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Zoonotic Infections

December 2015

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Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30329-4027, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

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On the Cover

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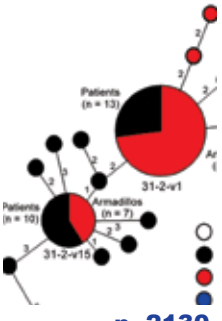
About the Cover p. 2282

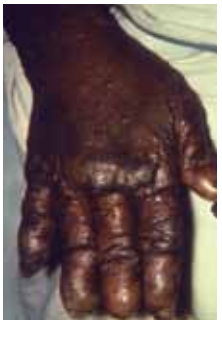
Synopses



p. 2130

Research

p. 2134



Historical Review

Dispatches

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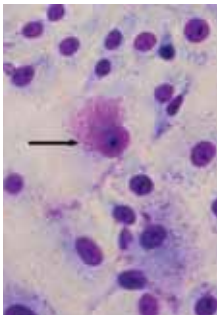

p. 2162

p. 2165

Medscape EDUCATION ACTIVITY

Letters

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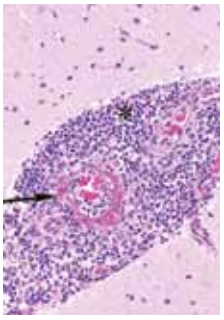
December 2015



p. 2183

p. 2210

Books and Media

About the Cover

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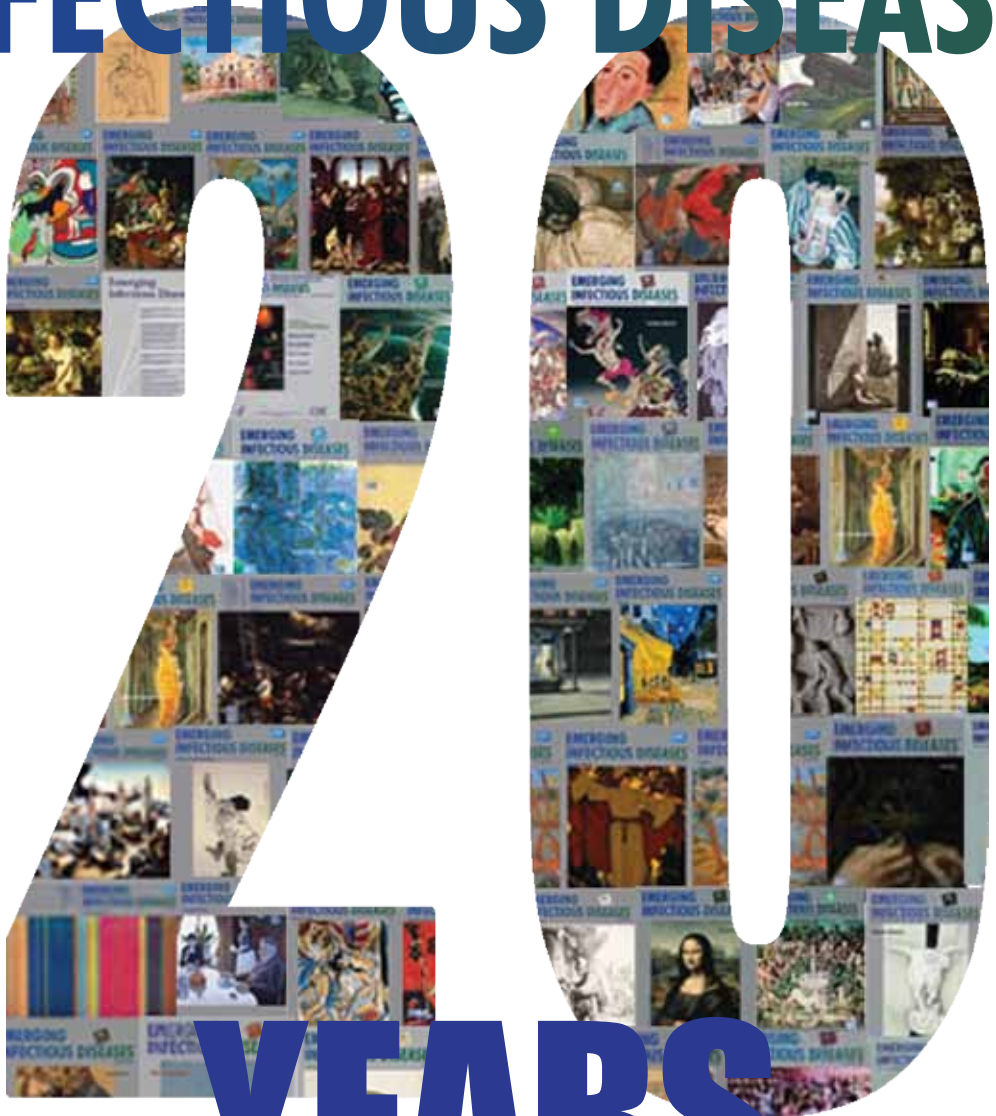

20 YEARS

Presenting the ongoing challenges that emerging microbial threats pose to global health

Identifying and Reducing Remaining Stocks of Rinderpest Virus

Keith Hamilton,[1,2] Dawid Visser,[1] Brian Evans, Bernard Vallat

In 2011, the world was declared free from rinderpest, one of the most feared and devastating infectious diseases of animals. Rinderpest is the second infectious disease, after smallpox, to have been eradicated. However, potentially infectious rinderpest virus material remains widely disseminated among research and diagnostic facilities across the world and poses a risk for disease recurrence should it be released. Member Countries of the World Organisation for Animal Health and the Food and Agricultural Organization of the United Nations are committed to destroying remaining stocks of infectious material or ensuring that it is stored under international supervision in a limited number of approved facilities. To facilitate this commitment and maintain global freedom from rinderpest, World Organisation for Animal Health Member Countries must report annually on rinderpest material held in their countries. The first official surveys, conducted during 2013–2015, revealed that rinderpest material was stored in an unacceptably high number of facilities and countries.

Rinderpest, also known as cattle plague, is a highly contagious viral disease of cattle. Until global freedom from rinderpest was declared in 2011, it was one of the most devastating and feared infectious diseases of animals (*1*). Infection with rinderpest virus (a morbillivirus) led to severe illness and death. Mortality rates in susceptible cattle populations could exceed 90%. Outbreaks have led to food shortages, economic losses, social unrest, and disrupted transport networks in regions where agriculture was dependent on draft cattle (*1*).

It has been suggested that rinderpest originated in central Asia. Over the centuries, the disease swept through Asia and was subsequently introduced into Africa, resulting in "the great African rinderpest pandemic of the 20th century" (*2*,*3*). Apart from an isolated outbreak in Brazil in 1920 and one in Australia in 1923, rinderpest has not affected countries in the Americas or Australia (*4*).

During the 20th century, control efforts became better coordinated and more effective, greatly facilitated by the availability of improved diagnostics and vaccine technologies (*5*). After a concerted international eradication campaign, success was finally achieved at the beginning of the 21st century; global freedom was declared in 2011, a decade after the last reported case of rinderpest had been detected in wildlife in Kenya in 2001 (*6*). After smallpox, rinderpest is the second infectious disease to have been eradicated through the efforts of mankind.

Throughout the eradication campaign, in affected and nonaffected countries, rinderpest material became widely disseminated in diagnostic laboratories, vaccine production facilities, and research institutes. While efforts were focused on eradication, less thought was probably given to what would happen to this material after eradication. In 2015, although natural infections in animals have been eradicated, live rinderpest virus, vaccines, and genetic material remain stored in scientific institutes across the world.

Today an outbreak of rinderpest could occur only if infectious material held in these laboratories and other institutions were accidentally released into a susceptible animal population or if animals were deliberately infected. The social and economic effects of a recurrence for the international community would be substantial. Vaccination against rinderpest has been prohibited (*7*). Therefore, cattle populations are fully susceptible and infection would spread rapidly if the virus were reintroduced. Recurrence of the disease would seriously damage agricultural economies, would paralyze trade in animals and animal products in affected regions, and would undermine the decades of investment and effort that went into its eradication. Accidental inoculation of cattle with a rinderpest vaccine would also be disruptive because the detection of seropositive animals would lead to suspicion of rinderpest recurrence (*7*). To ensure that rinderpest remains confined to the history books, international efforts are now focused on ensuring that all remaining stocks of infectious material are destroyed or stored safely in a minimum number of approved high-containment facilities.

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DOI: http://dx.doi.org/10.3201/eid2112.150227

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In 2010, for the purpose of regulating postrinderpest eradication activities, the World Organisation for Animal Health (OIE) and the Food and Agricultural Organization of the United Nations (FAO) Advisory Committee (a specialist body of selected laboratory and rinderpest experts) described potentially infective material (the material that needs to be regulated and safeguarded to prevent a recurrence) as "rinderpest virus–containing material." The material was defined as follows: rinderpest virus–containing material means field and laboratory strains of rinderpest virus; vaccine strains of rinderpest virus including valid and expired vaccine stocks; tissues, sera, and other clinical material from infected or suspect animals; and diagnostic material containing or encoding live virus. Recombinant morbilliviruses (segmented or nonsegmented) containing unique rinderpest virus nucleic acid or amino acid sequences are considered to be rinderpest virus. Full-length genomic material, including virus RNA and cDNA copies of virus RNA, is considered to be rinderpest virus–containing material. Subgenomic fragments of morbillivirus nucleic acid that are not capable of being incorporated in a replicating morbillivirus or morbillivirus-like virus are not considered as rinderpest virus–containing material (*8*).

