, China

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To determine risk for avian influenza virus infection, we conducted serologic surveillance for H5 and H9 subtypes among poultry workers in Beijing, China, 2009–2010, and assessed workers' understanding of avian influenza. We found that poultry workers had considerable risk for infection with H9 subtypes. Increasing their knowledge could prevent future infections.

Avian influenza A viruses (AIVs), subtypes H5N1 and H9N2, are endemic to poultry in the People's Republic of China and have often infected humans. During early 2009, several cases of subtype H5N1 infection were found in China (*1*), and on January 6, a case was confirmed in a girl in Beijing. Clinical data showed that the girl had contact with slaughtered ducks, which were bought from a farm product market in Yanjiao, Langfang, Hebei Province, which neighbors Beijing. To assess the risk for AIV infection among poultry workers, we conducted serologic surveillance in Beijing from May 2009 to March 2010. Using a questionnaire, we also assessed the knowledge, attitudes, and practices (KAPs) of poultry workers regarding avian influenza infection. The Ethics Committee of Beijing Municipal Bureau of Agriculture approved this study, and all participants signed informed consent documents.

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The Study

A total of 305 serum specimens were collected from 305 workers who were in close contact with poultry populations during May 2009–March 2010. Influenza strains A/duck/Huabei/01/2007 (H5N1), belonging to clade 2.3.4, and A/chicken/Shangdong/ZB/2007 (H9N2) of the F/98 genotype were used for the microneutralization assay, which was performed as described (*2*,*3*). The F/98 genotype (H9N2) and clade 2.3.4 (H5N1) viruses had been demonstrated to be the predominant strains circulating in poultry in this region and were responsible for most cases of human infection during the period of the survey (*4*,*5*). Therefore, we only used the 2 viral strains in the MN assay.

Serum samples were considered positive if titers were ≥80, and all results were generated from at least 2 independent assays. Simultaneously, the 305 surveyed workers were administered questionnaires to ascertain avian influenza–related KAPs. Among the distributed questionnaires, responses from 207 were considered valid and were used for further analysis. Epi Info software, version 3.5.4 (Centers for Disease Control and Prevention, Atlanta, GA, USA), was used to analyze the survey data. The Pearson χ^2 test was used to compare differences between groups. Differences were considered significant if p value was <0.05.

Of the 305 poultry workers, 155 (50.8%) were duck keepers from 8 farms, 114 (37.4%) were chicken keepers from 5 farms, and 36 (11.8%) were chicken butchers who worked at an abattoir. The duck and chicken farms were located in different districts in Beijing. One hundred and fifty-five (50.8%) workers were male, and 150 (49.2%) were female; 147 (48.2%) participants were 36–45 years of age, 76 (24.9%) were 18–35 years, and 82 (26.9%) were >45 years. All participants had no history of vaccination for seasonal influenza in the past 3 years. MN assay revealed that no workers were positive for antibodies against influenza virus subtype H5, whereas 14 (4.6%) were positive for antibodies against subtype H9; titers ranged from 80 to 640 (Figure). Further analysis indicated that proportions of seropositive workers were 2.6% (4/155) for men and 6.7% (10/150) for women. By age, the proportions of seropositive poultry workers were 9.2% (7/76) for those 18–35 years, 2.7% (4/147) for those 36–45-years, and 3.7% (3/82) for those >45 years of age. These results suggest that subtype H9N2 virus infection was more prevalent among persons 18–35 years of age. The proportions of seropositive duck keepers, chicken keepers, and chicken butchers were 3.9% (6/155), 3.5% (4/114), and 11.1% (4/36), respectively. No significant differences were found in the infection rate among the 3 groups.

A total of 207 poultry workers completed a questionnaire regarding KAPs related to avian influenza.

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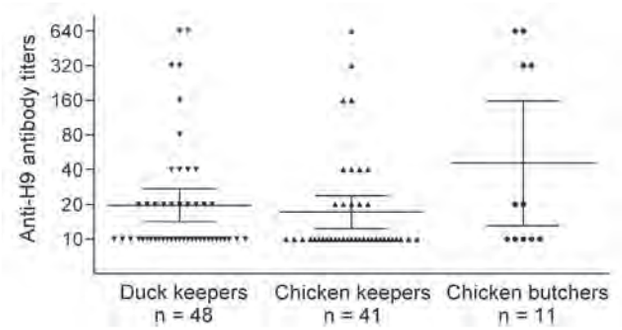

Figure. Avian influenza A (H9N2) virus microneutralization titers of workers with occupational exposure to poultry, Beijing, China, 2009–2010. A total of 305 serum specimens were tested by microneutralization assay, serum samples were considered positive with titers >80, and titers <10 were not included in this figure. Geometric mean titers and 95% CIs of subtype H9N2 microneutralization titers in various groups are indicated by long and short horizontal lines.

In terms of knowledge of avian influenza, 181 (87.4%) of workers recognized the transmission route through the respiratory tract, 113 (54.6%) recognized the transmission route through the gastrointestinal tract, and 117 (56.5%) recognized the transmission route through the mucosa. Nearly half of the participants ignored the latter 2 major transmission routes. In terms of knowledge of poultry housing practices, 135 (65.2%) and 160 (77.3%) of the workers had the correct understanding that chickens and ducks should not be raised with pigs in the same backyards and that poultry should not have contact with wild birds. Furthermore, 181 (87.4%) of the participants knew that eating and selling dead birds was against health regulations, and 167 (80.7%) knew that improving vaccination coverage and quality is an effective strategy for preventing AIV infection. Significant discrepancies were observed among groups with different educational levels in knowledge of avian influenza (except for those who understood that infection occurred through the respiratory tract and mucosa) ($p<0.05$) (Table 1). Workers with a high level of education (senior high school, university or college, and above) had more correct answers to the corresponding questions. Significant differences were also found between groups of different ages and occupations regarding knowledge of avoiding mixed housing practices ($p<0.05$). Most (79.0%–95.0%) young persons, 18–35 years of age, knew that poultry should not be kept in mixed housing with pigs nor kept in contact with other species of birds. Also concerning the above 2 risks, chicken keepers had more accurate knowledge than duck keepers (Table 1). No significant differences were found between men and women in terms of general knowledge ($p>0.05$).

Table 1. Knowledge of avian influenza among 207 poultry workers, Beijing, China, 2009–2010*

Risk variable†	OR‡	95% CI	p value
AIV infection through the respiratory tract			
Age, y (<36/36–45/>45)	–/1.97/1.11	–/0.66–6.11/0.27–4.46	–/0.18/0.87
Education (low/high)	–/0.57	–/0.21–1.53	–/0.23
Job (chicken keepers/duck keepers)	–/1.11	–/0.44–2.79	–/0.81
AIV infection through the gastrointestinal tract			
Age (<36/36–45/>45)	–/1.03/0.94	–/0.52–2.04/0.42–2.11	–/0.93/0.88
Education (low/high)	–/0.49	–/0.26–0.91	–/**0.02**
Job (chicken feeders/duck feeders)	–/1.52	–/0.83–2.78	–/0.14
AIV infection through mucosa			
Age (<36/36–45/>45)	–/0.85/0.64	–/0.43–1.68/0.28–1.44	–/ 0.61/0.24
Education (low/ high)	–/0.51	–/0.27–0.95	–/**0.02**
Job (chicken feeders/duck feeders)	–/1.58	–/0.86–2.89	–/0.11
Avoiding mixed housing with pigs			
Age, y (<36/36–45/>45)	–/2.54/2.69	–/1.17–5.6/1.11–6.6	–/**0.01**/**0.02**
Education (low/ high)	–/0.21	–/0.10–0.44	–/**<0.01**
Job (chicken feeders/duck feeders)	–/3.97	–/1.96–8.14	–/**<0.01**
Avoiding touching wild birds			
Age (<36/36–45/>45)	–/7.95/12.87	–/2.14–34.91/3.24–59.52	–/**<0.01**/**<0.01**
Education (low/high)	–/0.00	–/0.00–0.11	–/**<0.01**
Job (chicken feeders/duck feeders)	–/–	–/–	–/ **<0.01**
Forbidding eating and selling dead birds			
Age, y (<36/36–45/>45)	–/1.1/2.82	–/0.33–3.70/0.87–9.44	–/0.87/**0.05**
Education (low/high)	–/0.05	–/0.01–0.22	–/**<0.01**
Job (chicken keepers/duck keepers)	–/1.63	–/0.63–4.33	–/0.28
Improving vaccination coverage and quality			
Age, y (<36/36–45/>45)	–/0.79/0.93	–/0.33–1.90/0.34–2.52	–/0.57/0.87
Education (low/high)‡	–/0.14	–/0.04–0.44	–/**<0.01**
Job (chicken keepers/duck keepers)	–/1.03	–/ 0.48–2.21	–/0.93

*OR, odds ratio; AIV, avian influenza virus; –, OR of variable itself is not calculated; **boldface** indicates that p value is significant.
†Low education indicates junior high school, elementary school, and below; high education indicates senior high school, university or college, and above.
‡ORs are calculated as follows: for different age groups, we calculated 2 ORs—OR1 = odds (<36 y)/odds (36–45 y), OR2 = odds (<36 y)/odds (>45 y); for different education groups, OR = odds (low)/odds (high); for different job groups, OR = odds (chicken keepers)/odds (duck keepers).

Regarding attitudes toward avian influenza, 116 (56.0%) of 207 surveyed workers did not consider that AIVs pose a public health threat. They also rarely showed concern for the consequences resulting from avian influenza.

Analysis of practices concerning avian influenza prevention among 207 poultry workers is shown in Table 2. Although 184 (88.9%) respondents said they wore specific work clothing, wearing personal protective equipment was not a routine practice among poultry workers: only 112 (54.1%) wore gloves, and 95 (45.9%) wore masks. We also found that 165 (79.7%) participants routinely washed their hands after work and that 174 (84.1%) workers regularly used disinfectant. Significant differences were found between chicken keepers and duck keepers; the former were more likely to follow good hygiene practices than were the latter ($p<0.01$) (Table 2).

Conclusions

Transmission of AIVs from poultry to humans probably results from contact with infected poultry or contaminated materials (*6*–*9*). Workers in the poultry industry are at high risk for AIV infection. We found that 4.6% of poultry workers in Beijing had antibodies against influenza virus subtype H9. These findings indicate that viruses of subtype H9 may have previously infected a considerable number of persons in China, thus highlighting the potential public health risk for H9 AIV. None of the poultry workers in our study had positive test results for H5. Similarly, previous serologic surveillance studies in China showed that the prevalence of antibodies against H5 strains was significantly lower than that for antibodies against H9 (*10*,*11*).

In assessing the KAPs about avian influenza in poultry workers, we found that knowledge of avian influenza needs to be increased among poultry workers, especially among workers who are older, less educated, and duck keepers, and that the use of protective measures against AIVs should be enhanced among poultry workers, especially those on duck farms. Improving the KAPs of poultry workers could provide an effective means of preventing AIV infection in humans.

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Table 2. Practices of avian influenza among 207 poultry workers, Beijing, China 2009–2010*

Risk variable†	OR‡	95% CI	p value
Wearing work clothing			
Age, y (<36/36–45/>45)	–/3.08/11.24	–/0.58–21.83/2.22–76.60	–/0.19/**<0.01**
Education (low/high)	–/0.70	–/0.25–1.91	–/0.45
Job (chicken keepers/duck keepers)	–/0.2	–/0.07–0.58	–/**<0.01**
Wearing gloves			
Age, y (<36/36–45/>45)	–/0.51/0.62	–/0.26–1.02/0.27–1.38	–/**0.04**/0.20
Education (low/high)	–/0.67	–/0.36–1.23	–/0.17
Job (chicken keepers/duck keepers)	–/0.43	–/0.23–0.78	–/**<0.01**
Wearing mask			
Age, y (<36/36–45/>45)	–/0.66/0.7	–/0.33–1.33/0.31–1.58	–/0.21/0.35
Education (low/high)	–/0.71	–/0.39–1.29	–/0.23
Job (chicken keepers/duck keepers)	–/0.42	–/0.23–0.77	–/**<0.01**
Washing hands after finishing work			
Age, y (<36/36–45/>45)	–/0.91/2.58	–/0.35–2.34/0.99–6.76	–/0.83/0.31
Education (low/high)	–/0.69	–/0.31–1.50	–/0.31
Job (chicken keepers/duck keepers)	–/0.29	–/0.14–0.63	–/**<0.01**
Regular disinfection			
Age, y (<36/36–45/>45)	–/1.52/4.29	–/0.49–4.86/1.39–13.77	–/0.43/**<0.01**
Education (low/high)	–/0.48	–/0.19–1.19	–/0.08
Job (chicken keepers/duck keepers)	–/0.11	–/0.04–0.30	–/**<0.01**

*OR, odds ratio; –, indicates that OR of variable itself is not calculated; b**oldface** indicates that p value is significant.
†Low education indicates junior high school, elementary school and below; high education indicates senior high school, university or college and above.
‡ORs are calculated as follows: for different age groups, we calculated 2 ORs—OR1 = odds (<36 y)/odds (36–45 y), OR2 = odds (<36 y)/odds (>45 y); for different education groups, OR = odds (low)/odds (high); for different job groups, OR = odds (chicken keepers)/odds (duck keepers).

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High Seroprevalence for Typhus Group Rickettsiae, Southwestern **Tanzania**

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Rickettsioses caused by typhus group rickettsiae have been reported in various African regions. We conducted a cross-sectional survey of 1,227 participants from 9 different sites in the Mbeya region, Tanzania; overall seroprevalence of typhus group rickettsiae was 9.3%. Risk factors identified in multivariable analysis included low vegetation density and highway proximity.

Murine, or endemic, typhus is primarily caused by *Rickettsia typhi* (typhus group rickettsiae [TGR]) and is usually manifest as a benign disease. A systemic vasculitis causes a clinical triad of fever, headache, and maculopapular rash (*1*). Because these signs and symptoms are nonspecific, the disease is often misdiagnosed or overlooked and can frequently be misclassified as malaria (*2*,*3*). In rare cases, murine typhus can lead to severe systemic complications such as acute renal failure, interstitial pneumonia, and complications of the central nervous system. The case-fatality-rate is <5% (*2*), in contrast to the situation for epidemic, or louse-borne, typhus caused by *R. prowazekii*, which can produce severe disease and fatality rates up to 30% if untreated. Serologic tests cannot distinguish these 2 infections, however. We assume that the antibodies detected in Tanzania in this study were caused by *R. typhi*, because, to our knowledge, no severe or epidemic illness compatible with louse-borne typhus has been described in the study region.

Murine typhus is found throughout the world, widely distributed in subtropical and tropical regions, and is most apparent in port cities with large rat populations (*2*,*4*), which provide a reservoir for the pathogen and its main vector, the rat flea (*Xenopsylla cheopsis*). Additional transmission cycles have been described in Texas and California, USA, which involved mainly suburban cats and opossums as reservoir hosts and the cat flea (*Ctenocephalides felis*) as vector (*5*). Other yet unknown cycles may exist.

In Tanzania, information on typhus is sparse. A seroprevalence study among pregnant women from the port city of Dar es Salaam found a seropositivity prevalence of 28% (*4*). In the landlocked northern Tanzanian town of Moshi, murine typhus was detected in 0.5% of febrile patients (*6*).

A predictive risk model for endemic typhus based on environmental conditions has not been established, but because plague is also transmitted by *X. cheopsis* fleas, some of the findings regarding plague transmission might also apply to murine typhus. However, no data are available on the vector flea *C. felis*, the predominant flea harvested from rodents in a study in Uganda (*7*).

The Study

We conducted a cross-sectional seroprevalence study among 1,227 persons from the Mbeya region in southwestern Tanzania to estimate TGR seroprevalence rates and to assess associated sociodemographic and environmental risk factors. This study was conducted as a substudy within the EMINI (Evaluation and Monitoring the Impact of New Interventions) longitudinal cohort study. Briefly, in 2005 we conducted a census at 9 study sites (Figure) to collect baseline data, and 10% of census households were chosen by geographically stratified random selection to participate in the 5-year EMINI longitudinal cohort study (http://www.mmrp.org/projects/cohort-studies/emini.html). From these, serum specimens for this substudy were selected by stratified disproportionate random sampling of stored samples from 17,872 persons who took part in the second EMINI survey in 2007. Stratification was done for age (7 categories), altitude of residence (2 categories), and ownership of domestic mammals (2 categories) and resulted in 28 strata of roughly similar size, described in detail elsewhere (*8*). Serum samples were tested for IgG against *R. typhi* by indirect immunofluorescence assay (IIFA) with the same batch of a commercially available test (Rickettsia typhi Spot IF; Fuller Laboratories, Fullerton,

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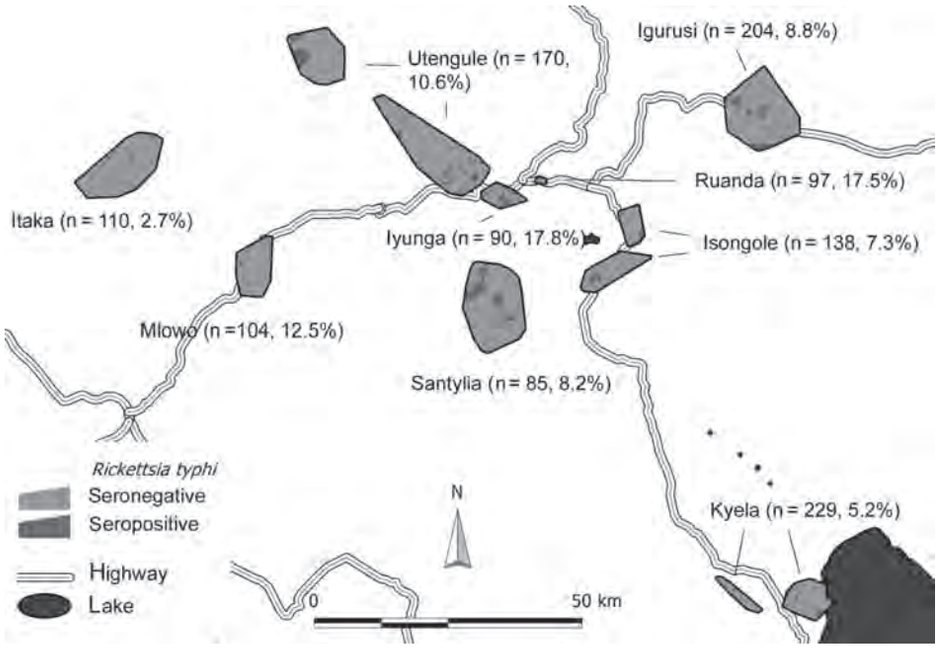

Figure. Study sites in Tanzania, showing positivity and negativity for IgG against *Rickettsia typhi* displayed in Voronoi polygons. Every polygon represents 1 household. Numbers in parentheses indicate site prevalence.