Hereafter, we refer to the above-described material as "rinderpest material." By adopting 3 resolutions (nos. 18, 23, 25), all OIE Member Countries committed to destroying remaining stocks of rinderpest material or ensuring that the material would be stored securely in a minimum number of approved facilities (*8,9*) (http://www.oie.int/en/about-us/key-texts/resolutions-and-recommendations/resolutions-adopted-by-the-oie-international-committee/). The OIE and the FAO launched a work program to help Member Countries fulfill this commitment.

To safeguard remaining rinderpest material and to facilitate and monitor its destruction, knowledge of where the material is stored and close monitoring of the status of these stocks are crucial. Updated international standards on rinderpest in the OIE Terrestrial Animal Health Code make it a legal requirement for countries to report annually to the OIE on the nature, whereabouts, and quantity of rinderpest material held in each country (*7*).

During 2013–2014, the OIE conducted the first official survey to identify the precise location of remaining stocks of rinderpest material, and during 2014–2015, the second official survey was conducted. This article summarizes the results from these 2 surveys.

Methods

The following countries were selected to participate in the survey: 180 OIE Member Countries and Territories as of 2014 (which includes all countries that have a large livestock population) (*10,11*), and 2 other non–OIE Member Countries that may potentially have held rinderpest material. When the first survey was initiated in 2014, there were 178 OIE Member Countries; however, 2 additional countries were adopted as OIE Members in May 2014, bringing the total to 180 before the survey was completed. These 2 countries had submitted reports as non-OIE Members in 2014 before their adoption as Members.

Data on the number of countries that had reported an outbreak of rinderpest and the date of the last reported infection were collected from the OIE World Animal Health Information Database (*4*). To maintain confidentiality and to prevent identification of individual countries, the data in this article have been anonymized.

To facilitate reporting, OIE developed a standard questionnaire and a secure electronic system for returning completed questionnaires. A username and unique secure password were issued to the OIE Delegate in each Member Country and the Delegate identified as the responsible person in the National Veterinary Service to oversee completion of the questionnaire. The OIE Delegate is the official OIE representative for an OIE Member Country and is usually the Chief Veterinary Officer or equivalent.

Countries were required to answer an introductory question: "Does your country currently hold rinderpest virus–containing material?" Only 1 of 4 predetermined options could be selected: "yes," "no," "unknown," or "never held rinderpest virus–containing material." Responders who answered "yes" were asked to provide further information, including details about the nature and quantity of rinderpest virus held, the name and address of the facility where it was held, and the biosafety/biocontainment level of the facility. When 1 of the other 3 options (no, unknown, or never held) was selected, then no further responses were required, and the questionnaire was considered complete.

Countries that reported having rinderpest material were required to provide details about the nature and quantity of material held for the following categories:

- live virus, including field isolates and genetically modified viruses but excluding stocks of approved/registered vaccines;
- vaccine stocks, including seed stocks;
- other potentially infectious materials;
- other noninfectious rinderpest virus–containing materials.

Responders were asked to provide information about rinderpest material currently held, material destroyed during the previous 12 months, and material that had been transferred to or from another institute. Questions also asked whether the institute had conducted any manipulation of rinderpest material in the previous 12 months and whether they intended to destroy material or transfer it to another institute for safer keeping.

After responders had submitted the completed questionnaire to the OIE, they still had access to their respective completed questionnaire in a noneditable PDF format. If institutes held a large number of different strains of virus, or types of tissue, then the country could return information in an Excel (Microsoft, Redmond, WA, USA) spreadsheet format.

The OIE Terrestrial Animal Health Code specifies that the deadline for submitting the annual OIE rinderpest report each year is the end of November (*7*). However, because countries were unable to meet this deadline, deadlines for the first 2 surveys were extended until May 26, 2014, and June 11, 2015, respectively. A weekly Excel report was exported from the electronic database for evaluation of information received and to enable the OIE to follow up on erroneous reports and nonresponders. Weekly follow-up with nonresponders included telephone calls and email correspondence. When countries were unable to use the electronic reporting system, they were asked to submit a hard copy (paper) report to the OIE on a template provided. Countries were given 2 opportunities to validate their data: 1) Member Countries that had submitted their report to the OIE were sent a noneditable PDF version of their completed questionnaire and asked to confirm the accuracy of their data; and 2) during the May 2014 and May 2015 OIE General Sessions, all Member Countries were given the complete datasets and a final opportunity for comment.

Results

For the first survey (completed in 2014), 171 (95%) of 180 OIE Member Countries responded to the survey, and for the second survey (completed in 2015), all 180 OIE Member Countries responded. Additionally, 2 countries that are still (as of September 2015) not OIE Members reported in 2014 but they did not report again in 2015. In 2014, of the 173 countries that responded to the survey, 23 (13.3%) reported holding stocks of rinderpest material; and in 2015, of 180 countries, 24 (13.3%) reported holding stocks of rinderpest material. All countries that reported holding stocks of rinderpest material in 2014 reported still holding rinderpest material in 2015. One country that had reported not holding stocks of rinderpest material in the 2014 survey subsequently discovered that it did hold stocks and reported holding rinderpest material in 2015. For 1 country that reported in both surveys that it held rinderpest material, whether the material (a subgenomic fragment of DNA) constituted the intended meaning of rinderpest material was in doubt. All countries that reported holding stocks of rinderpest material were OIE Member Countries.

Of the 24 countries that reported holding rinderpest material, 1 country reported holding it in 5 institutes in 2014; this country subsequently destroyed all the stocks that were held in 1 institute. Another country reported holding rinderpest material in 2 institutes in 2014 and consolidated the material to 1 institute in 2015. All other countries reported holding the material in only 1 institute. By 2015, a total of 27 institutes in 24 countries reportedly held rinderpest material. In 9 facilities, rinderpest material was stored at Biosafety Level 2 (BSL-2), and in 18 facilities the material was stored at BSL-3 or BSL-4.

The regions with the greatest number of institutes holding rinderpest material were Asia, Pacific, and Oceania (*9*), followed by Africa (*7*), Europe (*7*), the Americas (*3*), and the Middle East (*1*). OIE regions are described at http://www.oie.int/en/about-us/wo/regional-commissions/.

At least 23 of the 24 countries reporting having rinderpest material held the live virus (including wild strains of virus, vaccine seed virus, and attenuated virus). Because of the questionnaire design, it was not possible to differentiate between vaccine seed strains and packaged and manufactured vaccine, unless this differentiation was specified by the country. Also not provided by some countries was complete information on passage history of virus isolates. By 2015, a total of 22 facilities indicated that they stored vaccine seed virus (which would be classified as live virus). According to data provided in 2015, a total of 7 facilities indicated that they stored manufactured and packaged vaccine.

Discussion

The annual rinderpest survey serves several purposes that support and facilitate the destruction and safeguarding of remaining stocks of potentially infective rinderpest material.

- It identifies the whereabouts of remaining rinderpest material so that action can be taken to ensure that these stocks are destroyed or stored safely.
- It monitors and evaluates progress of the rinderpest destruction and sequestration program.
- It locates stocks of rinderpest vaccine that could be mobilized in the event of a recurrence of disease.
- Because the whole dataset is shared with all OIE Member Countries, it is hoped that transparency will encourage OIE Member Countries to comply with their commitment to destroy stocks or to store them safely.

As of June 2015, the survey response rate was 100%, indicating that all OIE Member Countries had fulfilled their obligation to report on remaining stocks of rinderpest virus. Four years after the declaration of global freedom, rinderpest material remains stored in at least 27 facilities in 24 countries. Responses indicate that one third of these stocks are stored in facilities equivalent to BSL-2. Considering the potential consequences of a recurrence of rinderpest, this situation represents an unnecessarily high risk.

The data obtained from the surveys may underestimate the real number of facilities holding rinderpest

material because there are several potential sources of underreporting. Rinderpest material might be stored in some countries without the knowledge of the reporting authorities, which was confirmed when 1 country submitted a negative report in 2014 and a positive report in 2015. Countries with strong and well-governed official National Veterinary Services should have a system to regulate the shipment, handling, and storage of dangerous pathogens (rinderpest virus is considered a dangerous pathogen for animals). In theory, these systems should identify where stocks of rinderpest virus are being held; however, on a global level, National Veterinary Services are not universally strong. Many countries have a weak regulatory framework, and even in countries with a strong regulatory framework, mistakes occur. Therefore, a country's Veterinary Services may be unaware of material that is held in facilities outside of their direct jurisdiction, such as in universities or private laboratories. Also, samples containing rinderpest material may have been poorly identified or not included in a laboratory inventory. Pathogen inventories and quality management systems are unlikely to have been in place in all facilities receiving and storing rinderpest virus several decades ago. Archived pathology and surveillance samples collected from animals for reasons other than rinderpest diagnosis may also contain rinderpest virus if these samples were collected in areas where rinderpest was prevalent and stored under conditions suitable for virus survival.

All countries should be encouraged to continue to search for rinderpest material in any places where it may have been held with or without the institute's knowledge; such places might include laboratories outside the direct control of the official National Veterinary Services, including private laboratories and universities. Institutes that have less contact with National Veterinary Services might even be unaware that rinderpest has been eradicated and unaware of the international commitment to destroy or safeguard remaining stocks. The surprise discovery of smallpox virus at the US National Institutes of Health in 2014 highlights the possibility that unidentified dangerous material may lie in storage unnoticed for years and underscores the need to maintain current and accurate laboratory inventories (*12*).

In addition, no information is available about the viability of live virus for those countries that reported holding stocks of rinderpest material. For rinderpest virus to remain viable during storage over long periods, the material must be continuously kept under suitable conditions. Rinderpest virus is relatively labile, and some institutes with stores of rinderpest have probably experienced power supply disruption, leading to thawing and destruction of the virus. Therefore, some reported stocks of live rinderpest virus might not contain viable virus.

Two countries holding stocks of rinderpest virus have never experienced an outbreak of rinderpest. Because each of these countries has substantial agricultural and veterinary research sectors, it can be assumed that virus was held for research and for preparedness (e.g., diagnostics, vaccine manufacture) purposes. Other countries will have probably kept rinderpest material for the same reasons. However, in a postrinderpest era, the value and justification for maintaining rinderpest material for research are minimal.

The risk for pathogen release from containment laboratories into susceptible animal populations, albeit low, is real. This risk was highlighted in 2007 when a biosecurity breach at a site in the United Kingdom resulted in an outbreak of foot-and-mouth disease among cattle (*13,14*). Fortunately, infection was detected early and the source was identified quickly. These actions, combined with an effective response, prevented a wider outbreak, which could have severely hurt the economy (*15*). Tragically, the last case of smallpox was also caused by an escape of the virus from a laboratory (in Birmingham, UK, in 1978), resulting in a human death (*16*). Action must be taken to ensure that a future case of rinderpest does not occur through an avoidable laboratory escape of virus.

On a positive note, 6 institutes in 6 countries had destroyed some rinderpest material during 2013–2015. It is hoped that other countries holding rinderpest material will take similar action.

If countries comply with their commitment to destroy rinderpest material or ensure that it is secured in 1 of the facilities approved by the OIE and FAO, the risk for disease recurrence after a laboratory escape or deliberate release of virus can be substantially reduced. This risk can be further reduced if all known stocks of potentially infective rinderpest material (particularly live virus) worldwide are totally destroyed. To address concerns about the loss of historical data, entire genes of rinderpest virus isolates could be sequenced before destruction—a process commonly referred to as sequence and destroy—and archived with data about the epidemiology and pathology of those viruses. The sequence-and-destroy procedure for rinderpest virus will be used as an additional incentive to encourage scientists to destroy high-risk biological material while retaining academic and historical data that may have research value.

OIE Member Countries should fulfill their international obligation to continue to report to the OIE on an annual basis so that the OIE can monitor and transparently report progress on sequestration and destruction over time. The OIE and FAO have been working with the World Health Organization to apply lessons learned from the smallpox posteradication era to rinderpest. It is hoped that the experience gained during the rinderpest posteradication era will support future programs for eradication of other diseases.

Acknowledgments

We thank the OIE Delegates of all OIE Member Countries who submitted data to the OIE, Bertrand Flahault for his development of the electronic reporting system and database, Stephanie Beau for administrative support, and Michael Baron for his help with survey design.

Although this survey was conducted solely within the framework of the OIE, we acknowledge the partnership between OIE, FAO, and the FAO-OIE Joint Advisory Committee, which contributes to other postrinderpest eradication activities.

Dr. Hamilton is a veterinarian specializing in disease control. At the time of the study, he had been working with the OIE in the field of biological threat reduction. He is now working at Kansas State University.

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Opportunistic Pulmonary *Bordetella hinzii* Infection after Avian Exposure

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We report 2 cases of pulmonary *Bordetella hinzii* infection in immunodeficient patients. One of these rare cases demonstrated the potential transmission of the bacteria from an avian reservoir through occupational exposure and its persistence in humans. We establish bacteriologic management of these infections and suggest therapeutic options if needed.

Bordetella hinzii bacteria cause respiratory infections in birds and have been isolated from rodents on rare occasions (*1*,*2*). Pulmonary infection, digestive infection, and bacteriemia in humans have been reported (*3*–*5*). *B. hinzii* can persist for ≥1 years in the respiratory tract of humans (*5*), but its transmission from birds has not been proven. Through 2 new cases and a brief review of the literature, we show a possible association between occupational risk and pulmonary colonization by *B. hinzii*. Then, we suggest how to manage these infections in humans, although pathogenicity of this bacterium remains unclear.

Clinical Cases

Case-Patient 1

In April 2013, a 43-year-old man was admitted to the Pneumology Service of the University Hospital Centre of Rennes (Rennes, France) because of fatigue, fever, and exacerbation of bronchiectasis. He had undergone an allograft bone marrow transplantation and therapy with corticosteroids and chemotherapy for an acute myeloid leukemia diagnosed 15 months earlier but was in remission during the infectious episode. After the leukemia was diagnosed, he stopped working; his occupation had involved cleaning pipes and waste tanks with high-pressure water in poultry abattoirs.

The patient was a former smoker with a medical history of type 1 diabetes, vascular hypertension, and nonsymptomatic chronic bronchiectasis before the allograft. He was hospitalized for 2 episodes of pulmonary infections in October 2012 and February–March 2013, during which *Escherichia coli* was isolated and for which he received ciprofloxacin.

On admission, he was febrile (39°C) despite chemoprophylaxis with trimethoprim/sulfamethoxazole, posaconazole, and valaciclovir. The patient had dyspnea and dry cough without sputum production. Physical examination found crackles at bases of both lungs. Thoracic radiograph showed a cardiomegaly, an interstitial syndrome, and pleural effusion.

Laboratory analyses showed anemia (hemoglobin 92 g/L [reference range 130–170 g/L]) and hyperleukcocytosis (12.4 G/L [reference range 4–10 G/L]) with 8.3 G/L (reference range 2–7.5 G/L) polynuclear neutrophils (PNN). The inflammatory syndrome was confirmed by the elevated C-reactive protein concentration (44.7 mg/L [reference range <5 mg/L]). Calculated antimicrobial therapy was started on the second day after admission with piperacillin/tazobactam and ciprofloxacin.

Bacteriologic cultures of sputum collected on admission yielded 10^9 CFU/mL *B. hinzii*. Bronchial aspiration and bronchoalveolar lavage fluid were collected for microbial investigations 2 days later and showed 10^7 CFU/mL *B. hinzii* and 3×10^5 CFU/mL *Staphylococcus epidermidis*, respectively. Polymicrobial flora were present in all cultures performed, indicating that those samples were contaminated by oropharyngeal flora.

Laboratory analyses for nocardiosis, pneumocystosis, aspergillosis, and tuberculosis were negative. Multiplex PCR for herpes simplex virus, varicella zoster virus, cytomegalovirus, and Epstein-Barr virus and culture for influenza viruses A and B, human herpesvirus 6, adenovirus, metapneumovirus, and parainfluenza viruses 1–3 did not detect any of these viruses.

Because of the persistence of symptoms, the patient's antimicrobial therapy was changed, following a decision

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DOI: http://dx.doi.org/10.3201/eid2112.150400

after 5 days to target *Staphylococcus*, to parenteral vancomycin for 10 days. Clinical improvement of pulmonary signs and symptoms was observed after 15 days. In addition, physical rehabilitation was initiated to support the malnutrition and muscle atrophy. The patient returned home after 1.5 months of rehabilitation.

Case-Patient 2

In September 2014, a 74-year-old man hospitalized in a private clinic for partial laryngectomy and a tracheotomy because of a second recurrence of laryngeal cancer was transferred to the intensive care unit of the University Hospital Center of Bordeaux (Bordeaux, France) for decompensation of chronic obstructive pulmonary disease 11 days later. His medical history included vascular hypertension, dyslipidemia, prostate cancer in 2007 that required total surgical excision, and ischemic heart disease in 2011. He also had chronic obstructive pulmonary disease that had not been documented or treated and a laryngeal cancer for which he underwent a cordectomy in 2002 and a partial laryngectomy in 2007. The patient was a former airline pilot living in the city; he had not had any pets for many years and had had only rare contact with poultry during childhood.

On admission, he had fever (38.7°C), respiratory distress with hypoxemia, and purulent tracheal secretions discharging from the tracheotomy orifice. Physical examination found a high heart rate (107 bpm). Pulmonary auscultation was normal. Thoracic radiograph showed systematic alveolar images on the right. Results of laboratory tests revealed anemia (88 g/L [reference range 130–170 g/L]) and an inflammatory syndrome with hyperleucocytosis (11.8 G/L [reference range 4–10 G/L]) with 11.3 G/L (reference range 2–7.5 G/L) PNN.