CA, USA.). Samples with an IgG titer of ≥64 or higher were regarded as positive; because IIFA for antibody testing against rickettsiae has a high sensitivity and specificity, as shown by different researchers and with different antigen preparations (*9*). Comparison of the commercial IIFA with a commercial ELISA in our laboratory confirmed this approach (G. Dobler, unpub. data).

To identify possible risk factors for TGR IgG positivity, we analyzed seropositivity as the binary outcome of uni- and multivariable Poisson regression models with robust variance estimates adjusted for household clustering. Initial univariable models for all factors that we deemed as possibly related to TGR infection (Table) were used to identify variables with a univariable p value ≤0.1 for further multivariable evaluation. Stepwise backward and forward regression, the Akaike and Bayesian information criteria, and various assessments of model fit were used to identify the best multivariable model, in which only variables with a multivariable p value <0.1 were retained.

Of the 1,227 analyzed serum specimens, 114 specimens (9.3%) were positive for TGR IgG. This finding translates into an estimated overall population prevalence of 8.4% (95% CI 6.8%–10.1%) when findings are extrapolated from our stratified sample to the underlying population of the 9 sites by using direct standardization. We found local maximum prevalence in the urban sites, Ruanda (17.5%) and Iyunga (17.8%), and in semiurban Mlowo (12.5%; Figure). The prevalence at other sites ranged from 2.7% to 10.6%. The highest seropositivity rate was found in the age quintile from 42.1 to 55.2 years, with a decline thereafter. In univariable analysis, several environmental covariates showed a significant inverse association with TGR IgG (Table), which included vegetation density, rainfall, minimum and night temperatures, whereas population density, cattle density, and socioeconomic status were positively associated with seropositivity. The geographic distribution of seropositive participants (Figure) led us to include distance to the nearest highway as a variable in the analysis. Distance was found to be inversely associated with seropositivity. The final multivariable model included age, vegetation density, and distance to the nearest highway as significant predictors of TGR IgG. Other factors were not included in the multivariable model because their lack of multivariable significance.

Although significant in univariable analysis, the association of population density, rainfall, socioeconomic status, and cattle density became nonsignificant in the multivariable model when vegetation density was included (p = 0.66 for population density; data not shown). Other factors, including sex, livestock ownership, day and night average land surface temperatures, and other environmental factors, were unrelated to TGR seropositivity.

Conclusions

In contrast to results of a recent study of febrile patients from inland northern Tanzania (*6*), site-specific seropositivity prevalences of up to 17.8% in our study suggest that TGR contributes substantially to febrile illness in some areas of the Mbeya region. Our study provides data on environmental risk factors for TGR seropositivity, which might be useful

Table. Covariates associated with seropositivity for typhus group rickettsiae, Mbeya region, southwestern Tanzania, 2007*

Covariate/stratum	No. specimens (% positive)	Univariable†‡ PR (95% CI)	Univariable†‡ p value	Multivariable†§ PR (95% CI)	Multivariable†§ p value
Age, y					
5–<13.6	245 (5.3)	1	–	1 (–)	–
13.6–<27.8	245 (6.5)	1.23 (0.60–2.51)	0.568	1.28 (0.63–2.58)	0.495
27.8–<42.1	243 (12.3)	2.33 (1.23–4.39)	0.009	2.40 (1.28–4.49)	0.006
42.1–55.2	248 (14.9)	2.81 (1.53–5.18)	0.001	2.73 (1.49–4.99)	0.001
>55.2	246 (7.3)	1.38 (0.69–2.76)	0.365	1.41 (0.71–2.80)	0.331
Distance to nearest highway, km	1,227 (9.3)	0.96 (0.94–0.99)	0.012	0.97 (0–0.99)	0.011
Enhanced vegetation index, per 0.1 units	1,227 (9.3)	0.58 (0.44–0.76)	<0.001	0.60 (0.46–0.79)	<0.001
Persons/km^2, per 1,000 persons	1,227 (9.3)	1.08 (1.04–1.12)	<0.001		
Sex					
F	672 (8.6)	1	–		
M	544 (9.9)	1.15 (0.81–1.63)	0.430		
Unknown	11 (18.2)	2.11 (0.59–7.58)	0.254		
SES rank, per unit¶	1,227 (9.3)	1.08 (1.02–1.15)	0.008		
Cattle/km^2, per 100 cattle	1,227 (9.3)	1.28 (1.05–1.56)	0.017		
No. cows owned	1,227 (9.3)	0.96 (0.84–1.09)	0.526		
No. goats owned	1,227 (9.3)	0.94 (0.83–1.07)	0.367		
Dogs owned					
No	820 (8.8)	1	–		
Yes	191 (8.4)	0.95 (0.54–1.67)	0.869		
No information	216 (12.0)	1.37 (0.89–2.11)	0.150		
Minimum ambient temperature, °C	1,227 (9.3)	0.92 (0.87–0.97)	0.004		
Average day land surface temperature, °C	1,227 (9.3)	1.37 (0.71–2.63)	0.351		
Average night land surface temperature, °C	1,227 (9.3)	0.61 (0.38–0.99)	0.044		
Elevation, per 100 m	1,227 (9.3)	1.04 (1.00–1.08)	0.025		
Rainfall, per 1,000 mm	1,227 (9.3)	0.53 (0.30–0.92)	0.025		

*PR, prevalence ratio; SES, socioeconomic status. Blank cells indicate that the variables were not included in the multivariable analyses due to lack of multivariable significance.
†Results of univariable and multivariable Poisson regression adjusted for household clustering by using robust variance estimates.
‡Results of separate models for each of the covariates below.
§Multivariable model, including only age, distance to nearest highway, and enhanced vegetation index as covariates.
¶SES rank, rank (from 0 [lowest] to 10 [highest]), according to socioeconomic score.

to inform a predictive model for disease occurrence. The inverse association of vegetation density with seropositivity has been described for plague in Uganda, a disease that is also transmitted by the rat flea (*X. cheopsis*) (*10*). That study and early laboratory data suggest that dryness is not the driving factor behind the link between vegetation and disease transmission, because increasingly dry conditions in the laboratory adversely affect vector lifespan (*11*). In our study, rainfall was not significantly associated with seropositivity in the multivariable model. The urbanity of a settlement, expressed by population density and closeness to highways, may still be a relevant factor through providing more favorable habitats for the mammal reservoir hosts, and sparse vegetation could just be a proxy for urbanization. The positive univariable association of seropositivity with socioeconomic status appears to be a product of the higher socioeconomic status in urbanized communities. Our results suggest that TGR incidence may increase with deforestation and increasing urbanization. Additional research is needed to detect the pathogen in acute infection and to describe the local transmission cycle to validate the identified risk factors prospectively. We further hypothesize that remote sensing data could be used to design a model for prediction of *R. typhi* infection, which could be used to direct public health interventions in the future.

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Acute Schmallenberg Virus Infections, France, 2012

To the Editor: After unexpected emergence of bluetongue virus serotype 8 in northern Europe in 2006 (*1*), another arbovirus, Schmallenberg virus (SBV), which is transmitted by *Culicoides* spp. biting midges, emerged in Europe in 2011 and caused disease outbreaks among ruminants (*2*). Nonspecific clinical signs such as fever, decreased milk production, and diarrhea were associated with acute infection in cattle, and late abortions and birth defects in newborns were associated with infection of pregnant cows, ewes, and goats (*2*,*3*).

SBV, which belongs to the family *Bunyaviridae* and genus *Orthobunyavirus*, was detected in Germany, the Netherlands, and Belgium in 2011. This virus was later detected in the United Kingdom, France, Italy, Luxembourg, Spain, Denmark, and Switzerland (*4*). As of August 1, 2012, a total of 5,701 infected farms were reported in Europe (2,498 sheep farms, 3,124 cattle farms, and 79 goats farms) (www.survepi.org/cerepi/). France has been the country most affected: it had 2,650 SBV-infected farms (*5*), (i.e., in which ≥1 malformed offspring was positive for SBV by real-time reverse transcription PCR [RT-PCR] on 1,128 sheep farms, 1,505 cattle farms, and 17 goat farms).

Abnormalities detected in offspring in 2011 and in early 2012 were caused by infections acquired in 2011 (*4*). At that time, it was unclear whether SBV could survive the 2011–2012 winter and remain a threat to Europe. We report data suggesting that SBV overwintered or was reintroduced in France.

On May 16, 2012, a herd of 75 dairy cows in southwestern France (Pyrénées-Atlantiques) had hyperthermia and decreased milk production. Of 18 cows tested by the Agence Nationale de Sécurité Sanitaire (Maisons-Alfort, France), 9 were positive for SBV by PCR (*6*) (cycle threshold [C_t] range 17–36.5) and negative for SBV by ELISA (IDVet, Montpellier, France), 1 was positive by PCR and ELISA, and 8 were negative by PCR and ELISA (Figure).

On May 23, a week after the first samples were collected, all cows tested were positive by ELISA and only 1/18 cows were positive by RT-PCR. On June 29, all 18 cows tested were negative by RT-PCR. Detection of SBV-neutralizing antibodies by virus neutralization assay in serum samples obtained on May 23 confirmed SBV ELISA results and showed that a commercial IgG ELISA is suitable for detection of acute cases of SBV.

Viremia, as measured by RT-PCR, occurs during the first 5 days after acute infection (*2*). Antibody response against SBV, as measured by ELISA, is detected during or after the first 10 days after experimental infection (C. Sailleau et al., unpub. data). Accordingly, serologic and molecular data showed that acute SBV infection occurred in cattle in southwestern France in May 2012, suggesting that SBV overwintered or was reintroduced. Moreover, in July 2012, another case of acute SBV infection was identified in Finistère (Brittany). A cow with hyperthermia and diarrhea was SBV positive by RT-PCR (C_t 31) and negative by ELISA, which indicated a recent SBV infection.

Three blood samples (each 170 μL) from RT-PCR–positive cows (C_t range 18–13) (1 from the Finistère and 2 from Pyrénées-Atlantiques) were injected into 3 adult IFNAR$^{-/-}$ mice. Seventy microliters of each sample was injected intraperitoneally and 100 μL was injected into the neck scruff of the same mice. After 4 days, the mice showed no clinical signs and their weights were unchanged. Blood samples were collected and tested for SBV by RT-PCR. All mice were positive for SBV RNA (C_t range 18–31). These data showed that blood samples from cows contained virus RNA and confirmed that SBV reemerged in 2012.

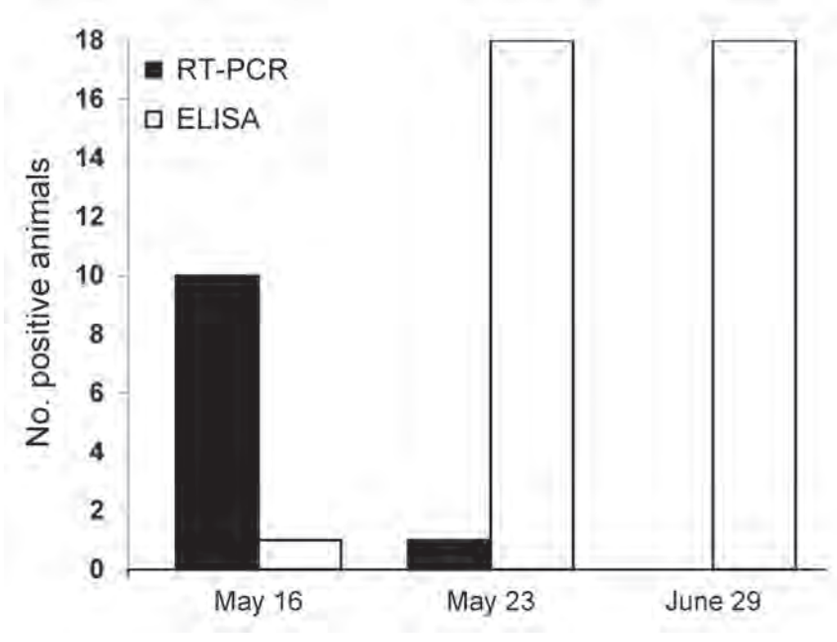

Figure. Number of cows positive for Schmallenberg virus according to reverse transcription PCR (RT-PCR) and ELISA, France, 2012.

On July 25, 2012, SBV infection was identified in a cow in Jura Canton in the northwestern, French-speaking region of Switzerland (Romandie) (*7*). A serologic study conducted in the United Kingdom showed that several cattle and sheep seroconverted for SBV in 2012 (*8*). However, our data show that SBV survived the winter, when midge numbers decreased. The precise mechanisms of SBV overwintering are not known and need to be explored.

The consequences of SBV recirculation should be investigated, particularly in pregnant cows, ewes, and goats. The 2 SBV-positive farms described in this report are located in a previously SBV-free area (Finistère-Brittany) or an area in which the infection rate was low (Pyrénées-Atlantiques) in the winter of 2011–2012, during which seroprevalence for most herds was probably weak (C. Sailleau et al., unpub. data). Therefore, reemergence of cases of congenital forms of SBV infection in France and others areas of Europe can be expected.

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Toscana Virus Isolated from **Sandflies, Tunisia**

To the Editor: Toscana virus (TOSV; genus *Phlebovirus*, family *Bunyaviridae*) is transmitted by sandflies, mostly the species *Phlebotomus perniciosus* and *P. perfiliewi* (*1*). Initially discovered in central Italy, TOSV was recently identified in other European countries (i.e., Portugal, Spain, France, Croatia, and Turkey) (*2*). TOSV is a primary cause of aseptic meningitis during warm months (*2*). A seroprevalence study suggested that TOSV is present in Tunisia and may cause neuroinvasive infections, but definitive evidence of TOSV circulation has not been possible because it is difficult to distinguish from the antigenically related phlebovirus Punique virus (*3*,*4*); both viruses are members of the species *Sandfly fever Naples virus*. We investigated the prevalence of TOSV among sandflies in northern Tunisia.

A total of 5,288 sandflies (3,547 females, 1,740 males) were collected during June–October 2010 by using CDC light traps (John W. Hock Company, Gainesville, FL, USA) at Utique (37°08′N, 7°74′E), a focus for visceral leishmaniasis in northern Tunisia. Sandflies were separated by sex and trapping nights and pooled with ≥30 specimens by pool. Pools were processed as described (*4*) and subjected to PCR detection of phlebovirus RNA targeting 2 genes independently (*4*–*6*) and virus isolation onto Vero cells. Of 249 pools processed, 8 strains of phleboviruses were isolated: 2 TOSV, 3 Punique virus, and 3 other phleboviruses currently being characterized.

TOSV strains were obtained from 2 pools of sandflies trapped in September 2010, T152 and T166, consisting of 30 males and 30 females, respectively. These pools were positive for TOSV RNA by sequencing of

2 PCR products (201-nt and 280-nt sections in the large [L] and small [S] gene segments, respectively). Supernatant of the third passage was prepared for electron microscopy, which showed spherical and pleomorphic structures, 80–120 nm in diameter, compatible with viruses of the family *Bunyaviridae*. Complete genome sequencing was then done by using the Ion PGM Sequencer (Life Technologies SAS, Saint Aubin, France) (*7*); a total of 165,307 reads were obtained, of which 135,700 matched with the sequence of TOSV Iss.PhL3 used as reference. The viral genome of TOSV Tunisia-2010-T152 (GenBank accession nos. JX867534–JX867536) was composed of 12,488 nt; the complete sequence consisted of 1,869 nt, 4,215 nt, and 6,404 nt for the S, medium [M], and L RNA segments, respectively. The partial S sequence of the TOSV Tunisia-2010-T166 strain (GenBank accession nos. JX867537–JX867539) was identical to that of T152, but 1 synonymous mutation was observed in the partial L sequence and 1 nonsynonymous mutation in the partial M sequence (I906V).

The TOSV Tunisia-2010-T152 strain was aligned with homologous sequences retrieved from the GenBank database. Genetic distances were calculated at the amino acid and nucleotide levels by using the p-distance algorithm (online Technical Appendix Tables 1–3, wwwnc.cdc.gov/EID/article/12-1463-Techapp1/.pdf). Phylogenetic studies were performed by using the neighbor-joining method in MEGA5 (*8*) (Figure). The robustness of the nodes was tested by 1,000 bootstrap replications. We found that TOSV Tunisia-2010-T152 was most closely related to the prototype strain from Italy, Iss.PhL3, with nucleotide/amino acid distances of 0.031/0.052, 0.032/0.073, and 0.039/0.012 for the S, M, and L RNA sequences, respectively. Together, these genetic distances and phylogram topologies indicate that TOSV Tunisia-2010-T152 is most closely related to strains within the Italian lineage, although it may represent a distinct sublineage, more distantly related to strains belonging to the Spanish lineage (*9*).

Concomitantly with virus isolation, the phenology of sandfly species was studied during May–November 2010. Sandflies were identified, and the density was calculated as described (*10*). Most of the sandflies belonged to the subgenus *Larroussius* (98.3%). *P. perniciosus* sandflies were the most abundant species (71.74%), followed by *P. longicuspis* (17.47%) and *P. perfiliewi* (8.82%). Other sandfly species, such as *Phlebotomus* (*Phlebotomus*) *papatasi*, *Phlebotomus* (*Paraphlebotomus*) *sergenti*, *Sergentomyia minuta parotti*, *S. christophersi*, and *S. antennata* were found, but these were much less abundant. The phenology of 3 main sandfly species showed 2 main peaks: 1 small peak in June and a second, larger peak during September–October (online Technical Appendix Figure).

In summary, of a total of 5,288 sandflies collected, 2 pools were positive for TOSV, yielding an infection rate of 0.03%. A similar infection rate was observed in Spain (0.05%) (*9*); however, the infection rates in Italy (0.22%) and in France (0.29%) are substantially higher (*1*,*6*). The isolation of TOSV from male and female sandflies suggests transovarial transmission in nature, as reported

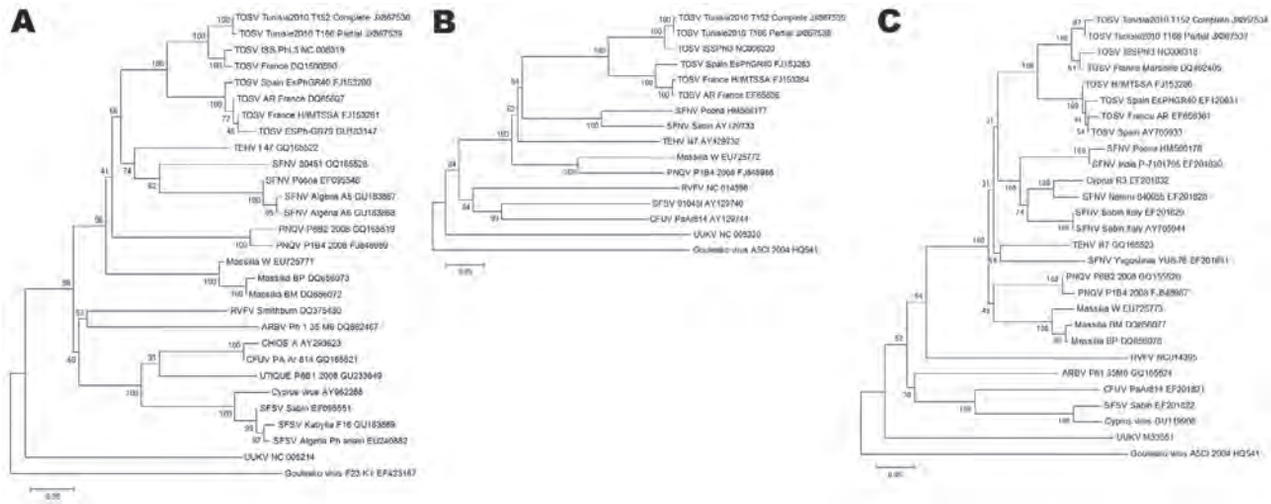

Figure. Phylogenetic analysis of 3 segments of Toscana virus (TOSV) isolates from pools of sandflies collected in Tunisia and homologous sequences of other selected phleboviruses. A) Large segments; B) medium segments; C) small segments. Sequences are identified by virus name or acronym, strain name, and GenBank accession number. Scale bars indicate nucleotide substitutions per site. TEHV, Tehran virus; SFNV, sandfly fever Naples virus; PNQV, Punique virus; RVFV, Rift Valley fever virus; ARBV, Arbia virus; CHIOS, phlebovirus Chios-A; CFUV, Corfou virus; SFSV, sandfly fever Sicilian virus; UUKV, Uukuniemi virus.

in Italy and Spain (*1*,*9*). In southern European countries, TOSV is mostly transmitted by *P. perniciosus* and *P. perfiliewi* sandflies (*1*,*6*,*9*), whereas *P. perniciosus*, *P. longicuspis*, and *P. perfiliewi* are the most abundant sandfly species in northern Tunisia. It is therefore probable that TOSV is transmitted by sandfly species of the subgenus *Larroussius*.

We found that 2 phleboviruses belonging to the *Sandfly fever Naples virus* species, TOSV and Punique virus, are cocirculating in northern Tunisia. This finding calls for further investigation of these viruses' potential effect on human health in this area.

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Seroprevalence of Dengue in American Samoa, 2010

To the Editor: Since the 1970s, regular dengue epidemics have caused considerable illness in the Pacific region (*1*). In 2009, an epidemic year, the incidence of reported clinical dengue cases in American Samoa reached 644 cases/100,000 population; in 2010, incidence decreased to 77 cases/100,000 population (*2*). Dengue surveillance in American Samoa is being developed, but the effects of this disease are unknown.

In 2010, blood samples were collected in American Samoa primarily for a leptospirosis seroprevalence study. Samples were also tested for IgG antibodies against dengue virus, and a seroprevalence of 95.6% was observed. We report this finding and advocate improved surveillance and integrated control programs to limit dengue transmission in American Samoa.

A cross-sectional seroprevalence study was conducted during May–July 2010 with the primary aims of identifying risk factors for human leptospirosis and providing an evidence base to direct public health interventions in American Samoa (*3*,*4*). During the study, investigators encountered community concern about dengue and were asked by health authorities to use the remaining collected serum for a dengue seroprevalence study. Amendments to the original human research ethics applications submitted to the American Samoa Institutional Review Board and the University of Queensland

Medical Research Ethics Committee (2010000114) were approved.

From the general population of the islands of Tutuila, Aunu'u, and Manu'a, 807 adults were recruited. Households were selected from Tutuila and Aunu'u Islands by using a spatial sampling design to facilitate geospatial analysis (*4*). One adult from each household was asked to volunteer for the study. The small size of villages on the Manu'a Islands meant that spatial sampling was not possible; thus, a convenience sample of volunteers was recruited. A 5-mL blood sample was collected from each participant, information on demographics and risk exposures was obtained by using a standardized questionnaire, and each participant's primary place of residence was georeferenced.

In October 2011, serum samples from 794 participants 18–87 years of age (median age 39.5 years) were tested at the Australian Army Malaria Institute (Brisbane, Queensland, Australia) for IgG antibodies against dengue virus. Thirteen participants were excluded from the original sample of 807 because of insufficient serum. Samples were screened by using the PanBio Dengue IgG Indirect ELISA Kits (Inverness Medical Innovations, Brisbane, Queensland, Australia) following the manufacturer's recommendations and protocols. PanBio Dengue IgG Indirect ELISA Kits can detect antibodies to all 4 dengue virus serotypes with a sensitivity of 99.2% and a specificity of 96.2% (*5*). However, these kits cannot identify the specific dengue serotypes responsible for infections. Results were calculated as counts and proportions of PanBio Units (PBU) and allocated a dengue IgG status accordingly: <9.0 PBU was a negative result, 9.0–11.0 PBU was an equivocal result, and >11.0 PBU was a positive result.

Serum samples from 759 (95.6%, 95% CI 93.9%–96.8%) of 794 study participants had IgG antibodies against dengue virus (Table). Seroprevalence for men and women was comparable and did not differ from overall results. As expected, the seropositivity rate was lower among persons 18–25 years of age (89.1%, 95% CI 84.0%–92.6%) because of less time exposed to dengue viruses, and the seropositivity rate was higher among persons 26–40 years of age (99.5%, 95% CI 97.5%–99.9%) than for the overall study population. Despite this study being limited by convenience sampling on the Manu'a Islands, it demonstrates almost universal exposure of sampled adults in American Samoa to dengue viruses.

Table. Prevalence of IgG against dengue virus among 794 adults, American Samoa, 2010*

Characteristic	No. positive/no. tested	% Positive (95% CI)
Dengue IgG status		
Negative, PBU <9.0	29/794	3.6 (2.6–5.2)
Equivocal, PBU 9.0–11.0	6/794	0.8 (0.35–1.6)
Positive, PBU >11.0	759/794	95.6 (93.9–96.8)
Sex		
M	402/418	96.2 (93.9–97.6)
F	357/376	94.9 (92.2–96.7)
Age, y		
18–25	179/201	89.1 (84.0–92.6)
26–40	216/217	99.5 (97.5–99.9)
41–53	182/187	97.3 (93.9–98.8)
54–87	182/189	96.3 (92.6–98.2)
Total	NA	NA

*PBU, PanBio units; NA, not applicable.

In the absence of a vaccine, timely and accurate dengue surveillance and consequent public health response is imperative. Current dengue surveillance in American Samoa is passive and relies on clinicians reporting suspected cases to public health authorities. Passive surveillance systems are typically insensitive, and barriers to treatment seeking by residents (distance to health care facility, financial costs, and encouragement from health authorities to stay at home unless symptoms are severe) further reduces their efficiency (*6*). Moreover, passive surveillance systems do not capture asymptomatic infections, which contribute to disease transmission in the community during the viremic stage of illness. Development of an active surveillance system incorporating geographic information systems would enable health authorities to better monitor distribution and intensity of acute infections, identify high-risk areas, and target dengue control activities (*7*).

These preliminary findings should be evaluated by additional study. Further research into dengue seroprevalence in American Samoa should involve identifying dominant and circulating virus serotypes, studying vector population dynamics, investigating dengue exposure among children, exploring environmental risk factors, and integrating these data into active geographically enhanced surveillance systems. In addition, we suggest implementing a sustainable vector control program similar to those undertaken in Vietnam to limit dengue transmission and reduce associated illness in American Samoa (*8*).

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Delayed Diagnosis of Dirofilariasis and Complex Ocular Surgery, Russia

To the Editor: *Dirofilaria repens* is a vector-borne, zoonotic, filarial nematode that infects dogs, cats, and humans. In humans, *D. repens* worms cause subcutaneous dirofilariasis, characterized by the development of benign subcutaneous nodules that mimic skin carcinomas (*1*), and ocular dirofilariasis in orbital, eyelid, conjunctival, retroocular, and intraocular locations (*2*). Intraocular and retroocular dirofilariasis causes considerable damage and discomfort in patients from the presence of the worms and from their surgical removal (*3*). Here, we report a retroocular *D. repens* nematode infection in a patient in Russia that illustrates the difficulties in clinical management and the inherent risks of surgical procedures to remove the worms.

A 20-year-old woman living in Rostov-na-Donu in southwestern Russia who had never traveled outside the city sought ophthalmologic consultation for pain and skin redness and swelling in the inner corner of the upper left eyelid. Swelling migrated successively to the temporal area, the lower eyelid, and the inner corner of the lower eyelid. The patient had no other ocular signs or symptoms, and her general condition was otherwise good. Results of ophthalmologic examination and routine laboratory tests were within normal limits. Four days of treatment with cefotaxime resulted in the remission of signs and symptoms. Approximately 2 months later, swelling in the inner corner of the upper eyelid appeared again, affecting the whole upper eyelid, without itching or tenderness. Allergies were diagnosed; cetirizine was administered for 4 days, and the signs remitted at the third day of treatment. One month later, marked upper left eyelid swelling occurred, resulting in ptosis. Cetirizine was prescribed again; edema subsided after 4 days of treatment but relapsed in the following 3–4 days.

At least 4 subsequent relapses occurred; thus, a computed tomographic scan of the paranasal sinuses and orbits was performed, 4 months after signs and symptoms began (Figure, panel A). The scan detected a soft tissue structure, 12 × 13 × 14 mm, behind the left eyeball, adjacent to and medially dislodging the optic nerve. No other abnormalities were found in the visible area of the brain and sinuses. Magnetic resonance imaging (MRI) performed 1 month later (Figure, panel B) corroborated the presence of a cyst-like structure with an irregular, rounded shape and clear, smooth borders closely adhered to the eyeball and optic nerve. T2-weighted images showed that the lesion had a high-density core but the surrounding tissue was low density. Adjacent to the lesion, the retrobulbar tissue was slightly swollen, the optic nerve was displaced medially and downward, and the adjacent upper muscle was displaced medially and upward. The diagnosis was evidence of a retroocular cystic lesion in the left orbit with a well-defined capsule and high-density but heterogeneous core structure.

High-resolution ultrasound examination (Figure, panel C) revealed a well-defined, 3-mm, cyst-like wall containing fluid and dense, coiled-twisted linear internal structures that appeared to be actively moving (Video 1, Appendix, wwwnc.cdc.gov/EID/article/19/2/12-1388-V1.htm). Color Doppler examination (Figure, panel D; Video 2, Appendix, wwwnc.cdc.gov/EID/article/19/2/12-1388-V2.htm) revealed blood vessels in the wall but not inside the cystic structure. These additional examinations led to a diagnosis of a retroocular parasitic cyst in the left orbit, most likely a *Dirofilaria* spp. parasite. The parasitic cystic nodule was removed during a transpalpebral orbitotomy. A live, adult roundworm, 87 × 0.6 mm, was

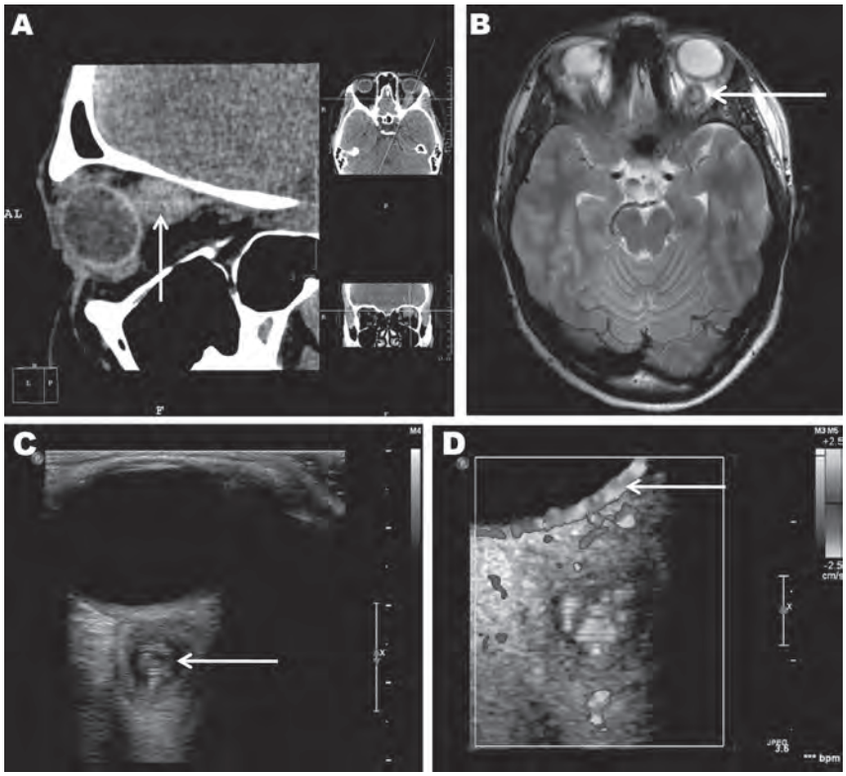

Figure. Retroocular nodule of a *Dirofilaria repens* worm detected in a 20-year-old woman, Rostov-na-Donu, Russia. The cyst (arrows) is shown by computed tomography scan (A) and magnetic resonance imaging (B). Ultrasonography image (C) shows a worm-like structure inside the cyst (arrow), and Doppler imaging (D) shows marginal vascularization of the lesion (arrow). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/2/12-1388-F1.htm).

discharged from the cyst. Conventional PCR identified the roundworm as *D. repens* (data not shown).

Although human subcutaneous *Dirofilaria* spp. nodules are benign, their detection may raise suspicion for a malignant tumor; thus, differential diagnosis is the key point in the management of human dirofilariasis (*4*). The case we described illustrates the difficulties of diagnosis when worms are in deep locations and patients experience unspecific and even unusual signs. These confounders resulted in a lengthy diagnostic procedure, with consequent detrimental physical and psychological effects on the patient. Even though the final diagnosis determined that the nodule was nonmalignant, its anatomic location required aggressive surgical intervention to remove it.

D. repens nematodes are spreading in Europe from the south toward the north and east (*5*–*7*) as a consequence of global warming, and prediction models have suggested incidence is increasing among animal and human hosts (*3*). Consequently, human ocular dirofilariasis will probably be found with increasing frequency in the future. Our experience illustrates that dirofilariasis should be included in the differential diagnosis of any nodule, independent of its anatomic location and the signs and symptoms shown by the patient. Moreover, ultrasonography represents a noninvasive technique that enables rapid preoperative identification of the parasitic origin of the nodules, thus avoiding unnecessary diagnostic delays. This technique is used for the diagnosis of cardiopulmonary dirofilariasis in animals (*8*) but has been used only sporadically for human dirofilariasis (*9*,*10*), which is habitually diagnosed postoperatively, after the surgical removal of the nodules or worms (*1*).