Tracheal aspiration was performed on admission and bronchoalveolar lavage 6 days later and samples were cultured. Tracheal secretions yielded 10^6 CFU/mL *B. hinzii* and 6×10^7 CFU/mL methicillin-resistant *S. aureus*. Bronchoalveolar lavage fluid gave 9×10^2 CFU/mL *B. hinzii* and 10^2 CFU/mL methicillin-resistant *S. aureus*. *B. hinzii* was also cultured on ESBL medium (bioMérieux, Marcy l'Etoile, France) from a rectal swab sample taken for systematic research for the carriage of resistant bacteria.

Antimicrobial therapy was started on admission to intensive care with piperacillin/tazobactam (7 days) and vancomycin (11 days). The patient was transferred after 14 days, following clinical pulmonary recovery, to the private clinic for otorhinolaryngeal care.

Microbiological Investigations

Direct microscopic examination of specimens showed substantial presence of PNNs and gram-negative short bacilli, except in the bronchoalveolar lavage fluid from case-patient 2, in which no bacteria was observed. In both case-patients, colonies were apparent after 24 or 48 hours of incubation (37°C, 5% CO_2) on PolyViteX chocolate agar. In case-patient 2, they also grew on trypcase soy agar with 5% horse blood and Haemophilus chocolate agar with bacitracin (bioMérieux). The colonies were medium sized (1–2 mm), smooth, round, convex, and grayish; those from case-patient 2 were very mucoid. Microscopic examination of colonies showed gram-negative short bacilli. Identification was inconclusive with Vitek 2 system IdGN cards (bioMérieux) and RapID NH (Remel, Lenexa, KS, USA). The Api 20NE strip (bioMérieux) identified *B. avium* based on the score 0000067 with high percentage (96.7%) and typicality (T = 1). Routine identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Brucker Daltonik GmbH, Germany) gave *B. hinzii* with good scores (T = 2.29 for case-patient 1 and T = 2.35 for case-patient 2). Final identification for both cases involved amplification and sequencing of 1,480 nt of 16S rRNA gene, then use of the blastn program (http://www.ncbi.nlm.nih.gov/BLAST/) and the GenBank database. The nucleotide sequences showed the best BLAST hit with *B. hinzii* (99.9% homology in both cases). Antimicrobial susceptibility testing was performed on Mueller-Hinton medium by an Etest method (bioMérieux) and MICs determined (Table).

Discussion

B. hinzii is found in the respiratory tract of poultry. Although it was initially described as a commensal bacterium in birds, some veterinary isolates appear to have pathogenic properties: Register et al. reported that it is associated with tracheal lesions in turkeys (*1*,*6*). *B. hinzii* has been sporadically described in rabbits (*6*) and rodents (*2*) and had been reported to cause pulmonary disease in laboratory mice (*7*) and bacteremia in rats (*2*).

Few cases have been described in humans. Most involved bacteremia or respiratory or digestive infections (*3*–*5*). *B. hinzii* was first isolated in 1957 from the sputum of a patient in France but was misidentified as *Alcaligenes faecalis* (*8*). In 1994, Cookson et al. described *B. hinzii* bacteremia in an AIDS patient (*4*). Two other cases of bacteremia in immunodeficient patients were associated with the isolation of *B. hinzii* from the pulmonary tract (*9*,*10*).

Two digestive infections have been reported. A fatal case was described in an immunocompetent man (*11*) with cholestasis and bacteremia. The second case was a biliary infection in a liver transplant recipient: *B. hinzii* was isolated from 4 bile samples during a 6-month period (*3*), demonstrating that colonization by *B. hinzii* can be long-lasting.

B. hinzii appears as an opportunistic pathogen causing respiratory infections in cystic fibrosis patients (*5*,*12*). Other respiratory infections in both immunodeficient and

Table. Antimicrobial drug susceptibility testing for human *Bordetella hinzii* isolates by the Etest method*

Antimicrobial drug	Case-patient 1†	Case-patient 2†	BC-306 (*11*)	BC-305 (*11*)	BL-3210 (*3*)	Hristov et al. isolate 1 (*10*)‡	Hristov et al. isolate 2 (*10*)‡	Hristov et al. isolate 3 (*10*)‡
Amoxicillin	ND	32	16§	16§	12§	ND	ND	ND
Amoxicillin–clavulanic acid	ND	ND	32	16	ND	ND	ND	ND
Ampicillin–sulbactam	ND	ND	16/8	16/8	ND	ND	ND	ND
Piperacillin	0.38	ND	ND	ND	ND	32	ND	ND
Piperacillin–tazobactam	0.25	0.75	1	1	ND	ND	ND	ND
Ticarcillin	ND	ND	ND	ND	ND	>32	>64	ND
Ticarcillin–clavulanic acid	ND	32	32	64	ND	ND	ND	ND
Cefoxitine	ND	>256	ND	ND	ND	ND	ND	ND
Cefotaxime	ND	>32	ND	ND	>32	ND	ND	ND
Ceftriaxone	ND	ND	64	64	ND	ND	ND	>32
Ceftazidime	1.5	2	4	4	4	ND	ND	ND
Cefepime	ND	6	8	8	ND	>16	>16	>16
Ertapenem	3	0.023	ND	ND	ND	ND	ND	ND
Imipenem	0.75	1.5	2	2	1	ND	ND	ND
Meropenem	1	0.125	ND	ND	ND	≤1	8	4
Doripenem	0.5	0.125	ND	ND	ND	ND	ND	ND
Aztreonam	ND	ND	ND	ND	ND	>16	>16	ND
Gentamicin	0.75	4	2	4	4	≤1	4	≤2
Tobramycin	4	48	ND	ND	ND	>8	>8	4
Amikacin	2	16	ND	ND	ND	16	≤4	≤8
Clarithromycin	4	ND	ND	ND	ND	ND	ND	ND
Clindamycin	>256	ND	ND	ND	ND	ND	ND	ND
Rifampin	1	>32	N	ND	ND	ND	ND	ND
Levofloxacin	>32	0.25	2	2	ND	ND	ND	ND
Ciprofloxacin	>32	0.75	4	4	>32	>2	>2	>2
Moxifloxacin	4	0.5	ND	ND	ND	ND	ND	ND
Cotrimoxazole	0.38	ND	0.047	0.023	ND	≤0.5/9.5	2/38	ND
Vancomycin	>32	>256	ND	ND	ND	ND	ND	ND
Teicoplanin	>32	>256	ND	ND	ND	ND	ND	ND
Daptomycine	>256	>256	ND	ND	ND	ND	ND	ND
Linezolid	>256	>256	ND	ND	ND	ND	ND	ND
Fusidic acid	>32	ND	ND	ND	ND	ND	ND	ND
Tetracyclin	ND	ND	ND	ND	0.38	ND	ND	ND
Minocyclin	1	ND	ND	ND	ND	≤2	>8	ND
Tigecyclin	0.5	0.38	ND	ND	ND	ND	ND	ND
Colistin	0.38	0.094	ND	ND	ND	ND	ND	ND
Fosfomycin	>1024	>1024	ND	ND	ND	ND	ND	ND

*Values are MICs as determined by Etest in μg/mL ND, not determined.
†This study.
‡Isolates 1, 2, and 3 of Hristov et al. case correspond to respiratory isolates (1 and 2) and blood isolate (isolate 3).
§Ampicillin rather than amoxicillin was tested.

immunocompetent persons have been reported. Gadea et al. isolated *B. hinzii* associated with *Nocardia asteroides* in a bronchoalveolar lavage sample from an AIDS patient (*13*). Palacián Ruiz et al. described a *B. hinzii* respiratory infection in an immunocompetent elderly woman that was associated with *Klebsiella oxytoca* (*14*). *B. hinzii* was isolated 8 times during a 1-year period from the respiratory tract of a cystic fibrosis patient (*5*).

B. hinzii infections are presumably underdiagnosed because of misidentification by the routine phenotypic identification procedures that are the basis for Phoenix (Phoenix BD Diagnostic Systems, Sparks, MD, USA) and Vitek (bioMérieux) automated systems and for the API 20NE strip manual system (bioMérieux). Indeed, *B. hinzii* is not referenced in the API 20NE database and often gives the same score as *B. avium*. The API 20NE database should be updated and the score 0000067 should be referred to as *B. avium–B. hinzii* complex. The mean score for 6 clinical isolates analyzed with the MALDI-TOF mass spectrometry system (Brucker Daltonik GmbH) by the French National Reference Centre of pertussis and other bordetelloses was T = 2.31, with T = 2.063 as the minimal score. MALDI-TOF is useful to discriminate *B. hinzii* from other *Bordetella* species (*15*). Sequencing the 16S rRNA gene is the most reliable technique to confirm the species, but the MALDI-TOF system is more suitable for routine identification and would enable more cases to be detected.