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Lymphocytic Choriomeningitis Virus Infections among American Indians

To the Editor: Lymphocytic choriomeningitis virus (LCMV) is a rodent-borne pathogen that causes a spectrum of disease in humans, ranging from self-limiting meningoencephalitis to congenital birth defects to severe disseminated illness in organ transplant recipients (*1*). It is not known how frequently cases of LCMV infection are diagnosed in the United States. We performed a retrospective case analysis of American Indian and Alaska Native (AI/AN) patients treated within the Indian Health Service (IHS) health care system who had an LCMV-associated diagnosis defined by the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM). Our goal was to estimate the incidence of LCMV-associated aseptic meningitis and encephalitis diagnosed within a well-defined population.

Inpatient and outpatient visit data from fiscal years 2001–09 were obtained from the IHS National Patient Information Reporting System (*2*). For each fiscal year, records for AI/AN patients with at least 1 inpatient or outpatient visit within the year that listed the ICD-9-CM code 049.0 (lymphocytic choriomeningitis and meningoencephalitis) were selected (*3*,*4*). A subset of medical records was reviewed for patients with the diagnosis code of interest in the IHS Southwest and Southern Plains regions. Health care facilities were located in Arizona, New Mexico, and Oklahoma, USA. Suspected LCMV infection was defined as a diagnosis of meningitis, choriomeningitis, encephalitis, or meningoencephalitis not explained by another etiologic agent. A confirmed case of LCMV infection required laboratory detection of antibodies, virus antigen, or virus. A patient with no evidence of suspected LCMV infection in his or her medical record was not considered to have a case of LCMV infection.

Annual population denominators were determined by using annual IHS Southwest and Southern Plains region user populations, which includes all registered AI/ANs who received IHS-funded health care at least once during the previous 3 years (*5*). The annual average incidence rate for diagnosed cases of infection with LCMV was determined. Rates were also determined for viral meningitis not otherwise specified (ICD-9-CM code 047.9); for unspecified causes of encephalitis, myelitis, or encephalomyelitis (323.9); and for unspecified non-arthropod–borne viral diseases of the central nervous system or viral encephalitis not otherwise specified (049.9) (*3*). Annual numbers were calculated on a patient basis, whereby the first time a diagnosis was coded for a given patient during each fiscal year was counted.

Twenty-six AI/AN patients received the diagnosis code of 049.0 during fiscal years 2001–09. Of these patients, 16 received the diagnosis in the Southwest or Southern Plains regions. Fourteen available medical charts from these 2 regions were reviewed, and 4 patients were classified as having signs and symptoms consistent with suspected LCMV infection (Table), although no patients were confirmed by diagnostic testing as having LCMV infection. All 4 suspected cases of LCMV infection were in women (age range 16–43 years) who had a 1–2-day history of headache, nausea, and vomiting. Photophobia and neck or back pain were present in patients 1–3. Cerebrospinal fluid from patients 2–4 had increased leukocyte counts that were lymphocytic.

The diagnoses of the 10 remaining patients were classified as miscodes because the written diagnoses in the charts did not mention lymphocytic meningitis. Two patients had central nervous system disease (Lyme encephalitis and tuberculosis meningitis) although an etiologic agent was confirmed that was not LCMV. Additional diagnoses mistakenly coded are shown in the Table.

Among the 4 patients identified as having clinical signs and symptoms of suspected LCMV infection in the Southwest and Southern Plains regions during fiscal years 2001–09, the average annual incidence rate was estimated to be 0.06 cases/100,000 persons. In the same population, viral meningitis not otherwise specified was reported for 971 patients (incidence rate 13.69 cases/100,000 persons/year). Unspecified causes of encephalitis, myelitis, or encephalomyelitis were diagnosed for 444 patients (incidence rate 6.26 cases/100,000 persons), and unspecified non-arthropod–borne viral diseases of the central nervous system or viral encephalitis not otherwise specified were diagnosed for 56 patients (incidence rate 0.78 cases/100,000 persons).

Using a population-based analysis of diagnoses for patients who visited

Table. Clinical features and diagnoses for 14 American Indian patients with an ICD-9-CM diagnosis code of 049.0 (lymphocytic choriomeningitis), Southwest and Southern Plains IHS regions, United States, fiscal years, 2001–09*

Patient no.	CNS signs and symptoms	Lymphocytes in CSF	Diagnosis	Status
1	Yes	Yes	Viral meningitis	Suspected LCM
2	Yes	Yes	Lymphocytic meningitis	Suspected LCM
3	Yes	Yes	Lymphocytic meningitis	Suspected LCM
4	Yes	Yes	Lymphocytic meningitis	Suspected LCM
5	Yes	Yes	Tuberculosis meningitis	Miscoded
6	Yes	No	Lyme encephalitis	Miscoded
7	No	No	Lymphocele	Miscoded
8	No	No	Lymphocytic cholitis	Miscoded
9	No	No	Lymphocytic leukemia	Miscoded
10	No	No	Lymphocytic leukemia	Miscoded
11	No	No	Atypical lymphocytes	Miscoded
12	No	No	Reactive lymphocytes	Miscoded
13	No	No	Charcot foot anomaly	Miscoded
14	No	No	Tuberculosis	Miscoded

*Diagnosis was the first listed or most clinically relevant written diagnosis from the medical record. Patient status (confirmed or suspected lymphocytic choriomeningitis [LCM] or miscode) was determined by the chart reviewer on the basis of the criteria. ICD-9-CM, International Classification of Diseases, 9th Revision, Clinical Modification; IHS, Indian Health Service; CNS, central nervous system; CSF, cerebrospinal fluid.

the IHS system at least 1 time as an inpatient or outpatient, we found that LCMV-associated aseptic meningitis and encephalitis were infrequently diagnosed and that confirmatory testing was not conducted. We also found that the code for LCMV was used incorrectly for several patients, probably because the virus is named ambiguously, and LCMV could be mistaken for other unrelated diseases with a lympho prefix. In addition, the definition for the code does not specify LCMV infection. We recommend that description of the ICD-9-CM and ICD-10 codes should be adjusted accordingly to clarify and reduce coding errors.

LCMV infection is considered to be underdiagnosed (*6*,*7*), although the virus was not detected in several studies that prospectively tested for LCMV in large numbers (>90) of clinical encephalitis or meningitis patients (*8*–*10*). Despite infrequently causing aseptic meningitis or mild disease in immunocompetent persons, LCMV has the potential to cause severe disease and should be considered for patients with a compatible illness and potential rodent exposure.

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Streptococcus suis Meningitis in Swine Worker, Minnesota, USA

To the Editor: *Streptococcus suis* is a major bacterial pathogen in swine worldwide. Historically, cases in humans have occurred sporadically, mostly in Asia (*1*,*2*). However, an outbreak in China involved 215 human cases and 39 deaths (*3*). Only 3 human cases of *S. suis* disease were documented in the United States before 2011: 2 domestically acquired cases in New York and Hawaii, and 1 case in a person in California who was probably exposed in the Philippines (*4*). We describe a case of *S. suis* disease in a swine worker in Minnesota, USA.

The case-patient was a previously healthy 60-year-old man (truck driver). On December 14, 2011, severe headache and chills developed, which he attributed to the onset of influenza. He had a history of migraine headaches, and used prescription medications to treat his headache. However, on December 15, he awoke with a severe headache that was unresponsive to treatment. Despite having to stop his truck several times because of the severe headache, he successfully completed his delivery route.

Early on December 16, his wife drove him to a nearby emergency department after he did not respond to ordinary commands. The patient had reduced coordination and behaved aggressively. His blood pressure was 92/52 mm Hg. He underwent intubation for 24 hours for airway protection, and a lumbar puncture was performed.

Cerebrospinal fluid (CSF) had a leukocyte count of 10,501 cells/mL (99% neutrophils), a protein level of 509 mg/dL, and a glucose level of 38 mg/dL. A few gram-positive diplococci were observed in CSF. Complete blood count showed a leukocyte count of 14,800 cells/mL (92% neutrophils), a hemoglobin level of 14.1 g/dL, and a platelet count of 157,000/mL. *Streptococcus suis* was isolated from CSF and 2 of 4 blood cultures. Identification of *S. suis* was confirmed by using 16S rRNA gene sequencing at the Minnesota Department of Health.

The patient was given decadron, ceftriaxone, ampicillin, vancomycin, and acyclovir. During hospitalization, antimicrobial drugs were tapered until he received only ceftriaxone. Major symptoms were severe headache and nausea. He was discharged in good condition on day 10 of hospitalization and then completed a 14-day course of ceftriaxone.

There are 35 known serotypes of *S. suis* (*1*). Of these serotypes, serotype 2 is most commonly identified in infected swine and humans (*2*). The *S. suis* isolate from this patient was identified as serotype 2 by coagglutination test at the International Reference Laboratory at the Université de Montréal (*5*). The sequence type was identified by PCR as type 25, a common type in North America (*6*,*7*). The isolate was positive by PCR for the gene encoding virulence-associated factor muraminidase-released protein and negative for genes encoding virulence-associated extracellular factor and suilysin (*8*). The isolate was genotyped by enterobacterial repetitive intergenic consensus PCR and compared with 750 swine isolates in the University of Minnesota Veterinary Diagnostic Laboratory database (*9*). The obtained fingerprint matched that of 15 *S. suis* isolates from swine meningitis cases in Minnesota and Indiana during 2006–2010.

The patient worked for a trucking company that transports swine throughout the Midwest. His daily work required traveling to swine farms in Minnesota and making occasional trips to South Dakota and Iowa. His job was to load slaughter-weight swine into the truck and deliver them to regional slaughterhouses. Approximately 1 month before illness onset, he reported moving swine from a farm on which the farmer reported pneumonia, a rare yet reported manifestation of *S. suis* infection in swine.

The patient reported always wearing coveralls, boots, and gloves while loading and unloading swine, but he wore a dust mask only occasionally. He had no recent foreign travel and no skin breaks. However, absence of open wounds has been noted in previous case-patients (*10*).

The reported incubation period for *S. suis* infection in humans ranges from hours to weeks, and open wounds are associated with shorter incubation periods (*2*). Case-patients in the United States reported known risk factors, including handling ill swine or slaughtering and processing swine for meat (*4*). In this instance, the patient only loaded and unloaded slaughter-weight swine from his truck. He reported transporting swine that had pneumonia, which is common in finishing stages of swine production. However, although *S. suis* can cause pneumonia, this disease in finishing swine is probably caused by other common pathogens such as *Pasteurella multocida*, *Mycoplasma hyopneumoniae*, influenza virus, and porcine reproductive and respiratory syndrome virus. A definitive source of infection for this patient was not identified. This case demonstrates a rare but potentially under-recognized occupational hazard for workers in the swine industry in the United States.

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Streptococcus suis and Porcine Reproductive and Respiratory Syndrome, Vietnam

To the Editor: *Streptococcus suis*, an opportunistic pathogen of swine, is an emerging zoonotic pathogen among humans (*1*). In Vietnam, *S. suis* is the leading cause of human acute bacterial meningitis (*2*). Infection in humans is associated with direct exposure to infected pigs or infected raw or undercooked pork products (*3*). Of the 35 *S. suis* serotypes, only a limited number are pathogenic for pigs, and clinical cases in humans have most frequently been attributed to serotype 2 (SS2) (*1*). In Vietnam during September 2006–November 2007, the carrier rate of *S. suis* among slaughterhouse pigs was 41% (222/542); SS2 was the most frequently identified serotype in 14% (45/317) of *S. suis* isolations (*4*).

Porcine respiratory and reproductive syndrome (PRRS) is a major disease affecting the swine industry globally; the severity of PRRS in pigs can be increased by co-infection with *S. suis* (*5*). In 2010, PRRS outbreaks in swine were reported in 49 of 63 Vietnamese provinces (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/2/12-0470-Techapp1.pdf) (*6*). To understand the potential implications of swine PRRS outbreaks for human *S. suis* disease, we investigated co-infections of *S. suis* and PRRS virus (PRRSV) in sick pigs in 3 provinces of Vietnam during the PRRS outbreaks in 2010 (online Technical Appendix Figure).

We sampled 108 farms reporting pigs that had a clinical syndrome consistent with PRRSV infections in the provinces of Thai Binh (May), Tien Giang (July), and Soc Trang (July). Samples were blood from sick febrile pigs and postmortem tissue from freshly culled pigs. To confirm swine PRRS outbreaks, we performed reverse transcription real-time PCR on 1 randomly selected plasma sample from each farm (*7*). A total of 103 (95%) plasma samples from 103 farms tested positive for PRRSV (Chinese genotype). We additionally selected 3 PRRSV-positive farms per province for comprehensive PRRSV screening of all 42 sampled pigs; 100% of samples from the 9 farms were PRRSV positive. After swine outbreaks ended, blood samples from 52 healthy pigs from 10 farms that had no recent history of PRRS were collected from Tien Giang Province (March 2011). None of the 52 plasma samples from the 10 control farms tested positive for PRRSV.

We investigated the presence of SS2 in blood and tissue samples from pigs on PRRS- and non-PRRS–affected farms by bacterial culture (online Technical Appendix Table). A total of 534 specimens from sick pigs yielded 9 (1.7%) SS2 isolates. One (2%) of 52 specimens from the healthy control pigs yielded a non-SS2 *S. suis* isolate. *S. suis* has been proposed to contribute to the spread of antimicrobial resistance genes to other human pathogenic streptococci (*8*). The antimicrobial susceptibility results of 9 SS2 isolates by disk diffusion (*9*) revealed a high prevalence (6/9, 66%) of resistance to tetracycline, tobramycin, enrofloxacin, and either marbofloxacin or chloramphenicol.

PCR amplification of the *16SrDNA* gene (*10*) and the *cps2J* gene (*2*)

was performed on all blood samples to detect *S. suis* and SS2, respectively. Ninety-two (18%) of 521 sick pigs from PRRSV outbreak farms were systemically infected with *S. suis*. In contrast, no healthy pigs from control farms were positive for *S. suis* by PCR (online Technical Appendix Table). The SS2-*cps2J*–specific PCR was positive for 58 (11%) of 521 samples, and the *S. suis-16SrDNA* PCR was positive for 55 (11%). Twenty-one of the *16SrDNA*-positive samples also were positive for *cps2J*-PCR, which indicated that 34 (7%) sick pigs were infected with non-SS2 strains. Therefore, SS2 accounted for most (58 [63%] of 92) *S. suis*–positive detections. The bacterial load of SS2 in blood ranged from 1×10^3 CFU/mL^{-1} to 8.3×10^6 CFU/mL^{-1} (median 9.2×10^3 CFU/mL^{-1}). Overall, SS2 was found in 58 (11%) sick pigs and on 33 (32%) PRRS outbreak farms. The higher prevalence (92 [18%]) of systemic infections of *S. suis* and SS2 with high bacterial load in pigs from PRRS outbreak farms compared with prevalence on nonoutbreak farms (1 [2%] of 52) suggests increased systemic *S. suis* infections during swine PRRS outbreaks (p = 0.001, Fisher exact test).

We investigated the possible association between swine PRRS outbreaks and human *S. suis* infection. Case reports of confirmed human infections during 2007–2010 at the 2 tertiary referral hospitals in Hanoi and Ho Chi Minh City were reviewed. The number of human *S. suis* infection cases increased in August 2010 in southern Vietnam and doubled in northern Vietnam during May–August and October–November 2010 (Figure). Swine PRRS outbreaks were reported during June–September and March–December 2010 in southern and northern provinces, respectively (*6*) (online Technical Appendix Figure). Most patients with *S. suis* infection during these periods resided in provinces reporting swine PRRS outbreaks. Our data suggest a possible temporal association between swine PRRS outbreaks and human *S. suis* infections.

We demonstrated increased prevalence of systemic *S. suis* and SS2 infection in pigs co-infected with PRRSV during the 2010 swine outbreaks in Vietnam. The results indicate an increased risk for potential zoonotic transmission of *S. suis* to humans during outbreaks of PRRS in swine.

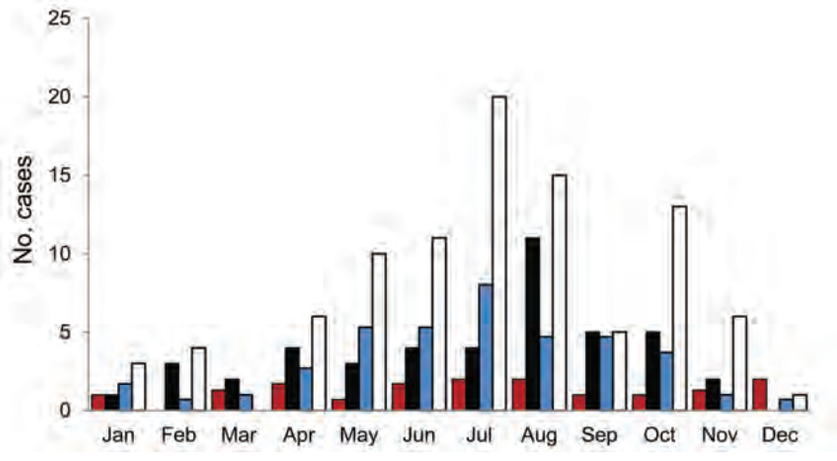

Figure. Monthly distribution of human *Streptococcus suis* infections in 2 referral hospitals, Vietnam, 2007–2010. Humans infected with *S. suis* during 2007–2009 are presented as mean total cases per month. Dark gray and black bars represent the number of *S. suis* case-patients at the Hospital for Tropical Diseases in Ho Chi Minh City during 2007–2009 and 2010, respectively. Light gray and white bars represent human *S. suis* cases at the National Hospital for Tropical Diseases in Hanoi during 2007–2009 and 2010, respectively.

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Hepatitis E Virus Seroprevalence among Men Who Have Sex with Men, United Kingdom

To the Editor: Immunosuppression might be associated with chronic carriage of hepatitis E virus (HEV) (*1*,*2*). HIV-infected persons could be at increased risk for HEV acquisition (*3*). If HIV infection is a risk factor for HEV, the risk will probably be mediated by associated behavioral factors. Men who have sex with men (MSM) are known to be at risk for transmission of enteric infection (*4*). Because of increasing prevalence of chronic liver disease induced by various causes among HIV-infected persons, it is necessary to determine whether these patients are at risk for HEV acquisition and possible hepatic decompensation (*5*).

We aimed to establish the contribution of HIV infection and MSM to seroprevalence of HEV among banked serum specimens. We used an unlinked, anonymous HIV seroprevalence survey of sexual health clinic attendees in England, Wales, and Northern Ireland, compared results from testing of residual serum samples collected for routine syphilis testing from sentinel clinics, and analyzed basic epidemiologic data (*6*). We examined serum samples collected during a 3-year period (2006–2008) and stored at –80°C. All samples were from male patients, 20–44 years of age. IgG against HEV was measured by using ELISA (Wantai; Fortress Diagnostics, Antrim, UK). To further increase the specificity for a seroprevalence analysis, and in accordance with previous work (*7*), we defined only samples with an optical density/cutoff value $\geq$1.5 as reactive and those in the range 1.0–1.5 as weakly reactive.

We analyzed 422 serum samples collected during 2008, comprising 146 samples from MSM with positive HIV test results, 135 from MSM with negative HIV test results, and 141 from heterosexual men with negative HIV test results. Thirty (7.1%) serum samples showed IgG reactivity against HEV and 3 (0.7%) additional samples showed weak reactivity. We examined the effect of HIV infection on prevalence of IgG against HEV by comparing samples from HIV-infected MSM with those from HIV-negative MSM . Seroprevalence rates did not differ significantly (HIV-positive MSM 7.5%; HIV-negative MSM 10.4%; p = 0.4).

We then examined the effect of being MSM as a risk factor for HEV infection. Prevalence of IgG against HEV among HIV-negative heterosexual men was 3.5%, significantly lower than that among MSM (odds ratio 3.1, p = 0.025, for comparison with non-HIV–infected MSM). We examined the relationship of status of IgG against HEV among MSM to the presence of an acute non-HIV sexually transmitted infection (STI) at the time of serum sampling. No association was found (acute STI, 14 [9.1%] of 154 vs. no acute STI, 11 [8.7%] of 127; p = 0.9). Similarly, no statistical association was found between HEV antibody status and the location of the clinic that provided the serum sample (London, 21 [10.0%] of 211; United Kingdom excluding London, 4 [5.7%)] of 70; p = 0.3). As has been observed for the general UK population (*7*), we

observed a trend toward increasing prevalence of antibodies against HEV with patient age (20–34 y, 9 [6.3%] of 142; 35–44 y, 16 [11.5%] of 139), although this trend did not reach significance (p = 0.13). Our samples were from persons who were younger than the previously described cohort of UK persons with increased prevalence of antibodies against HEV (born before approximately1960) (*7*). Multivariate analysis with the above variables showed that MSM (p = 0.044) and age group (p = 0.026) were independently associated with HEV seroprevalence. To explore recent temporal trends in HEV seroprevalence among MSM, we examined serum samples from 977 MSM collected during the 3-year study period. We observed an unexpected association between antibody prevalence and year of serum collection (2006, 4 [2.1%] of195; 2007, 26 [5.2%] of 501; 2008, 26 [9.3%] of 281; p = 0.003 (Figure).

We provide evidence that MSM might be at risk for HEV acquisition and confirm that HIV infection does not appear to be a risk factor. Although our study is of moderate size, and we have limited epidemiologic data owing to its unlinked, anonymized nature, the fact that patient groups are drawn from the same clinics should minimize the effect of unrecognized confounding factors. The pathologic mechanisms for HEV acquisition among MSM may plausibly include oro–anal sexual practices, which have been implicated in recent outbreaks of *Shigella flexneri* infection in this group (*8*). That ano–genital transmission of HEV is unlikely is supported by our finding that prevalence of antibodies against HEV was not more common among patients with an acute STI.

The shift in prevalence of antibodies against HEV among MSM occurred while HEV activity in the United Kingdom was increasing (*9*,*10*). The routes of transmission of indigenously acquired HEV infection in industrialized countries remain a subject of investigation, but our observations suggest that activity among MSM could expose this group to increased transmission. Thus, the putative combination of increased exposure in the general UK population and increased transmission among MSM suggests that HEV incidence and seroprevalence could increase for this group in the near future and couldbecome a substantial public health problem.

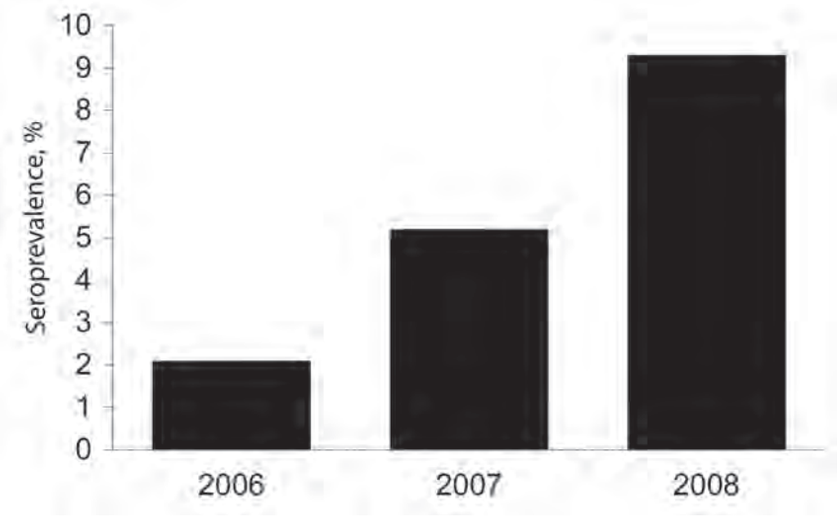

Figure. Hepatitis E virus seroprevalence among men who have sex with men, United Kingdom, 2006–2008.

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Ethical approval was obtained from Local Research Ethics Committees for the unlinked anonymized testing of archived residual serum samples for infectious diseases.

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Imported Hepatitis E Virus, Central African Republic, 2011

To the Editor: Hepatitis E virus (HEV) is endemic to India (*1*,*2*) and Central African Republic (*3*,*4*), although different strains circulate in the countries. In May 2011, a case of jaundice and fever in an expatriate Indian worker (a 33-year-old man) was reported to Institut Pasteur de Bangui, Bangui, Central African Republic. HEV RNA and IgM were detected in serum samples from the patient, and liver enzyme levels were raised (alanine aminotransferase 840 U/L, reference value 11–66 U/L). Symptoms lasted for ≈10 days and resolved without specific treatment. The patient was working and living at a construction site in Central African Republic with 51 other men (22–62 years of age) from India.

We investigated this case to determine whether it was linked to an outbreak and whether disease-control measures were needed. The protocol for surveillance and investigation was approved by the national ethical and scientific committee in Central African Republic.

Background information and blood and stool samples were obtained from the patient's coworkers. The Bioelisa HEV IgM 3.0 kit (Biokit, Barcelona, Spain), which has sensitivity >98%, was used to test serum samples for HEV IgM; real-time reverse transcription PCR (rRT-PCR) was used to test serum and stool samples for viral RNA (*5*). Test results provided evidence of early HEV infection. Liver enzymes (aspartate aminotransferase and alanine aminotransferase) were measured in serum by using an ABX Pentra 400 benchtop analyzer (Horiba Medical, Montpellier, France).

For genetic analysis of HEV strains from viremic study participants, we performed nested RT-PCR on serum and stool samples to amplify a 348-bp portion of the open reading frame 2 region (*6*). We directly sequenced the purified amplicons and compared the resulting sequences with HEV sequences in GenBank (*7*) and those from autochthonous HEV cases from 2008–2011. ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align sequences. MEGA5 (*8*) was used to construct a phylogenetic tree (300-nt sequences) by the neighbor-joining method. The genotypes and subtypes were identified as described (*7*).

The 52 men arrived in Central African Republic in several groups during July 2010–June 2011. During May–July 2011, a total of 40 (77%) men had a febrile illness; 9 illnesses were accompanied by digestive signs or symptoms, such as nausea and vomiting (Technical Appendix Table, wwwnc.cdc.gov/EID/article/12-0670-Techapp1.pdf). Only the patient whose case was reported was jaundice. Early HEV infection was biologically confirmed for 11 (21%) men, including the -patient whose case was reported; 8 of the 11 men had IgM only, 1 was HEV positive according to rRT-PCR and IgM negative, and 2 were HEV positive according to rRT-PCR and IgM positive. The 2 other men with viremia were asymptomatic, but liver enzyme levels were elevated in 1 of them.

Illnesses in infected and noninfected men did not differ, and, except for the notified case, we cannot say with certainty that the illnesses were caused by HEV. We found IgG against HEV in 14 (34%) uninfected men, which is close to the prevalence for the general population in India (*2*).

HEV subtype 1a isolates from the notified case-patient (serum-derived isolate) and a co-worker (stool-derived isolate) were sequenced (GenBank accession nos. JN863908 and JQ074213, respectively) and found to be 100% similar and to share 97%– 99% similarity with other HEV strains in India and Nepal (Technical Appendix Figure). The sequences obtained from persons with autochthonous HEV (GenBank accession nos. JN863909, JN863910, and JQ740782) clustered with HEV subtype 1e and type 2 strains and were closely related to strains from Africa and Mexico (93% and 82% similarity, respectively). Similarity between strains was not as high for the strain from the notified case-patient and those from persons with autochthonous infection (87% similarity with the subtype 1e strain and 77% similarity with the type 2 strain).

IgM titers typically rise after the incubation period, which is >3 weeks for HEV (*9*). Thus, for the purpose of this study, we assumed that men who

were positive for IgM against HEV or who had positive rRT-PCR results <3 weeks after their arrival in Central African Republic were probably infected while in India (Table, study participants 2–4 and 9). The notified case-patient and study participants 1, 5, 7, 8, and 10 had positive IgM or rRT-PCR results 4–20 weeks after arrival (Table) and, thus, could have become infected in India or Central African Republic. Study participant 6 was IgM positive >7 months after arriving in Central African Republic; thus, he was infected locally. Housing conditions for the workers were conducive to waterborne transmission of HEV, so it is likely that study participant 6 was infected with the imported strain. However, this participant was not viremic, and we cannot eliminate the possibility of autochthonous infection.

During the past few decades, concern that communicable diseases are emerging or re-emerging because of population mobility has focused mainly on mobility within industrialized countries. However, because of humanitarian and economic reasons, migration within low-income regions is also increasing. Resource-limited countries have weak infrastructures; thus, the consequences of imported outbreaks may be more serious in such countries. Our findings further demonstrate the need to improve cooperation among countries in terms of health policy, surveillance, and control, particularly in resource-limited countries. Such countries should immediately implement the International Health Regulations (2005) (*10*).

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Table. Chronology of events and clinical test results for 11 immigrants from India participating in a study of imported hepatitis E virus, Central African Republic, 2011*

Participant, region of origin	Arrived in Central African Republic	Symptomatic period, 2011	Biological specimen collected	Test result				
				IgM	rRT-PCR blood	rRT-PCR stool	AST, U/L†	ALT, U/L‡
1, Orissa	2011 Apr 29	NS	Mid Jun	+	+	+	852	791
2, Orissa	2011 Jun 4	NS	Mid Jun	–	+	–	22	14
3, Orissa	2011 Jun 4	Late Jun	Mid Jun	+	–	–	26	12
4, Orissa	2011 Jun 4	NS	Mid Jun	+	–	–	25	23
5, West Bengal	2011 Apr 5	Late Jun	Mid Jun	+	–	–	31	23
6, West Bengal	2010 Oct 10	Early Jul	Mid Jun	+	–	–	9	7
7, Orissa	2011 Apr 29	Late May	Mid Jun	+	–	–	23	18
8, Karnatanka	2011 Feb 23	Early May	Mid Jun	+	–	–	16	7
9, Orissa	2011 Jun 4	Early Jul	Mid Jun	+	–	–	19	12
10, West Bengal	2011 Apr 5	Early Jul	Mid Jun	+	–	–	24	23
NC, Orissa	2011 Apr 29	Early Jun	Early Jun	+	+	NA	2,100	840

*rRT-PCR, real-time reverse transcription PCR ; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NS, not symptomatic; NC, participant whose case was notified ; NA, not available.
†Reference value 5–34 U/L.
‡Reference value 11–66 U/L.

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Clustered Cases of *Rickettsia sibirica mongolitimonae* Infection, France

To the Editor: *Rickettsia sibirica mongolitimonae*, a member of the tick-borne spotted fever group (SFG) of rickettsia, was first isolated from *Hyalomma asiaticum* ticks in China (*1*). The first human case was described in France in 1996, and 7 new cases were described in 2005 (*1*). This rickettsiosis was named lymphangitis-associated rickettsiosis because lymphangitis was observed in 50% of the patients (*1*). Only 17 cases have been reported, for which 7 patients had lymphangitis, and 13 had inoculation eschars, including 2 patients with 2 eschars (*1*,*2*). We report a cluster of cases of *R. sibirica mongolitimonae* infection.

Patient 1, a 73-year-old man in France, had fever, rash, lymphadenopathies, and an axillary inoculation eschar in February 2011. A diagnosis of lymphangitis-associated rickettsiosis was suspected because of the season (most cases occur in spring in France) and clinical manifestations. The patient was confined to bed for several weeks after surgical placement of a knee prosthesis when his disease occurred; the domestic cat was suspected to have introduced ticks into the home.

In April, his wife (67 years of age) (patient 2) became febrile, had popliteal lymphadenopathies associated with lymphangitis, and had an eschar on the leg from which a swab specimen was obtained. Patient 3 was their neighbor; he had the same symptoms in March 2011 but samples were not collected from him.

None of patients reported tick bites, but they were in regular contact with animals, including a cat, a dog, horses, and birds. Both patients who lived with the cat reported that it would return home with ticks. Infections in these patients were successfully treated with doxycycline.

An immunofluorescence assay for antibodies against SFG antigens showed IgG/IgM titers of 128/0 for patient 1 and 64/16 for patient 2 (*3*). DNA was extracted from the skin swab specimen of patient 2 by using the QIAamp Mini Kit (QIAGEN, Hilden, Germany). A fragment of the citrate synthase gene of *Rickettsia* spp. was amplified by PCR and sequenced. The sequence show 99.7% homology with that of the same gene sequence of *R. sibirica mongolitimonae* in GenBank (accession no. DQ423370) (*4*).

Ticks were collected from the property of the 2 patients: from the garden by flagging vegetation (*3*), from animals, and near the cat litter (Table). SFG rickettsiae were detected by specific quantitative PCR. Species identification was confirmed by specific quantitative PCR for *R. massiliae* and sequencing of outer membrane protein A gene for others species (*5*). A negative control (sterile water) and positive control (DNA from *R. montanensis* or *R. massiliae*) were included in each PCR.

Ticks were morphologically identified as adult *Rhipicephalus sanguineus*. Molecular identification of these ticks harboring rickettsiae was performed by amplification of the 12S rRNA gene. DNA from *R. massiliae* was found in 3 ticks collected from the dog and near the cat litter morphologically identified as *Rh. sanguineus*. This DNA showed 98% homology with the sequence in GenBank (accession no. AY559843). *R. sibirica mongolitimonae* with 99.8% homology for the outer membrane protein gene sequence in GenBank (accession no. DQ097082) was isolated from 1 tick collected from the cat. This tick was identified as *Rh. pusillus* and showed 99.7% homology with the sequence in GenBank (accession no. FJ536547). *R. massiliae* was cultured from an *Rh. sanguineus* tick, and *R. sibirica mongolitimonae* was cultured from an *Rh. pusillus* tick.

A cluster of 1 documented case and 2 probable cases of lymphangitis-associated rickettsiosis in southern France was linked to a cat and *Rh. pusillus* ticks. Infection with *R. massiliae* for the 2 probable case-patients was unlikely because clinical findings

Table. Ticks collected from property of 2 patients infected with *Rickettsia sibirica mongolitimonae*, France, 2011*

Location of tick collection	No. ticks collected	Tick species identification†	No. ticks (*Rickettsia* species) Harboring rickettsial DNA	No. ticks (*Rickettsia* species) From which rickettsiae were cultured
Garden	2	ND	0	0
Dog	21	*Rhipicephalus sanguineus*	2 (*R. massiliae*)	0
Cat litter	7	ND	1 (*R. massiliae*)	1 (*R. massiliae*)
Cat	9	*Rh. pusillus*	1 (*R. sibirica mongolitimonae*)	1 (*R. sibirica mongolitimonae*)
Scrub land	5	ND	0	0

*ND, not done.
†Based on 12S rRNA gene. All adult ticks were morphologically identified as *Rh. sanguineus*.

were typical of lymphangitis-associated rickettsiosis, and most cases of rickettsioses in southern France in the spring are caused by *R. sibirica mongolitimonae*. Clustered cases of SFG rickettsiae infection have been reported in Europe, including southern France (*3*,*6*). In 2007, *R. conorii* and *R. massiliae* infections in humans were reported (*3*). In 2010, cases for which we were unable to discriminate between *R. conorii* and *R. massiliae* infections occurred in a family (*6*). In these 2 studies, clustered cases of SFG rickettsiosis involved *Rh. sanguineus* ticks. Clustered cases appeared to be related to an increase in aggressiveness of ticks toward humans during warmer periods (*3*). In our study, no correlation was identified with warmer weather.