Although poultry seems to be the major reservoir (*1*), we cannot exclude the possibility that mammals such as rabbits and rodents also could be potential reservoirs (*2*,*7*). Humans can become infected by aerosols from the avian reservoir, which probably was the route for case-patient 1, who had a pulmonary infection long after exposure. Survival in the digestive tract is another specificity of *B. hinzii* among the *Bordetella* species. This bacterial survival was

illustrated for case-patient 2, who had a positive culture from a rectal swab sample; the bacteria most likely was transmitted by the oral route, possibly after ingestion of contaminated poultry products (*3*) or deglutition of respiratory secretions.

Several reports have demonstrated the prolonged persistence of *B. hinzii* in the respiratory and digestive tracts (*3*,*5*); such persistence, at least in the respiratory tract, is also observed for *B. petrii* and *B. bronchiseptica* (*16*). This persistence may explain why infection can develop long after exposure and makes identification of the source difficult.

The characteristics of all the reported cases suggest that *B. hinzii* is an opportunistic pathogen in humans. Other pathogens were isolated in several cases such that the extent of its pathogenicity remains obscure. Surprisingly, *Staphylococcus* sp. was the most frequently associated pathogen, raising the possibility of synergy between these 2 bacteria (*5*). The role of *B. hinzii* is still unclear in immunocompetent patients, where it may act as colonizer. For case-patient 2, the mucoid aspect of the colonies is in accordance with prolonged carriage, as described for *Pseudomonas aeruginosa* (*17*).

Treatment of nonclassical *Bordetella* infections is not standardized. The interpretation of antimicrobial sensitivity testing is not established and is usually done by inference from other nonfermentative gram-negative rods. According to in vitro sensitivity testing for human isolates described in the literature and pharmacology, piperacillin/tazobactam and carbapenems (excluding ertapenem) may be effective (Table). Optimal duration of treatment has not been established but should be long enough to cure the infection and, if possible, eliminate the bacteria. Both patients reported here were initially treated with piperacillin/tazobactam without clinical improvement despite the sensitivity of the isolates to this association; presumably, the treatment duration was too short. The curative doses needed to eliminate *B. hinzii* appear to be high because *B. hinzii* was isolated from case-patient 1 despite prophylactic treatment with trimethoprim/sulfamethoxazole, a combination to which the isolate was susceptible. Numerous discrepancies exist between the results of disk diffusion and MIC tests (*5*), as observed for *B. bronchiseptica* (A. Le Coustumier, unpub. data). MIC testing should be performed to confirm the sensitivity of any such isolates. We can speculate about possible antimicrobial resistance acquisition, regarding fluoroquinolone resistance in case-patient 1 and his exposure to this pharmacologic class during previous hospitalizations, as described elsewhere (*3*), but data remain insufficient to prove it. Microbiological tests after the episode are required to evaluate the effectiveness of treatment.

In conclusion, we report here 2 cases of *B. hinzii* pulmonary infection in immunodeficient patients, probably after avian exposure. Although the transmission could not be clearly established, a potential link exists between the occupational exposure and the isolation of *B. hinzii* in the pulmonary tract. The respiratory or digestive carriage can be prolonged such that the infection might emerge only long after contamination, making identification of the source difficult. Although *B. hinzii* is well established as opportunistic, microbiologists and clinicians need to be aware of the difficulty in diagnosing infections by this species using routine methods. Identification of all clinical isolates belonging to nonclassical *Bordetella* sp. should be confirmed by a reference laboratory. Further clinical and microbial investigations are necessary to understand the epidemiology and the pathogenicity of *B. hinzii*. Optimal antimicrobial treatments need to be established and supported by pharmacology and antimicrobial in vitro sensitivity testing (bacteriostasis, bactericidy).

Acknowledgments

We thank the technicians who performed laboratory tests.

This study was supported by the Institut Pasteur Foundation and the Institut de veille sanitaire.

Dr. Fabre is a biologist in the laboratories of Cahors Hospital and Bordeaux University Hospital in France. Her research interests include infectious diseases in humans.

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Zoonotic Leprosy in the Southeastern United States

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Nine-banded armadillos (*Dasypus novemcinctus*) are naturally infected with *Mycobacterium leprae* and have been implicated in zoonotic transmission of leprosy. Early studies found this disease mainly in Texas and Louisiana, but armadillos in the southeastern United States appeared to be free of infection. We screened 645 armadillos from 8 locations in the southeastern United States not known to harbor enzootic leprosy for *M. leprae* DNA and antibodies. We found *M. leprae*–infected armadillos at each location, and 106 (16.4%) animals had serologic/PCR evidence of infection. Using single-nucleotide polymorphism variable number tandem repeat genotyping/genome sequencing, we detected *M. leprae* genotype 3I-2-v1 among 35 armadillos. Seven armadillos harbored a newly identified genotype (3I-2-v15). In comparison, 52 human patients from the same region were infected with 31 *M. leprae* types. However, 42.3% (22/52) of patients were infected with 1 of the 2 *M. leprae* genotype strains associated with armadillos. The geographic range and complexity of zoonotic leprosy is expanding.

Leprosy (Hansen disease), a chronic infectious disease caused by *Mycobacterium leprae*, primarily affects the peripheral nervous system and involves skin and other tissues (*1*). Although this disease is generally a rare disorder that occurs mainly in tropical and semitropical areas, the World Health Organization recorded 219,075 new leprosy cases globally in 2011, and 439,670 new cases were reported in the Western Hemisphere over the past decade (*2*,*3*). Although leprosy is curable by antimicrobial drug therapy, the treatment interval for this disease can require ≥2 years to complete, and underlying nerve damage caused by the infection might be irreversible. There are no established laboratory screening tests to detect leprosy; the disease must be diagnosed clinically. Therefore, physician awareness about leprosy and knowledge of populations potentially at risk for the infection, are paramount for early detection and treatment (*1*).

Leprosy was not present in the New World during pre-Columbian times and appears to have been introduced to the Western Hemisphere after colonization. Early case reports suggest the disease was well established in most countries surrounding the Gulf of Mexico by the 1750s (*4*,*5*). Genomic polymorphisms enable us to trace the spread of the disease worldwide and confirm the regional origins of most isolates (*6*). The disease is rare in the United States; only ≈13,000 cases have been recorded since the 1890s, and ≈200 new cases are reported each year. Most of these case-patients lived or worked outside the country in disease-endemic areas and might have acquired their disease abroad (*7*). However, approximately one third of all case-patients in the United States report no foreign residence history or known contact with another person who had leprosy. Therefore, they probably acquired the disease from local sources (*1*).

Leprosy is believed to be transmitted mainly from person to person through infectious aerosols or direct contact (*1*). However, there is a strong genetic component with regards to susceptibility to infection, and 95% of all persons appear to be naturally resistant to leprosy (*8*). *M. leprae* is an obligate intracellular parasite that can survive for only short periods unprotected in the natural environment (*9*), and few animals support experimental infection with this bacterium (*10*). The only known nonhuman reservoir of *M. leprae* is the nine-banded armadillo (*Dasypus novemcinctus*), and disease prevalence rates among armadillos may exceed 20% in some locales (*11*).

Armadillos are highly susceptible to *M. leprae* and can manifest massive burdens of bacilli in their tissues ($10^{10–11}$ organisms/g). This sylvatic infection was first detected in 1975 but is known to have occurred among armadillos for many decades before that time (*12*–*14*). Early surveys in the United States suggested that leprosy was

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DOI: http://dx.doi.org/10.3201/eid2112.150501

restricted mainly to armadillos in Texas and Louisiana. No evidence for infection was found among armadillos in Florida, Georgia, and Alabama (*12*,*14*,*15*). However, in recent times, the geographic range of the infection seems to be expanding (*16*).

We recently showed that armadillos over a 4-state area in the southern United States were infected with a single predominant *M. leprae* genotype strain (3I-2-v1), and we recovered this same strain from a large number of persons with leprosy in these same states. Leprosy is probably a zoonosis in the southern United States (*17*). Armadillos are common throughout the southern United States, and their geographic range extends through Latin America to northern Argentina (*18*). To better understand the geographic range of *M. leprae*–infected armadillos and the role that these animals might play in perpetuating leprosy, we surveyed armadillos for *M. leprae* and compared genoypes of *M. leprae* isolated from these animals with those from biopsy samples obtained from patients with leprosy in the southeastern United States.

Materials and Methods

Study Design

In an ecologic cohort study, we surveyed armadillos and patients in the southeastern United States for *M. leprae* and genotyped isolated bacilli. Patient samples were obtained from excess diagnostic materials after a category 4 exemption was granted by the institutional review board of Louisiana State University (Baton Rouge, LA, USA). Interviews with some patients were conducted by the Florida Department of Health, and some patients in Mississippi were interviewed according to a protocol approved by the institutional review board at Forrest General Hospital (Hattiesburg, MS, USA). Armadillos were collected according to established protocols approved by the Institutional Animal Care and Use Committee at the Valdosta State University (Valdosta, GA, USA) and the University of Georgia (Athens, GA, USA).