R. sibirica mongolitimonae is most frequently associated with *Hyalomma* spp. ticks (*1*,*2*,*4*). However, 1 case of infection with this bacterium was associated with *Rh. pusillus* ticks collected in Portugal (*7*); DNA from this bacteria was also identified in an *Rh. pusillus* tick collected from a mongoose. The European wild rabbit is the primary host of *Rh. pusillus* ticks. However, these ticks have been found on wild carnivorous animals, dogs, and domestic cats (*8*); these ticks can bite humans (*8*). Moreover, *R. massiliae* and *R. sibirica mongolitimonae* were found in *Rh. pusillus* ticks from Spain (*9*), and SFG rickettsiae were found in ticks from Sardinia (*10*). Therefore, *Rh. pusillus* ticks appear to be an emerging vector for *R. sibirica mongolitimonae* in Europe.

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Rickettsiae in Ticks, Japan, 2007–2011

To the Editor: Japanese spotted fever (JSF), caused by *Rickettsia japonica*, is the most prevalent tick-borne infectious disease in Japan (*1*), occurring most frequently in central and western regions (http://idsc.nih.go.jp/idwr/CDROM/Main.html [in Japanese]). Cases of unknown fever with rickettsiosis-like symptoms not associated with JSF have been reported in JSF-endemic regions of Japan (*2*). Several spotted fever group (SFG) rickettsiae (*R. japonica*, *R. heilongjiangensis*, *R. helvetica*, *R. tamurae*, *R. asiatica*, *Candidatus* R. tarasevichiae) and other related *Rickettsia* spp. have been identified in Japan (*1*,*3*–*6*). Human infections with *R. heilongjiangensis* and *R. tamurae* have been confirmed (*3*,*5*), and *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, known human pathogens, have been detected in ticks and deer in Japan. We conducted this study to determine the risk in central and western Japan for human exposure to ticks harboring SFG rickettsiae, *A. phagocytophilum*, or *Ehrlichia* spp.

In 2007–2011, we collected 827 *Haemaphysalis*, *Amblyomma*, and *Ixodes* spp. ticks (392 adults, 435 nymphs) by flagging vegetation in the prefectures of Shizuoka, Mie, Wakayama, Kagoshima, Nagasaki (Goto Island), and Okinawa (the main island and Yonaguni Island) (Technical

Appendix Figure 1, wwwnc.cdc.gov/EID/article1/12-0856-Techapp/.pdf). We extracted DNA from the salivary glands of each tick and performed PCR to amplify *gltA*, 16S rDNA, and *ompA* of SFG rickettsiae. To detect *A. phagocytophilum* and *Ehrlichia* spp., we performed nested PCR targeting the *p44/msp2* and *p28/omp-1* multigenes, respectively.

PCR *gltA* screening revealed SFG rickettsiae in 181 (21.9%) of the 827 ticks (Table). We obtained nearly full-length (1.1-kb) *gltA* sequences and classified them into 5 groups by phylogenetic analyses (Technical Appendix Figure 2). Sequences for groups 1 (prevalence 1.0%) and 2 (prevalence 3.2%) were identified as *R. japonica* YH (GenBank accession no. AP011533) and *R. tamurae* (GenBank accession no. AF394896), respectively (Table). Group 3 (prevalence 15.1%) sequences were identical to that of *Rickettsia* sp. LON (GenBank accession no. AB516964). The sequence for group 4 (prevalence 1.6%) was closely related to that for *R. raoultii* strain Khabarovsk (98.8% similarity), and a part of the sequence (342 bp) was identical to that of *Rickettsia* sp. Hf 151 (GenBank accession no. AB114815). Group 5 consisted of 4 newly identified rickettsiae (Technical Appendix Figure 2). Of these 4 rickettsiae, 3 (Mie311, Goto13, and Mie334) were closely related to *R. raoultii* strain Khabarovsk (98.0% identity) and 1 (Mie201) was similar to *Candidatus* R. principis (99.7% identity).

We further analyzed the 16S rDNA and *ompA* in *gltA*-positive tick samples. The 16S rDNA and *ompA* for group 1 samples shared 100% identity with 16S rDNA and *ompA* of *R. japonica* YH (AP011533). The 16S rDNA of group 2 was identical to that of *R. tamurae* (AY049981). In groups 3–5, some of the specific amplicons in 16S rDNA or *ompA* could be detected; their sequences were confirmed to be similar (but not identical) to those of several known rickettsial sequences.

We amplified the *p44/msp2* amplicons of *A. phagocytophilum* from 25 (3%) of 827 ticks (Table). By cloning (TA Cloning Kit; Life Technologies, Carlsbad, CA, USA) and sequencing these amplicons, we obtained and identified 60 new TA-clone sequences (366–507 bp) for *p44/msp2* (GenBank accession nos. JQ697880–JQ697950); these sequences may include a potentially novel *Anaplasma* species. (*7*). *Ehrlichia p28/omp-1* was detected from 2 (0.2%) of the 827 ticks. Of 5 TA-clone sequences (284–315 bp) obtained from the 2 ticks, 2 from an *A. testudinarium* tick (GenBank accession nos. JQ697886 and JQ697887) shared 83.3%–86.7% similarity with *E. ruminantium* Gardel Map-1 (GenBank accession no. YP196842), and 3 from an *H. longicornis* tick (GenBank accession nos. JQ697888–JQ697890) showed the closest relationship to *E. ewingii omp-1–15* (67%–73% similarity; GenBank accession no. EF116932).

We identified the tick species associated with *R. japonica* as *H. formosensis*, *H. hystricis*, and *H. cornigera*, and another study reported an association with *Dermacentor taiwanensis*, *H. flava*, *H. longicornis*, and *I. ovatus* (*4*). In our study and previous studies, the tick species associated with *A. phagocytophilum* in Japan were identified as *H. formosensis*, *H. longicornis*, *H. megaspinosa*, *A. testudinarium*, *I. ovatus*, and *I. persulcatus* (*8*). Thus, it appears that 3 tick species (*H. formosensis*, *H. longicornis*, and *I.*

Table. PCR survey results for *Haemaphysalis*, *Amblyomma*, and *Ixodes* spp. ticks tested for rickettsiae, central and western Japan, 2007–2011*

Tick species	No. ticks tested	Total no. (%) ticks positive	No. (%) ticks positive for *Rickettsia gltA*, by species group†					*A. phagocytophilum p44/msp2*	*Ehrlichia p28/omp-1*§
			Group 1	Group 2	Group 3	Group 4	Group 5		
H. formosensis	224	6 (2.7)	1 (0.4)	0	0	0	5 (2.2)	18 (8)	0
H. hystricis	97	19 (19.6)	6 (6.1)	0	0	13 (13.4)	0	0	0
H. longicornis	294	119 (40.5)	0	0	119 (40.5)	0	0	2 (0.7)	1 (0.4)‡
H. flava	55	6 (10.9)	0	0	2 (3.6)	0	4 (7.3)	0	0
H. kitaokai	10	0	0	0	0	0	0	0	0
H. megaspinosa	18	4 (22.2)	0	0	4 (22.2)	0	0	1 (5.6)	0
H. cornigera	11	1 (9.1)	1 (9.1)	0	0	0	0	0	0
A. testudinarium	112	26 (23.2)	0	26 (23.2)	0	0	0	3 (2.7)	1 (0.9)
A. geoemydae	1	0	0	0	0	0	0	0	0
I. ovatus	5	0	0	0	0	0	0	1 (20.0)	0
Total	827	181 (21.9)	8 (1.0)	26 (3.1)	125 (15.1)	13 (1.6)	9 (1.1)	25 (3.0)	2 (0.2)

*DNA was extracted from the salivary glands of each tick by using the DNeasy Mini Kit (QIAGEN Sciences, Germantown, MD, USA) and used as a template for PCR. The newly identified sequences of *gltA,* 16S rDNA, *ompA*, *p44/msp2*, and *p28/omp*-1 in this study were deposited into GenBank under accession nos. JQ697880–JQ697959. *A. phagocytophilum, Anaplasma phagocytophilum.*

†The PCR primers used, *gltA*–Fc (5′-CGAACTTACCGCTATTAGAATG-3′) and *gltA*–Rc (5′-CTTTAAGAGCGATAGCTTCAAG-3′), were designed in this study. Groups: 1, *Rickettsia japonica* YH (GenBank accession no. AP011533); 2, *R. tamurae* (GenBank accession no. AF394896); 3, *Rickettsia* sp. LON-13 (GenBank accession no. AB516964); 4, *Rickettsia* sp. Hf151; 5, other rickettsiae.

‡PCR primers of p3726 (5′-GCTAAGGAGTTAGCTTATGA-3′), p3761 (5′-CTGCTCT[T/G]GCCAA(AG)ACCTC-3′, p4183 (5′-CAATAGT[C/T]TTAGCTAGTAACC-3′), and p4257 (5′-AGAAGATCATAACAAGCATTG-3′) were used for detection of *p44/msp2*.

§PCR primers conP28-F1 (5′-AT[C/T]AGTG[G/C]AAA[A/G]TA[T/C][A/G]T[G/A]CCAA-3′), conP28-F2 (5′-CAATGG[A/G][T/A]GG[T/C]CC[A/C]AGA[A/G]TAG-3′), conP28-R1 (5′-TTA[G/A]AA[A/G]G[C/T]AAA[C/T]CT[T/G]CCTCC-3′), and conP28-R2 (5′-TTCC[T/C]TG[A/G]TA[A/G]G[A/C]AA[T/G]TTTAGG-3′) were used to detect *p28/omp-1*.

ovatus) are associated with *R. japonica* and *A. phagocytophilum*.

In addition, in an *H. formosensis* tick, we detected an SFG rickettsia that is closely related to *R. raoultii*, the etiologic agent of *Dermacentor*-borne necrosis erythema and lymphadenopathy in Europe and Russia (*9*). We detected *Candidatus* R. principis in *H. flava* in Japan; this species was previously detected in *H. japonica douglasi* and *H. danieli* ticks in Russia and China, respectively, (*10*). And, we found a high prevalence of *R. tamurae* in *A. testidinarium* ticks; Imaoka et al. (*5*) recently reported that *R. tamurae* causes local skin inflammation without general JSP-like symptoms. We did not detect the human pathogen *E. chaffeensis*, but we identified 2 potentially new *Ehrlichia* species.

Our findings contribute to the known risks for exposure to *Rickettsia*-related pathogens in central and western Japan. Further studies may be required for the surveillance of additional pathogens, such as *Candidatus* Neoehrlichia mikurensis (*2*), which was recently recognized as a human pathogen.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Tropheryma whipplei Genotypes 1 and 3, Central Europe

To the Editor: *Tropheryma whipplei* causes Whipple disease, a rare multisystemic disorder that affects mainly middle-aged white men and is most widely distributedin Europe and North America (*1*). In the general population of France, *T. whipplei* DNA was found in 2%–4% of stool samples and *T. whipplei*–specific antibodies were found in 51% of serum samples (*2*). Still, the prevalence of classic Whipple disease, which causes arthralgia, diarrhea, and weight loss, remains extremely low (*1*). Whipple disease has 4 known manifestations: 1) classic Whipple disease; 2) focused chronic infections, mainly endocarditis; 3) acute infections, such as gastroenteritis, bacteremia, and pneumonia; and 4) asymptomatic *T. whipplei* carriage in healthy persons (*1*–*5*). *T. whipplei* is thought to be transmitted through oral and oro–fecal routes by human-to-human contact (*2*,*6*).

The pathogen was cultivated in 2000, and 2 genomes were sequenced (reference strains Twist and TW08/27) (*7*,*8*). These events made possible a genotyping system based on 4 highly variable genetic sequences found by genome comparison (TW133, ProS, SecA, Pro184) (*9*). Since 2007, we have applied this system to patient samples positive for *T. whipplei* from central Europe and sub-Saharan Africa (*2*,*3*,*9*,*10*). The system showed a higher discriminatory power than previous typing methods and improved the genotyping resolution of *T. whipplei*, promoting better understanding of its epidemiology on the molecular level (*9*).

Since 2003, we have subcultured strain Twist every 3 weeks. In 2007 and 2012, we compared sequences for the subcultured strains with that for the 2003 strain. We found that the spacer sequence remained stable over the ≈10-year period. This finding suggests a high intrastrain genetic stability and highlights the value of the typing system, which is stable. Thus, a change in genotype in a patient with Whipple disease must be interpreted as an infection with a different strain and cannot be attributed to mutation of the original strain.

To date, 191 samples positive for *T. whipplei* collected from patients from central Europe (France, Germany, Switzerland, Austria, and Italy) have been typed, revealing a genetic diversity by identifying 72 different *T. whipplei* genotypes: 1–23, 25–45, 58–60, 76–77, 82–102, and 111–116. The discriminatory power was high (Hunter-Gaston discriminatory index 0.9298) for all samples from Europe. No connection between clinical manifestations and *T. whipplei* genotypes has been described. Reasons might be found either in an unknown link between genomics in *T. whipplei* and clinical outcome or might be because the typing system cannot identify possible virulence factors. Genotypes 1 and 3 are predominant (*1*,*3*), accounting for 35.1% of all tested *T. whipplei* samples from Europe.

Genotype 3 is the most common *T. whipplei* genotype in Europe (19.9% of all samples) and could be considered epidemic in and specific to France, Switzerland, and Italy. This genotype was proposed to be responsible for small outbreaks caused by clonal strains, such as gastroenteritis among young children or the strain carried by homeless persons in Marseille, France (*3*,*6*), but it has not been described in Germany or Austria (Figure).

Genotype 1 is found throughout central Europe and is the second most common genotype (15.2% of all samples) (Figure). Predominance of this genotype in Germany is high (46.2%, n = 13) and Austria (80%, n = 5). Infection with this genotype seems to be endemic in the population of the area, although no outbreaks have been reported.

Of 191samples, a total of 55 (28.8%) showed a unique genotype consistent with the high genetic variability in *T. whipplei* specimens. Phylogenetic analysis and clustering

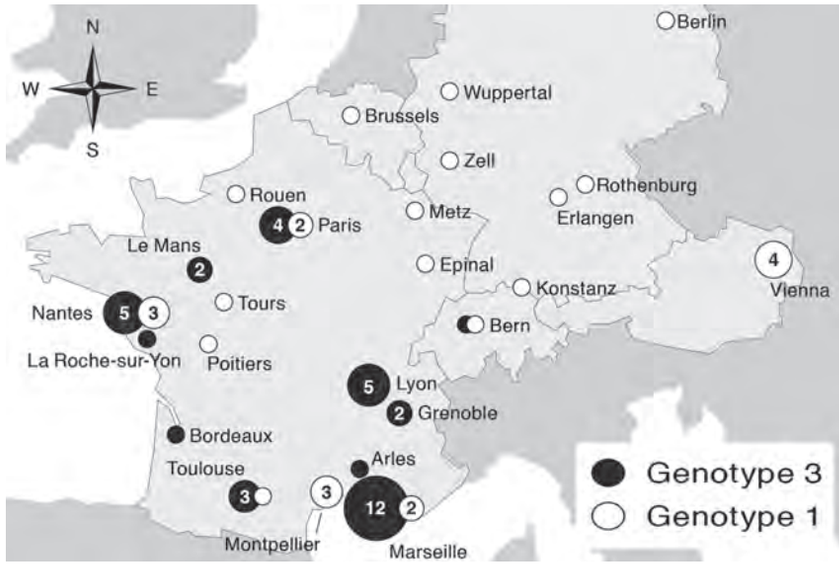

Figure. Geographic distribution of the 2 most common genotypes of *Tropheryma whipplei* in central Europe. Numbers in circles indicate number of cases with corresponding genotype; circles without a number indicate single cases. Cities are either the residence of patients or of their physicians; the capital of the country of residence is shown for persons whose city of residence was unknown. This map was made by using Epi Info 7 (wwwn.cdc.gov/epiinfo/).

of these singletons showed no correlation between clusters and geographic origin of samples.

Of the 191 samples, a total of 66 (34.6%) were from Marseille and represented 40 different genotypes. This finding underscores the broad heterogeneity in *T. whipplei*. Twelve (18.5%) of the 66 tested samples were genotype 3, which might be linked to the local outbreak among homeless persons mentioned above. Genotype 1, which is endemic to France, was found in only 2 (3.1%) persons in Marseille. The fact that Marseille is a metropolitan area with a high migration rate could play a role in the vast diversity of *T. whipplei* genotypes found there

Questions regarding the epidemiologic character of Whipple disease remain unanswered, such as why the bacterium is highly prevalent but the disease is not. Persons with the putative immunological defect probably responsible for classic Whipple disease (*1*) have the highest bacterial load in their stools. But these persons are unlikely to come into direct contact with one another. Thus, propagation of this bacterium on a large scale might be relatively limited, which could explain the high genetic diversity in the bacterial specimens assessed so far.

Two predominant genotypes seem to break out of this pattern: genotypes 1 and 3. Genotype 3 could be considered a genotype that causes small epidemics, whereas genotype 1 could be considered a genotype endemic to central Europe. Reasons for the success of these 2 genotypes remain unknown, but improvement of genotyping methods could provide the answers.