Collection of Samples from Wild Armadillos

Blood and reticuloendothelial tissue samples were collected from 645 armadillos at 8 locations in state and federal Wildlife Management Areas, Forests, and Refuges in Mississippi, Alabama, Georgia, and Florida during 2003–2012 (Figure 1). Armadillo serum or whole blood samples were dried on filter paper (Nobuto strips; Advantec, Dublin, CA, USA), and tissue samples were frozen or fixed in 70% ethanol. These specimens were shipped to the National Hansen's Disease Program (Baton Rouge, LA, USA) for testing. In addition, we reexamined 55 frozen serum samples from armadillos collected in Florida during 1983–1988 (*11*).

Biomarkers for *M. leprae* Infection

Serologic and molecular assays were used to identify armadillos infected with *M. leprae.* Serum samples were tested for IgM against phenolic glycolipid-1 (PGL1) antigen of *M. leprae* (BEI Resources, Manassas, VA, USA) and for leprosy IDRI diagnostic-1 (LID1) antigen (Infectious Disease Research Institute, Seattle, WA, USA) by using an ELISA as described (*19*). Positive results were determined according to optical density and by using limits described for the PGL1 assay (*13*,*19*). Interpretations for the LID1 ELISA were derived by inspecting the rank-ordered distribution of optical densities for deflection from linearity, and arbitrarily assigning a value limit. DNA was extracted from lymph nodes or spleens of animals seropositive by ELISA by using the DNA Easy Kit (QIAGEN, Valencia, CA, USA) and screened by using a PCR with primers specific for regions of the *M. leprae* multicopy repeat sequence and the heat shock protein gene encoding the 18-kD antigen as described (*20*). Amplicons were confirmed by sequencing.

Patient Samples

Skin biopsy specimens collected from patients attending the National Hansen's Disease Program outpatient clinic or referred for diagnosis were stored frozen in optimum cutting-temperature compound or archived as formalin-fixed, paraffin-

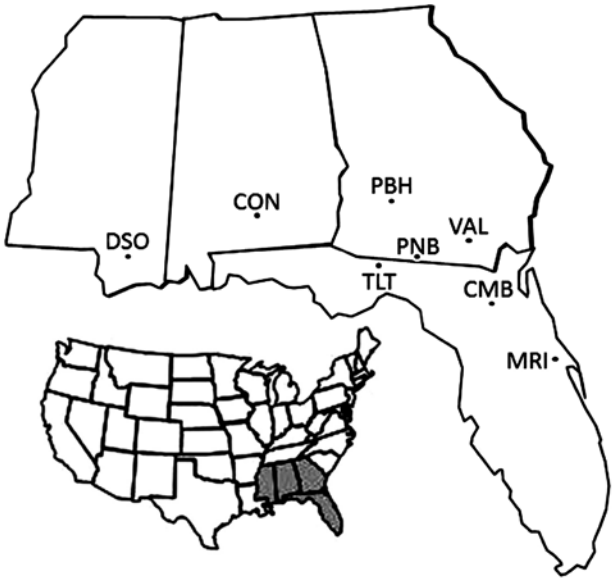

Figure 1. Eight locations in 4 states in the southeastern United States where armadillos were sampled and tested for infection with *Mycobacterium leprae*. Inset (shaded region) indicates location of the 4 states. DSO, DeSoto National Forest, Mississippi; CON, Conecuh National Forest, Alabama; PBH, Pebble Hill Plantation, Thomasville, Georgia; PNB, Pinebloom Plantation, Albany, Georgia; VAL, Valdosta, Georgia; TLT, Tall Timbers Research Station and Land Conservancy, Tallahassee, Florida; CMB, Camp Blanding, Florida; MRI, Merritt Island National Wildlife Refuge, Florida.

embedded blocks and occasionally fixed in 70% ethanol. To assess *M. leprae* genotype strains in the region, we used 52 biopsy specimens from cases-patients with leprosy during 2007–2012. Samples consisted of 47 fixed in formalin and embedded in paraffin, 4 fixed in ethanol, and 1 frozen.

Genotyping of *M. leprae* from Armadillos and Patients

We genotyped *M. leprae* isolated from 52 patients and selected armadillo samples, and assigned their phylogenetic affiliation by using an algorithm associating 16 major single-nucleotide polymorphisms (SNPs) as described (*6*,*17*) (Figure 2). Because SNP-type 3I predominates in North America, we first sequenced SNP7614 and insertion/deletion_17915. Samples with a single copy of insertion/deletion_17915 and a T at SNP7614 were confirmed as 3I and further discriminated as 3I-1 or 3I-2 on the basis of SNP-1527056. Non-3I isolates were typed for SNPS as described (*6*,*17*).

To enhance discrimination of isolates with an identical SNP type, we determined the copy number of 10 variable number tandem repeats (VNTRs) in a lineage dependent manner as described (*17*). Multiplex nested PCR amplified all 10 VNTR loci, and these loci were used as a template for individual assessments (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0501-Techapp1.xlsx). VNTRs <5 bp were sequenced to determine copy number, and those >5 bp were determined by fragment analysis. A representative number of amplicons were sequenced to confirm the fragment size (Genelab, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA, USA). The array of genotypes determined for patient and armadillo isolates was plotted by using minimum spanning tree analysis in BioNumerics 7.1 software (Applied Maths NV, Sint-Latem, Belgium) in a lineage-dependent manner. VNTR further discriminated the SNP lineage (Figure 3).

Genome Sequencing

The *M. leprae* genome sequences from 4 armadillos harboring the 3I-2-v15 genotype were obtained by fragment library sequencing by using the Ion Proton System Libraries Kit (Life Technologies, Grand Island, NY, USA). DNA quality and integrity were validated by using the Agilent 2000 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and sequenced with an Ion PI Chip Kit v2 (Life Technologies). The sequence data were compared with the published genome of the *M. leprae* TN reference standard (*21*), and variant calls were generated by using Partek 4.0 software (Partek, St. Louis, MO, USA). Variants with frequency >90%, and a minimum 10× coverage were compared with 3I-specific variants of the armadillo-associated *M. leprae* genotype strain 3I-2-v1 (online Technical Appendix Table 2) (*17*). The 13 unique variants that differentiated 3I-2-v15 from 3I-2-v1 were confirmed by direct sequencing of additional human (n = 10) and armadillo (n = 15) isolates of both strain types (primer sequences, online Technical Appendix Table 3).

Results

Expanded Geographic Range of *M. leprae* Infection among Armadillos

We screened blood and tissue samples to determine the prevalence of *M. leprae* infection among 645 armadillos obtained at 8 locations in the southeastern United States

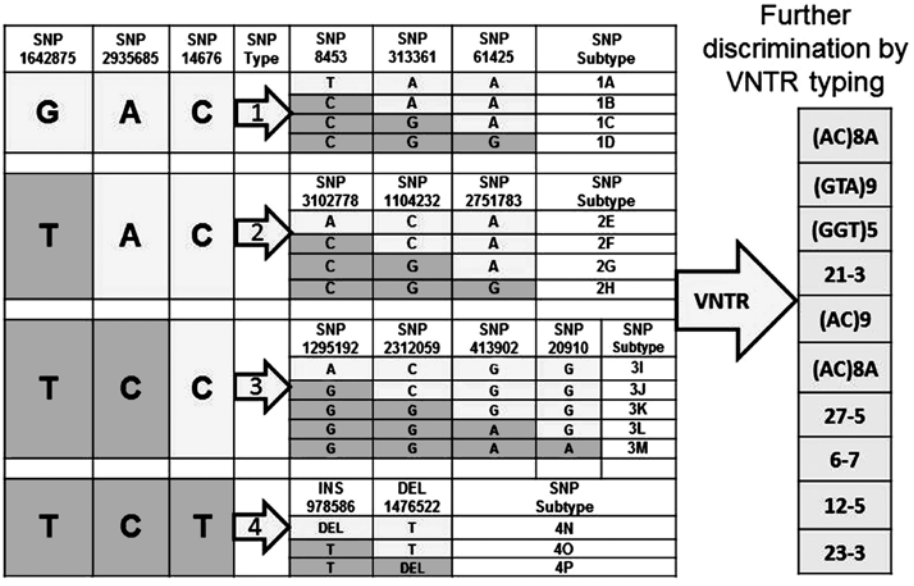

Figure 2. Genotyping scheme for *Mycobacterium leprae* determined by using single-nucleotide polymorphisms (SNPs) and variable number tandem repeat (VNTR) polymorphisms, southeastern United States. Shading indicates the base that differentiates SNP type and subtype of *M. leprae*. The algorithm used for strain typing of *M. leprae* is based on specific SNP location and type and VNTR copy number at the various locations identified along the chromosome. After identification of the major SNP subtype, *M. leprae* is further discriminated by using allele numbers at 10 VNTR loci. INS, insertion; DEL, deletion.