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Yersinia pestis Plasminogen Activator Gene Homolog in Rat Tissues

To the Editor*: Yersinia pestis* causes plague, which primarily affects rodents, but is an invasive and virulent pathogen among humans. *Y. pestis* infection is endemic in small rodent populations in different parts of the world, and the bacterium is considered a potential bioweapon because it can be easily isolated, produced, dried, and dispersed as an aerosol. Antimicrobial drug treatment can be lifesaving during the early stages of illness; hence, rapid and sensitive methods for *Y. pestis* detection in environmental and clinical samples are required. Multiple PCR assays for *Y. pestis* detection that primarily detect markers located on plasmids have been developed (*1*–*6*). The plasminogen activator/coagulase (*pla*) gene, located on plasmid pPCP1, is incorporated into most *Y. pestis* PCR assays, and in several studies it was the prime or sole marker (*1*,*2*,*5*,*7*–*9*). Reasons for including *pla* in these assays are its occurrence in multiple copies, its absence from closely related *Yersinia* species, and its role in *Y. pestis* virulence (*1*,*4*,*5*).

While validating the specificity of a multiplex qPCR assay for the detection of *Y. pestis* (*6*), we obtained DNA from the dissected peritoneum of a black laboratory rat (*Rattus rattus*), which tested positive for the *pla* gene. Two other *Y. pestis* signature sequences were not amplified. Additional samples were analyzed from black (n = 11) and brown (*Rattus norvegicus* [n = 4]) rats that had been caught on poultry and pig farms in the southeastern region of the Netherlands during 2008. Positive indicators for *pla* were found in samples from 8 of these black rats and in samples from 2 of the brown rats. Samples from 2 laboratory rats tested negative for *pla*. Inferences of the incidence of *pla*-positive rats cannot be made because of low sample numbers and potential bias in capturing rats that had putative infections.

To exclude the possibility of contamination of host DNA with DNA from intestinal flora during isolation of the peritoneum, we examined the occurrence of *pla* in other tissues. Lung and liver samples were available from all 17 rats, and leg tissue samples were available from 7 rats, 5 of which had positive peritoneal tissue test results. The leg and lung tissues of 1 rat and the leg tissue of another rat tested positive, albeit at considerably lower quantities (higher quantification cycles) than *pla* values measured in peritoneal samples. These results did not support the likelihood of contamination during sampling or the occurrence of local infections; they did support the hypothesis of a systemic infection in the rats. To investigate whether the presence of the *pla* gene sequences indicated the presence of the carrier pPCP1 plasmid in *Y. pestis*, we designed PCR assays for the amplification of 3 conserved regions of this plasmid (online Technical Appendix, wwwnc.cdc.gov/EID/article1/12-0659-TechApp/.pdf). Each assay produced PCR products from *Y. pestis*; only the transposase gene was amplified from *Y. pseudotuberculosis*. None of the PCR assays amplified DNA from samples collected from rats.

Pla genes obtained from 2 of the peritoneum samples collected from black rats were sequenced and appeared to be identical (GenBank accession no. HQ606074). Alignment with *Y. pestis pla* genes, which are highly conserved among *Y. pestis* isolates, revealed 11 nt differences in 880 bp (98.8% similarity). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search retrieved such highly similar genes only from *Y. pestis* sequences; the next most similar sequences of other *Enterobacteriaceae* were at 78% similarity.

The genewalking PCR procedure was used to explore sequences adjacent to the *pla* gene (online Technical Appendix). One PCR product was sequenced and appeared to be in part homologous to the *pla* gene, but the adjacent sequence displayed high homology to genes coding for replicon (*rep)* proteins in several bacterial genera in the family *Enterobacteriaceae*, e.g., *Escherichia*, *Shigella*, and *Salmonella*. The existence of a concatenated *pla-rep* sequence in rat tissue samples was confirmed by amplification of a PCR product from a primer targeting the *pla* gene, combined with a primer targeting the *rep* gene sequence that was acquired by using the genewalking procedure. The resulting 223-bp PCR product (GenBank accession no. JQ756394) consisted of a 141-bp sequence identical to the *Y. pestis pla* gene, linked to a 72-bp sequence that was 97% similar to enterobacterial *rep* protein genes. Attempts to obtain more sequence information from *rep* sequences by using primers derived from conserved domains in enterobacterial *rep* genes were unsuccessful. This suggests that the *pla-rep* sequence is derived from uncharacterized bacteria. *Rep* proteins function as replication activators of their carrier plasmids.

The *rep* sequences identified in this study were most similar to those of plasmids involved in bactericidal activity, a function that is also ascribed to the bacteriocin pesticin gene clusters of *Y. pestis* pPCP1 plasmids. The occurrence in unknown organisms that have *pla* genes that are similar to *Y. pestis pla* genes has consequences for the detection of *Y. pestis*. To prevent false positive results, detection protocols should include at least 1 supplemental target to confirm the presence of *Y. pestis* (*6*). In addition, investigators using *pla* gene analysis, for instance, while reconstructing ancient plague epidemics (*10*), should be aware of the occurrence of these homologs.

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Coxiella burnetii in Ticks, Argentina

To the Editor: The Gammaproteobacterium *Coxiella burnetii* is the causative agent of acute Q fever and chronic endocarditis in humans worldwide. It is transmitted primarily by aerosol route or by ingestion of fomites from infected animals, mostly from domestic ruminants (*1*). Although >40 tick species can be infected with *C. burnetii*, direct transmission of this agent to humans from infected ticks has never been properly documented. However, ticks may play a critical role in the transmission of *C. burnetii* among wild vertebrates (*1*). Only a few studies, mostly related to human clinical cases or seroepidemiogic evaluation of healthy animals, have reported *C. burnetii* in South America (*2*–*4*). However, to our knowledge, *C. burnetii* has never been reported in ticks in the continent.

During ecologic studies on *Amblyomma parvum* and *A. tigrinum* ticks in the Córdoba Province of Argentina, engorged nymphs were collected from the common yellow toothed cavy (the rodent *Galea musteloides*) (*5*,*6*). In the laboratory, engorged nymphs molted to adults (92 *A. tigrinum*, 13 *A. parvum*), which were individually submitted to the hemolymph test with Gimenez staining for detection of rickettsiae-like organisms (*7*). By the hemolymph test, 1 *A. tigrinum* female, and 2 *A. parvum* male ticks were found to contain red-stained rickettsiae-like structures. These 3 ticks were processed individually by the shell vial technique, with the purpose of isolating intracellular bacteria in Vero cell culture (*7*). Inoculated cells were always incubated at 28°C. Intracellular bacteria were successfully isolated from all 3 ticks and established in Vero cell culture, as demonstrated by Gimenez staining of infected cells from at least 10 subsequent passages, which all infected 100% of the cells (Figure, panel A). Infected Vero cells contained multiple vacuoles (Figure, panel B) that enclosed a seething mass of microorganisms (online Video; wwwnc.cdc.gov/EID/article/19/02/12-0362-F1.htm), compatible with *Coxiella* organisms. Such vacuoles were not seen in uninfected control Vero cells incubated under the same conditions as those of infected cells (Figure, panel C).

For molecular analyses, DNA from the infected cells of each of the 3 isolates was extracted by boiling at 100°C for 10 min; it yielded products of the expected size through PCR protocols selective for portions of 3 genes of the genus *Coxiella*: primers QR-FO (5′-ATTGAAGAGTTT GATTCTGG-3′) and QR-RO (5′-CG GCCTCCCGAAGGTTAG-3′) for the 16S rRNA gene (*8*); priers CAPI844F (5-ATTTAGTGGGTTTCGCG CAT-3′) and CAPI844R (5′-CAT CAGCATACGTTTCGGGAA-3′) for the *cap* gene (*9*); and primers Cox-F-pry2 (5′-TTATTTACCAAGTTCC GAGCCG-3′) and Cox-R-pry2 (5′ TTTATCCCGAGCAAATTCA ATTATGG-3′) for the *pyrG* gene (*9*). PCR products underwent DNA sequencing in an automatic sequencer (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) according to the manufacturer`s protocol. We sequenced 1,386, 557, and 545 nt of the genes 16S rRNA, *cap*, and *pyrG*, respectively, which were identical to each other for each gene amplified from the 3 tick isolates. By BLAST analyses (www.ncbi.nlm.nih.gov/blast), these sequences were 99.9% (1,384/1,386 nt), 99.6% (556/558 nt), and 99.6% (452/454 nt) identical to the corresponding GenBank sequences of the North American *C. burnetii* genes 16S rRNA, *cap*, and *pyrG*, respectively (HM208383, CP001020, CP001020). Partial sequences (16S rRNA, *cap*, *pyrG*) from *C. burnetii* generated in this study were deposited into GenBank and assigned nucleotide accession nos. JQ740886–JQ740888, respectively.

Infected Vero cell monolayers were fixed in a modified Karnovsky solution, stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope according to standard procedures. Ultrastructurally, *Coxiella* organisms were identified by morphologic features within heavily infected Vero cells. The organisms possessed typical bacillary morphologic characteristics and were observed inside vacuoles (phagolysosomes) of different sizes, proportional to the

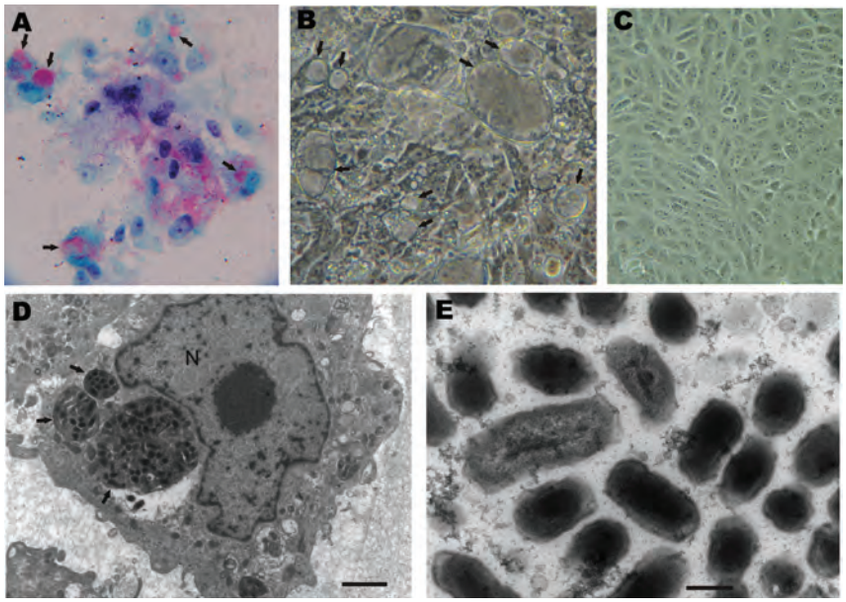

Figure. Vero cells inoculated with *Amblyomma* spp. tick extracts for isolation of rickettsiae. A) Rickettsiae-like organisms stained by Gimenez staining (original magnification ×400). B) Inoculated monolayer photographed under phase-contrast microscopy (original magnification ×400). C) Uninfected control monolayer under phase-contrast microscopy (original magnification ×400). D) Transmission electron microscopy of infected cells. Bar indicates 2 µm. E) Transmission electron microscopy image of intravacuolar bacteria. Bar indicates 250 nm. N, nucleolus. Arrows indicate vacuoles containing bacteria. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/2/12-0362-F1.htm).

number of organisms (Figure, panels D, E). Intravacuolar organisms had a mean length of 0.55 ± 0.13 µm (range 0.42–0.85 µm) and a mean width of 0.25 ± 0.03 µm (range 0.22–0.32 µm).

Ticks negative for rickettsiae-like organisms by hemolymph testing were subjected individually to DNA extraction by the guanidine isothiocyanate-phenol technique (*10*) and screened for *Coxiella* spp. by PCR that targeted the *pyrG* gene, as described above. Although no *A. parvum* tick yielded amplicons, 40 *A. tigrinum* ticks yielded amplicons of the expected size for the *pyrG* gene. DNA sequences generated from these ticks were identical to the *pyrG* partial sequences obtained from the *C. burnetii* isolates mentioned above.

We found 41 (44.6%) of 92 ticks and 2 (15.4%) of 13 of the *A. tigrinum* and *A. parvum* adult ticks, respectively, to be infected by *C. burnetii*. Because these ticks were collected as engorged nymphs from wild rodents in a natural biome of Argentina, namely, the Chaco phytogeographic domain (*5*,*6*), our results indicate that *C. burnetii* is established in this part of the country where ticks possibly play an essential role in the enzootic cycle. Serologic evidence of *C. burnetii* infection has been found among goats and cattle in several areas of Argentina (3,4). Because free-ranging domestic cattle and goats are considered among the most likely hosts for *A. parvum* adult ticks in the Chaco domain (*6*), humans are likely being exposed to *C. burnetii* as well.

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Epizootic Hemorrhagic Disease in Brocket Deer, Brazil

To the Editor: In February 2008, a 2-year-old female gray brocket deer (*Mazama gouazoubira*) at Pomerode Zoo in Santa Catarina, Brazil, exhibited sublingual swelling, drooling, lethargy, prostration, glossitis, slight cyanosis, and blood on the perineum. The animal showed progressive anorexia, ataxia, dyspnea, marked cyanosis, hypothermia (34.5°C), and severe anemia (hematocrit 4.6%). The deer died 5 days after the first clinical signs. Necropsy indicated that the gray brocket deer had pulmonary congestion and edema, mild renal congestion, and renal focal necrosis. Some intestinal vessels along the serosa presented with hyalinization of the walls with mild leukocytic infiltrate of neutrophils featuring vasculitis.

Seventeen days after this first case, a 1-year-old male pygmy brocket deer (*Mazama nana*) from the same zoo suddenly died. Findings on necropsy were cyanosis and petechiae in the oral mucosa, tongue, and gastrointestinal mucosa. There was bloody intestinal content, petechiae in the mucosa of the urinary bladder, and also petechiae and ecchymoses in the pericardium and epicardium. The spleen was contracted; lymph nodes and kidney medullae were hemorrhagic. The lungs showed congestion and petechiae, and the airways had a frothy fluid content. Histopathologic lesions included mild diffuse congestion in the pygmy brocket deer's kidneys and extensive subendocardial hemorrhage.

To identify the suspected disease agent (members of the species *Bluetongue virus* or *Epizootic hemorrhagic disease virus*), we performed virus isolation and reverse transcription PCR on tissues (heart, liver, lung, and bowel) from the pygmy brocket deer. (Virus isolation was not carried out on specimens from the gray brocket deer because brain tissue samples were inadequate and results of PCR were negative for epizootic hemorrhagic disease virus [EHDV] or bluetongue virus [BTV].) Infection with EHDV and BTV was first diagnosed by virus isolation. Pooled specimens from spleen-liver and heart tissue were inoculated in embryonated chicken eggs and thereafter in BHK-21 cells (*1*). Chicken embryos inoculated with tissues of the pygmy brocket deer died from 1 to 6 days after inoculation. All embryos showed swelling and hemorrhage throughout the skin and extensive areas of hemorrhage in the brain and heart. Cytopathic effects started 48 hours postinoculation in the BHK-21 monolayer. An indirect immunoperoxidase test, using anti-BTV/EHDV polyclonal antiserum of porcine origin (VMRD Inc., Pullman, WA, USA) and direct fluorescence assay using an anti-BTV monoclonal antiserum fluorescein conjugate (VMRD) were performed for virus identification (*1*). The isolates were then identified as EHDV.

To confirm the EHDV serogroup, we performed reverse transcription PCR. A fragment of ≈260 bp, which encodes the partial NS3 gene of EHDV, was detected from pooled tissue and the BHK-21 monolayer with cytopathic effect (*2*). No amplification was obtained in the S10 gene PCR for BTV (*3*). An amplified fragment was sequenced and identified as strain LDVA (GenBank accession no. GU014478). The phylogenetic relationship was assessed by using the neighbor-joining Mega.5 (*4*) method with the BTV sequence as an outgroup. The phylogenetic tree shows that the partial sequence of the S10 gene segregates EHDV serogroup into 2 clusters, with LDVA (GU014478) grouping together with North American EHDV samples (Figure).

EHDV and BTV (*Reoviridae: Orbivirus*) are involved in outbreaks of hemorrhagic disease, which occur mainly in late summer and early autumn in various parts of the world, probably because of the increase in the *Culicoides* population, the biologic vectors of these viruses. Clinical signs of EHDV and BTV infections are indistinguishable in deer and are characterized by severe depression, respiratory distress, anorexia, and blood-tinged oral and nasal discharge. Gross lesions are mainly edema and hemorrhage caused by vascular injury (*5*).

Clinical reports of EHDV infection have occurred in Australia, Asia, Africa, and North America (*6*). In Brazil, serologic research in marsh deer (*Blastocerus dichotomus)* populations in São Paulo and Mato Grosso do Sul revealed that 74% of animals were seropositive for EHDV,

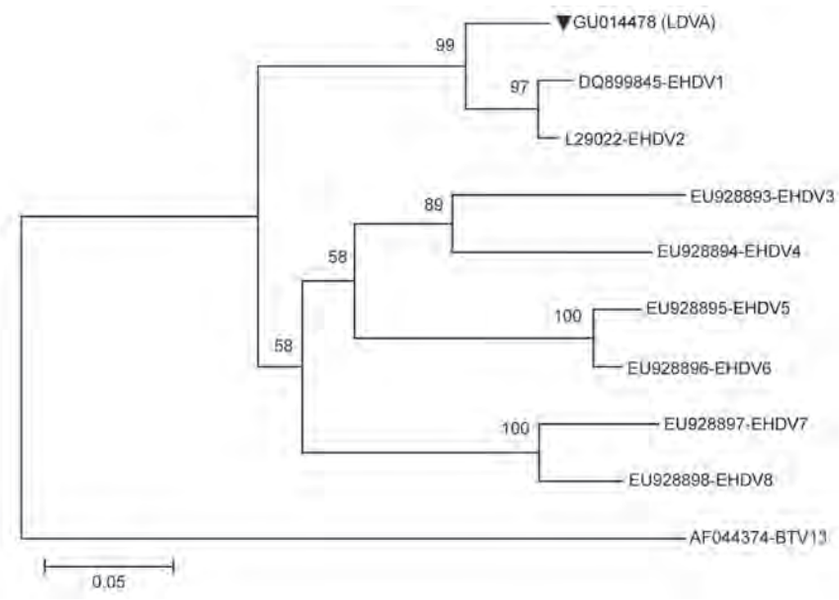

Figure. Phylogenetic tree based on a 244-bp epizootic hemorrhagic disease virus (EHDV) NS3 gene. GU014478 (GenBank accession no.; LDVA) strain (triangle) isolated from pygmy brocket deer in the present study (southern Brazil, 2008), New Jersey strain DQ899845 (EHDV1), Alberta strain L29022 (EHDV2), Nigeria strain EU928893 (EHDV3), Nigeria strain EU928894 (EHDV4), Australia strain EU928895 (EHDV5), Australia strain EU928896 (EHDV6), Australia strain EU928897 (EHDV7), Australia strain EU928898 (EHDV8), and bluetongue virus (BTV) 13 outgroup USA strain AF044374. Scale bar indicates nucleotide substitutions per site.