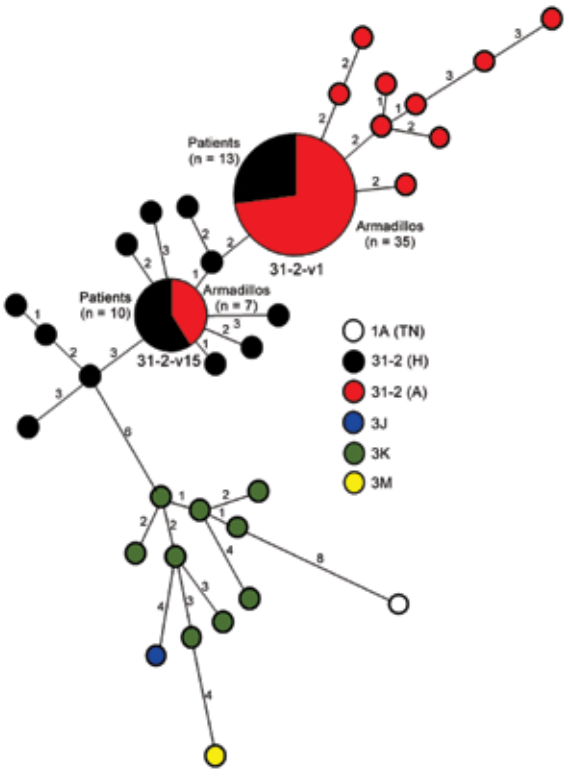

Figure 3. Minimum spanning tree constructed by using single-nucleotide polymorphism (SNP) and variable number tandem repeat (VNTR) polymorphism profiles for *Mycobacterium leprae* samples from patients and armadillos from the southeastern United States. Each circle represents a single strain genotype of *M. leprae*. Large circles indicate that >1 sample (number shown) had a common genotype. Values along lines indicate number of differences between allelic profiles. Human and armadillo samples of common SNP subtype 3I-2 are indicated by different colors. Only 2 genotypes were present in >1 sample, and both genotypes were present among armadillos and patients.

(Mississippi, Alabama, Georgia, and Florida) (Figure 1). We detected antibodies to *M. leprae*–specific antigens at each location and in 16.4% (106/645) of all the samples screened: 10.1% (65/645) had antibodies to PGL1, and 9.9% (64/645) had antibodies to LID1. Only 23 samples showed positive results in both assays. These samples included LID1 antigen–enhanced serologic detection of infection versus screening with PGL1 alone (Table 1).

M. leprae was not found among armadillos in this region before 2009 (*11*,*16*). Two of the areas surveyed (Tall Timbers Research Station and Land Conservancy, Tallahassee, Florida, and Pinebloom Plantation, Albany, Georgia) also had been sampled in earlier studies (*14*). In addition, we examined 55 serum samples collected from armadillos in nearby regions of Florida. These samples had been stored frozen since 1983–1988 (*11*). Rescreening these samples by using the current PGL1 and LID1 ELISAs, we again found no serologic reactivity, which confirmed the earlier findings.

Lymph node tissues were available from 95 of the 106 animals considered serologically positive by either ELISA. DNA was extracted from tissues and tested by PCR for *M. leprae*–specific multicopy repeat sequence and heat shock protein 18 gene fragments. All 95 samples amplified in ≥1 *M. leprae*–specific PCR, and 75/95 (80%) amplified with both PCRs (Table 1). Amplicon sequencing confirmed specificity for *M. leprae.*

M. leprae Isolated from Armadillos and Patients

Sufficient DNA was available to genotype the *M. leprae* recovered from 42/95 armadillos and from the biopsy samples of 52 patients who had leprosy in the same geographic region. Among armadillos, only 2 *M. leprae* genotype strains were recovered. We found 35/42 (83%) of the animals harbored *M. leprae* SNP-VNTR type 3I-2-v1, which we had identified as infecting patients and armadillos in Texas, Louisiana, Mississippi, and Arkansas (*17*). Therefore, type 3I-2-v1 can be found among armadillos in Mississippi, Alabama, Georgia, and northern Florida. However, in southern Florida, we found 7 armadillos infected with an *M. leprae* genotype strain not previously observed among armadillos. Designated 3I-2-v15, this new armadillo-associated genotype strain differed from 3I-2-v1 by having multiple allele changes at 3 VNTR loci. The 3I-2-v15 allele profile was unique and had not been previously identified in a global database of *M. leprae* VNTR strain types (*22*). According to allele frequencies derived from that database, the 3I-2-v15 genotype had only a 1:3,700 probability for random recombination within any of these 7 samples. Subsequent deep sequencing of 3I-2-v15 isolates from 4 armadillos showed that this genotype was uniform and consistent among all animals examined and had multiple SNP differences between 3I-2-v15 and 3I-2-v1 *M. leprae.* Four SNPs common among 3I-2-v1 isolates were not present in 3I-2-v15, and 9 additional common SNPs were unique to 3I-2-v15 (Table 2). These same 13 polymorphisms were confirmed by direct PCR of *M. leprae* from an additional 10 human and 15 armadillo isolates. 3I-2-v15 is the most diverse representative of the 3I-2 lineage sequenced to date.

Patient Samples

In contrast to SNP-VNTR analysis of *M. leprae* from armadillos, analysis of *M. leprae* from patient biopsy specimens discriminated multiple *M. leprae* genotypes. The 3I-2 lineage, which predominates in North America (*17*), was most common and found in 41 samples. The other samples had genotypes found more commonly in other parts of the world; 9 were 3K, and 1 each were 3J or 3M lineages (*6*). SNP-VNTR genotyping showed that 30

patients were infected with entirely unique *M. leprae* genotypes. However, 22 patients, as well as armadillos, had identical *M. leprae* genotypes: 12 patient biopsy samples harbored *M. leprae* type 3I-2-v1, and 10 samples harbored newly identified type 3I-2-v15. In this study, only the 2 *M. leprae* genotype strain types recovered from armadillos were present in >1 patient. Overall, 42% (22/52) of the patients were infected with *M. leprae* genotypes that were found associated with armadillos (Figure 3). All patients harboring type 3I-2-v1 had residence histories in areas of the southern United States where they may have been exposed to *M. leprae* through armadillos. All 10 patients infected with 3I-2-v15 resided and consulted physicians in southern Florida, the only region where armadillos with this same *M. leprae* genotype strain type also had been found.

None of the patients in this study reported any previous contact with another person who had leprosy. In separate studies, small groups of patients in Florida and Mississippi were interviewed about their medical history and exposure to armadillos. Only 4 of the patients in Florida interviewed could be fully typed: 3 had *M. leprae* 3I-2-v15 and 1 had 3I-2-v1. In Mississippi, all 4 patients were infected with 3I-2-v1. None of the patients interviewed in Mississippi or Florida recalled direct contact with armadillos. All patients were familiar with armadillos in their environment, and many reported gardening and other outdoor activities that might have provided some exposure to environments possibly contaminated by *M. leprae* from armadillos.

In this study, patients with no foreign residence history had 16 times greater odds of being infected with 1 of the 2 armadillo-associated *M. leprae* genotype strain types than with any other type of *M. leprae* (odds ratio 16.8, 95% CI 3.881–73.374, p<0.0001). Patients with residence histories in areas where they may have been exposed to *M. leprae* from armadillos also had 41 times greater odds of being infected with 1 of the 2 armadillo-associated types than with any other *M. leprae* genotype (odds ratio 41.3, 95% CI 2.297–742.68, p<0.0001). Although leprosy has not previously been recognized among armadillos in Florida, 16% of the animals that we studied in the region harbored *M. leprae*, and 22/52 patients that we examined also were found to be infected with 1 of the same 2 *M. leprae* genotype strain types that we recovered from armadillos in the region.

Discussion

Leprosy appears to be an emerging infection of armadillos throughout the southeastern United States. Most armadillos are infected with a single predominant *M. leprae* strain type (3I-2-v1), which has been associated with probable zoonotic transmission of leprosy to humans (*17*). However, armadillos in southern Florida, as well as several patients from that region, are infected with a distinctly different *M. leprae* genotype strain (3I-2-v15). Armadillos must have acquired *M. leprae* from humans within the past 400 years, after the disease was introduced into the Western Hemisphere. The 3I-2-v15 strain type was not used for in vivo propagation of *M. leprae* in armadillos. With its multiple genomic polymorphisms, this train type does not appear to have evolved recently from the 3I-2-v1 strain type. Armadillos must have acquired these infections from humans who originally harbored the strains in the region, and *M. leprae* appears to have been naturally transferred to armadillos on >1 occasion and in >1 location. Interspecies transfer of *M. leprae* between humans and armadillos appears to be rare and inefficient. However, emergence of the infection among armadillos in southeastern states, which were previously believed to be free of *M. leprae*, suggests that the disease will eventually be detected among animals throughout North America, and additional *M. leprae* genotype strains might also be acquired by animals in other locations over time.