88% were seropositive for BTV, and 60% were seropositive for both viruses (*7*). Deaths of 6 marsh deer that showed clinical signs and gross and microscopic lesions resembling EHDV/BTV infection were described, but not confirmed, in São Paulo (*8*). Although EHD is endemic in the country (*7*,*8*), in the southern region of the Brazil, no serologic reports of EHDV have been made, and the serologic prevalence of BTV in domestic ruminants is low, ≈0.2% (*9*). Climatic factors can contribute to this distribution because temperatures in southern Brazil are low in autumn and winter and thus unfavorable to the development and survival of competent species of *Culicoides* biting midges. This low serologic prevalence probably produces an area of enzootic instability in which epidemic cases occur that are associated with the presence of infected vectors and susceptible animals (*9*).

In summary, we describe the isolation of EHDV that is involved in hemorrhagic disease affecting pygmy brocket deer in Brazil. The hazard that this virus infection could pose to the local and national Cervidae populations remains unknown, but it is noteworthy that *M. nana* deer are the most threatened species in Brazil (*10*). For better control and prevention measures to be developed, research is needed to characterize Brazilian EHDV isolates and variations in resistance at the species and subspecies level.

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Spillover: Animal Infection and the Next Human Pandemic

David Quammen

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Pages: 487; Price: US $28.95

Spillover is a single event during which a pathogen from 1 species moves into another species; such movement can result in an outbreak. In 9 chapters, David Quammen chronicles various spillover events by using personal anecdotes and multiple stories to recount these events for the expert and novice alike. He frames the events within an ecologic sense of the pathogen, the host, and the increasing human population. He focuses recurrently on the NBO (next big one) and how, if HIV or Ebola virus were more easily transmissible, no one would remain to read his book.

Quammen's analogies are superb. Instead of trying to turn the reader into a scientist with dry explanations, he uses analogies that have universal relevance. For viral morphology, Ebola and Hendra virions together would resemble a "capellini in a light sauce of capers." Mathematical modeling can be appreciated in translation, just as Dostoevsky can be appreciated in translation instead of in the original Russian. Quammen compares combining specific antibodies with their virus to splashing holy water on a witch. Regarding airborne transmission, he says that pathogens can "waft into a nearby village as easily as the pleasant, autumnal smell of smoke from a pile of leaves." Throughout the book, the subjects of human and animal diseases are ". . . strands of one braided cord."

The last chapter, "It Depends," is particularly sobering. If, in an ecologic sense, an outbreak is a rapid and explosive increase in the abundance of a particular species, then maybe humans are the current outbreak in the world. We have become a dense forest; tinder is dry; and the NBO is around the corner.

Who should read this book? Anyone interested in science can enjoy it—those who make their living at the bench, teach, or study—and anyone just looking for a good read.

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Georges de La Tour (1593–652) ***La Femme à la puce (The Flea Catcher)*** (1638) Oil on canvas (90 cm × 120 cm) Musée Lorrain, Nancy. Photo P. Mignot

The Iconography of Vermin

Polyxeni Potter

"Haughty, sharp-tongued, self-assured, unbearably self-sufficient, stingy, and violent beyond measure," is how Georges de La Tour was described by his contemporaries. Municipal records confirm that he refused to pay his share to feed the hungry during times of famine. He assaulted an officer, beat a peasant, and made himself obnoxious to everyone by "sending his dogs after hare into the standing crops which they trample down and ruin."

A "painter" is how he described himself in the marriage contract in 1617. Shortly after the wedding, he moved to Lunéville, a prosperous town near Nancy, in Lorraine, now France, where he lived and worked. His early life and training remain otherwise ambiguous. He was influenced by the style of Caravaggio, either from travel to Italy or from contact with the Dutch followers of the Italian master. He found fame and fortune in his lifetime and was known as "Painter to the King." He had 10 children, three of whom lived to adulthood. His son Étienne studied under him. La Tour died suddenly, possibly of the plague, within a few days of the deaths in his household of his wife and servant. He was soon forgotten to be discovered hundreds of years later and become an icon, anointed among the greats of his generation.

The 1630s was a turbulent period for Lorraine, a region contested by France and Germany for centuries. The 30 Years' War and consequent epidemics, famine, and destruction, compounded by a fire in 1638 that burned Lunéville to the ground, contributed to the loss of much of La Tour's legacy, as many as 400 works. A few remaining paintings were variously thought to be the work of Ribera, Zurbarán, Murillo, Velázquez, Rembrandt, and always, Caravaggio. Some paintings are still emerging from oblivion. *The Flea Catcher*, on this month's cover, was not attributed to him until 1955.

In following Caravaggio, La Tour rejected Baroque classicism, the art movement of his age. He abandoned architectural backdrops and complicated scenes for solitary figures in dark tones. But his kinship with the master went beyond the dramatic chiaroscuro. The two shared, along with a troubling inability to cope with ordinary life, an incongruous gift for capturing its poetry on canvas. Well acquainted with darkness in the world, they brought into their paintings light. And though their choice of subjects wandered into the rogue—criminals, thieves, beggars—the light cast an aura of spirituality, driving the mood, the character, and the message.

In a departure from Caravaggio, La Tour introduced in his works the actual source of light, usually a candle, and became famous for his religious night scenes, often referred to as nocturnes. In these, he moved away from the traditional halos and wings, injecting an earthy holiness into his figures: Sebastian, patron saint of plague victims, pious women who nursed the wounded, and several versions of penitent Mary Magdalene. Alternating light and dark built mystery and stillness into these scenes, which, stripped of extraneous background, attained an almost geometric simplicity well ahead of the times.

The Flea Catcher has been unanimously accepted as the work of La Tour, although initially the subject matter caused confusion. Seventeenth-century art was filled with flea-searching figures painted by European masters. The Dutch often included daily bodily search for parasites in their repertoire, but flea and louse iconography was not part of French art. And although clearly in La Tour's nighttime style, *The Flea Catcher* is different from his other works and from Dutch works on this subject. True to genre, the Dutch offerings were playful tongue-in -cheek, even erotic presentations, along the lines of, if not inspired by, poetry from Ovid to John Donne. "Madam, that flea, which crept between your breasts / I envied that there he should make his rest; / The little creature's fortune was so good / That angels feed not on so precious food."[1]

The phase of La Tour's career during which *The Flea Catcher* was painted is not known, nor are the circumstances of naming the work. The dating is also approximate. Its execution, whether in the 1630s or 1640s, did coincide with instances of the plague in Lorraine and with troop movements through the region. But the cause of plague was unknown. Flea hunting was a mindless sport intended to

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[1]"On a Flea on His Mistress' Bosom," ascribed to John Donne.

provide relief from annoying bites and itching. And, of course, there was the issue of cleanliness.

The Dutch were notorious advocates of cleanliness. Gerard ter Borch and others often paid tribute to the dictum "spiritual purity starts with a clean body." A woman was considered the "moral laundress" of the household and the guardian of proper child care: "Lazy mother, lousy heads." Although French artists were not under as much pressure as the Dutch to couch moral messages in genre scenes, a moral or spiritual interpretation of La Tour's woman's search for fleas is intriguing. Certainly morality is present in the background of other well-known La Tour paintings (*The Fortune Teller, The Card Sharp with the Ace of Clubs*). In *The Flea Catcher,* the woman's focus on the task rivals any Dutch example. And whatever it may have lost from abandoning the lighthearted approaches preceding it, this work made up in silence and intensity.

Unlike La Tour's other work, mostly religious and genre scenes, *The Flea Catcher* has a complicated, even mysterious, aura. Not so much in its intimacy and introspection, which are found in most all his work, but in the discrepancy between these and the mundane task described. The enigmatic nature of the painting has attracted multiple interpretations. Some observers view the flea crushing as ancillary to the quiet contemplation and sadness implicit in the figure's posture. Others take a religious approach. They see a fallen woman, possibly expecting a child, a penitent Magdalene pondering the excesses of her past life. Some viewers sense spiritual contemplation, a form of asceticism that comes from introspective concentration on a mundane task. And yet others suggest that the woman is not hunting fleas at all but praying the rosary or inspecting her garment, as the candlelight may be attracting fleas. Whatever the interpretation, the staging is pure La Tour: passive with an air of personal transcendence.

In this treatment of flea hunting, the viewer is given privileged access. The setting is sparse, fluid, linear, and intimate. The woman in plain wrap is in a stage of undress, her hair concealed, head downcast, bust and chest pathetically exposed, belly engorged. The hands, frozen in the all too familiar flea-crushing curl, are tightened to trap the undesirable guest between the thumbnails. In a characteristic maneuver, La Tour contrasts the impassive face with active, engaged hands. A smoking flame, the artist's signature, exudes an otherworldly calm as it exposes red tones in the adjacent chair and settles lightly on the woman's face. The emphasis here is not on charm but on drama, and the intense introspection implies deeper concerns.

Historical and religious references aside, La Tour's *femme à la puce* begs for a contemporary interpretation, one informed by current knowledge of fleas, plague, and human frailty relieved by introspection. For as she absently tried to rid her body of fleas, La Tour's woman became one more human protest against the ubiquitous pests. Whatever the distracting affliction she might have contemplated during her hunt, it would pale in the face of what she did not know about their behavior as vectors of disease.

Long a favorite of poets, the flea was an allusion to all sorts of mischief, a kind of literary pet. "If you turn me into anything, let it be in the likeness of a little pretty frisking flea that I might be here and there and everywhere."[2] Far from its reputation during La Tour's lifetime as a harmless, even amusing insect, the flea has since emerged as enemy of public health, no less from its role in the spread of plague, a most dreaded scourge.

Fleas may be some of the most modern insects. Part of their success as vectors comes from their strong, but not absolute, preference for the blood of a good reservoir species (rodents, especially rats globally, prairie dogs and squirrels in the United States), along with a willingness to feed on humans when opportunity and need arise. Fleas that prefer prairie dog blood will feed on and infect a person who wanders through a prairie dog village after a plague-associated prairie dog die off. This less than desirable feed transforms an unrecognized sylvatic cycle into human illness and death. Fleas have demonstrated admirable staying power, causing pandemics, beginning in antiquity, and maintaining to this day a sylvatic cycle that bubbles along with few or no human cases but does not go away.

What does La Tour's downcast woman have to do with modern fleas? She is collateral damage to a natural cycle of infection that engages rodent reservoir hosts and the flea vectors. Whatever her emotional situation, it probably had less to do with her worth as a person than with her status in the world, as misfortune generally does. She is in some way as much a host of her calamity as of the flea between her thumbs.

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[2]*Doctor Faustus*. Christopher Marlowe (1588–1592).

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Article Title:
Eastern Equine Encephalitis in Children, Massachusetts and New Hampshire, USA, 1970–2010

CME Questions

1. You are evaluating a 10-year-old girl with a 2-day history of fever and malaise. The girl's parents are worried because of reports of 2 confirmed cases of Eastern equine encephalitis (EEE) in the state during the past 3 weeks. Based on the current study by Silverman and colleagues, which of the following statements regarding the clinical presentation of EEE is most accurate?

A. Cases were diagnosed between November and February
B. Half of patients had fever at the time of presentation for medical care
C. All patients had symptoms or signs of meningoencephalitis at the time of presentation for medical care
D. The mean age of patients was approximately 10 years

2. What should you consider regarding findings on ancillary studies of children with EEE in the current study?

A. Neutrophilic cerebrospinal fluid (CSF) pleocytosis was present in all patients
B. CSF protein levels were generally normal
C. Epileptiform activity was universal among patients who received EEG
D. Lesions were most common in the pons on neuroimaging studies

3. The patient is diagnosed with EEE. Which of the following variables was most important in predicting a worse outcome in the current study?

A. Younger age
B. Seizure at the time of presentation
C. Higher levels of total protein in CSF
D. Shorter prodrome

4. The patient's parents are very concerned regarding the prognosis for their daughter. What was the most common outcome of EEE in the current study?

A. Death or severe neurologic deficits
B. Some form of mild neurologic impairment only
C. Seizure disorder only
D. Complete recovery with no sequelae

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: Laboratory-based Surveillance for Hepatitis E Virus Infection, United States, 2005–2012

CME Questions

1. You are a consultant advising an HMO regarding the percentage of hepatitis E among US patients with hepatitis. Based on the study by Dr. Drobeniuc and colleagues, which of the following statements would most likely appear in your report?

A. Hepatitis E was present in more than half of patients who were seronegative for acute hepatitis A and B
B. Among patients with hepatitis E, only one quarter had recently traveled abroad
C. Among patients with hepatitis E, half the patients had acute and half the patients had chronic hepatitis
D. Hepatitis E virus (HEV) infection was determined by testing for IgM and IgG anti-HEV and for HEV RN

2. Based on the study by Dr. Drobeniuc and colleagues, which of the following statements about group characteristics of nontravelers vs travelers with hepatitis E is most likely correct?

A. Nontravelers were older than travelers
B. Nontravelers were more likely than travelers to be jaundiced
C. Nontravelers comprised fewer South Asians than travelers
D. Nontravelers were less likely than travelers to be solid organ transplant recipients

3. Based on the study by Dr. Drobeniuc and colleagues, which of the following statements about HEV genotypes among nontravelers vs travelers with hepatitis E is most likely correct?

A. Nontravelers were infected exclusively by HEV genotype strains
B. Nontravelers were infected by HEV genotype 3 and 4 strains
C. Travelers were infected exclusively by HEV genotype 3 strains
D. The findings suggest that the nontravelers were infected by HEV that was circulating autochthonously in the United States.

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Measles Elimination Efforts and 2008–2011 Outbreak, France

Parallels in Amphibian and Bat Declines from Pathogenic Fungi

Treatment Outcomes for Extensively Drug-Resistant Tuberculosis and HIV Co-infection

Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities, United States, 1998–2008

Epidemiologic Trends of Human Leptospirosis, the Netherlands, 1925–2008

Increasing *Pneumocystis* Pneumonia, England

Fly Screens and *Campylobacter* spp. Prevalence among Chickens

Emergence and Spread of Extensively Drug-Resistant and Totally Drug-Resistant Tuberculosis, South Africa

Clinical and Therapeutic Features of Pulmonary Nontuberculous Mycobacterial Disease, Rio de Janeiro, Brazil, 1993–2011

Effects of the Pandemic Influenza A (H1N1)pdm09 Virus Vaccine Program, United States, 2009–10

Tuberculosis and HIV Syndemic, California, 1993–2008

Lack of Norovirus Replication and Histo-Blood Group Antigen Expression in 3-Dimensional Intestinal Epithelial CellsDispatches

Mycobacterial Lineages Causing Pulmonary and Extrapulmonary TB in Ethiopia

Ecology of Swine Influenza in Sri Lanka

Tuberculosis in a Primary School, Milan, Italy

Vibrio cholerae Non-O1, Non-O139 Serogroups and Cholera-like Diarrhea, Kolkata, India

Unexpected Increase of Alveolar Echincoccosis, Austria, 2011

Lymphogranuloma Venereum in Men Screened for Pharyngeal and Rectal Infections, Germany, 2009–2010

Cryptococcus gattii, Florida

Multidrug-resistant *Mycobacterium tuberculosis*, Somalia

Coronaviruses associated with Human Betacoronavirus 2c EMC/2012 in African and European Bats

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

Upcoming Infectious Disease Activities

February 15–18, 2013
4th International Meeting on Emerging Diseases and Surveillance (IMED)
Vienna, Austria
http://imed.isid.org

February 25–27, 2013
2013 ASM Biodefense and Emerging Diseases Research Meeting
Washington, D.C.
www.asmbiodefense.org

March 3–7, 2013
The Conference on Retroviruses and Opportunistic Infections (CROI) 2013
Georgia World Congress Center
Atlanta, GA, USA
http://www.retroconference.org

May 1–4, 2013
The Society for Healthcare Epidemiology of America (SHEA)
Spring 2013 Conference
Atlanta, GA, USA
http://shea2013.org

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

September 10–13, 2013
ICAAC 2013 (Interscience Conference on Antimicrobial Agents and Chemotherapy)
Denver, Colorado, USA
http://www.icaac.org

Announcements

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Announcements may be posted on the journal Web page only, depending on the event date.

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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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 http://wwwnc.cdc.gov/eid/pages/author-resource-center.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 (http://wwwnc.cdc.gov/eid/pages/translations.htm).

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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. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

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 in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

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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.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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 (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

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.

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