Table 1. Serologic and molecular detection of *Mycobacterium leprae* infections among armadillos from various locations in the southeastern United States*

Location	No. blood or serum samples	Serologic screening, no. samples positive for *M. leprae* antigen				No. lymph node samples tested	PCR, no. samples positive for *M. leprae* DNA			
		LID1	PGL1	LID1 or PGL1	LID1 and PGL1		RLEP locus	*hsp 18* locus	1 site	2 sites
CMB	31	2	2	2	2	2	2	0	2	0
VAL	8	1	0	1	0	0	0	0	0	0
MRI	65	9	16	18	7	17	16	14	17	13
CON	38	5	2	7	0	5	5	2	5	2
DSO	23	7	0	7	0	7	6	3	7	3
PNB	117	11	13	20	4	19	19	19	19	19
PBH	23	5	1	6	0	3	3	2	3	2
TLT	340	24	31	45	10	42	41	36	42	36
Total	645	64	65	106	23	95	92	76	95	75

*LID, leprosy IDRI diagnostic 1 antigen; PGL, phenolic glycolipid 1 antigen; RLEP, mutlicopy repeat sequence; *hsp*, heat shock protein; CMB, Camp Blanding, FL; VLD: Valdosta, GA; MRI, Merritt Island National Wildlife Refuge, FL; CON, Conecuh National Forest, AL; DSO, DeSoto National Forest, MS; PNB, Pinebloom Plantation, Albany, GA; PBH, Pebble Hill Plantation, Thomasville GA; TLT, Tall Timbers Research Station and Land Conservancy, Tallahassee, FL.

Table 2. Next-generation whole-genome sequencing of *Mycobacterium leprae* strain 3I-2-v15 derived from wild armadillos from the southeastern United States compared with that for armadillo-associated strain 3I-2-v1

Sample no.	Average coverage (% genome covered)*	No. variants partially shared†	No. variants only in 3I-2-v15‡	No. 3I-2 variants absent in 3I-2-v15‡	No. variants in both strains	No. TN strain variants in both strains
US-36	19.8 (88.72)	7	9	4	20	37
US-95	97.99 (98.36)	1	9	4	14	37
FL-26	119.32 (99.00)	0	9	4	13	37
MRI-9	25.3 (92.08)	6	9	4	19	37

*Average no. of consensus sequence reads obtained from the particular specimen covering the entire genome.
†No. variants reported to be specific for single-nucleotide polymorphism (SNP) type 3I-2 that are present in all samples of the 3I-2-v1 genotype and that were found in some, but not all, samples of the 3I-2-v15 genotype.
‡Resequencing referenced the *M. leprae* TN standard genomic sequence, identified 13 variants present only in 3I-2-v1 (2 SNP, 2 insertion/deletions) or 3I-2-v15 (9 SNP), and differentiated the strains.

Three epidemiologic case studies in the United States (*23*–*25*) and 1 in Brazil (*26*) have implicated contact with armadillos as a risk factor for leprosy infection. Leprosy is not highly communicable, and knowledge about potential transmission of the infection through armadillos can help reduce the overall risk for disease among persons who come in contact with these animals or environments contaminated by them. *M. leprae* may be spread through direct or indirect routes, but long-term direct contact with an infectious source is believed to be the most effective means to transmit the infection (*1*). None of the patients interviewed in this study recalled any direct contact with armadillos, although they may have had indirect exposure to *M. leprae* through gardening or other outdoor activities. Because leprosy is a rare disease, any risk for infection attributable to indirect exposure to armadillos would have to be extremely low overall. Nevertheless, persons concerned about exposure to *M. leprae* from armadillos in their environment might be advised to wear gloves while gardening or use similar general hygienic practices commonly recommended for avoiding exposure to other pathogens in the environment (*27*). Physicians caring for patients with possible exposure to *M. leprae* through armadillos should retain leprosy in their differential diagnoses for cutaneous lesions, especially for patients who do not respond well to most common therapies.

The range of armadillos in the Western Hemisphere is the southern United States, Central America, and northern Argentina. Biomarkers of *M. leprae* have been reported among armadillos in Argentina, Brazil, and Colombia (*28*–*30*). However, reports of detection of the infection have been inconsistent in different locales (*14*,*31*). Disease prevalence rates among animal populations might be influenced by the season and local variations in animal density or population structure that can affect detectability of disease (*32*). Among armadillos, typically only small numbers of animals can be screened from any given location, and relatively high prevalence rates are required to reliably detect the infection. The role that armadillos might play in helping to perpetuate leprosy throughout the Western Hemisphere merits consideration.

There are currently no established laboratory tests to aid in the diagnosis of leprosy, and the disease can only be detected once persons have clinical disease. Serologic screening for PGL1 antibodies has shown only limited utility, and effective tools to aid diagnosis or monitor progress of individual infections are needed (*33*). Wild armadillos showed considerable diversity in their response to LID1 and PGL1 antigens. Use of the antigens in combination markedly enhanced serologic detection of *M. leprae* infection among armadillos, and PCR analysis of matching tissue samples showed those reactions were highly specific for *M. leprae.* For infection of armadillos initiated by intravenous administration of 1×10^9 *M. leprae*, antibodies against LID1 and PGL1 become detectable only after a delay of several months, and it appears that relatively well-established infections are required before either antibody is produced (*19*). Naturally transmitted infections would involve much lower initiating doses, and the amount of bacilli required to elicit T cell–dependent IgM responses against PGL1 might be higher that needed to initiate T cell–dependent IgG responses to LID1. Trials are underway to discern the efficacy of using these antigen combinations in screening human populations, and in 1 leprosy-endemic region, LID1 antibodies appeared to be more prevalent than PGL1 antibodies (*33*–*35*).

Elimination of an infectious mycobacterium from a wildlife species is extremely difficult and costly. Authorities have struggled for decades with bovine tuberculosis in the United Kingdom and Ireland, where the badger (*Meles meles)* plays a role in spread of the disease (*36*); in New Zealand, where the opossum (*Trichosurus vulpecula*) is responsible (*37*); and, more recently, in the northern United States, where white-tailed deer (*Odocoileus virginianus*) and other cervids are involved (*38*). It is unlikely that any effort to remove armadillos from large areas would be effective, and the removal process might provide even greater risks to humans for exposure to *M. leprae* from animals. Public education about the risk for exposure to infectious agents through animals can be highly effective. The greatest potential for exposure to *M. leprae* through armadillos would probably be direct contact with the flesh of animals

hunted or prepared as food. However, armadillos can also shed leprosy bacilli into the environment in bodily secretions, and bacilli might survive extracellularly in the environment for short periods, or may even be sustained within encysted amoeba or other reservoirs for 8 months (*39*). In addition, potential involvement of insects in leprosy transmission has never been fully discounted, and the role that biting insects might play in mechanically transmitting *M. leprae* between hosts also merits attention (*9*). A better understanding of the specific risk factors that might be involved in transmission of *M. leprae* between armadillos and humans is needed.

Current leprosy control efforts focus on use of multiple antimicrobial drugs to treat clinically active human cases. The decreases in global leprosy prevalence reported over the past decade seem to validate this approach because millions of persons have been cured of leprosy. However, as 1 source of infection is brought under control, other major sources might arise. Evidence is now accumulating that leprosy is a zoonosis in North America, and the infection could extend throughout the range of the armadillo. New strategies to detect leprosy and prevent its spread will be needed. Molecular genotyping of *M. leprae* enables application of modern public health principles of infectious disease control to identify sources of infection and related clusters of new cases (*40*). Insight into the dynamics of leprosy transmission in different populations will help clarify the proportional risk related to nonhuman reservoirs and could facilitate objective development of new methods to ultimately eliminate leprosy.

Acknowledgments

We thank the participants in this study and Anne Burdick, T.P. Gillis, Barbara Stryjewska, and Ramesh Subramanian, for providing advice and support; and P. Andrews, M. Kearney, S. Keas, G. McCormick, N. Robbins, R. Stevenson, and H. Zhang for providing expert assistance.

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etymologia

Leprosy [lep'rə-se]

From the Greek *lepros*, "scaly," leprosy is a chronic infectious disease of man caused by *Mycobacterium leprae* and principally affects the peripheral nerves and skin. The earliest known skeletal evidence for leprosy has been found in India and dates to 2000 BCE. This finding suggests that the first textual references to leprosy are in ancient Sanskrit hymns of the *Atharva Veda*. The armies of Alexander the Great may have brought leprosy from India to western Asia circa 326 BCE, and it spread further west when Roman armies campaigning in Asia Minor and Syria returned home (62 BCE). The Romans referred to leprosy as *elephantiasis graecorum* and could distinguish between the similar symptoms of lymphatic filariasis, or *elephantiasis arabum*.

Photo from the Public Health Image Library, Dr. Andre J. Lebraun, 1969

Norwegian physician Armauer Hansen identified the causative agent, *Mycobacterium leprae*, in 1873; however, it was successfully identified as a bacterium only in 1879 by a young German physician, Albert Neisser, who attempted to take credit for the discovery. Today, leprosy is also known as Hansen disease to avoid stigma.

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DOI: http://dx.doi.org/10.3201/eid2112.ET2112

Infection Risk for Persons Exposed to Highly Pathogenic Avian Influenza A H5 Virus–Infected Birds, United States, December 2014–March 2015

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Newly emerged highly pathogenic avian influenza (HPAI) A H5 viruses have caused outbreaks among birds in the United States. These viruses differ genetically from HPAI H5 viruses that previously caused human illness, most notably in Asia and Africa. To assess the risk for animal-to-human HPAI H5 virus transmission in the United States, we determined the number of persons with self-reported exposure to infected birds, the number with an acute respiratory infection (ARI) during a 10-day postexposure period, and the number with ARI who tested positive for influenza by real-time reverse transcription PCR or serologic testing for each outbreak during December 15, 2014–March 31, 2015. During 60 outbreaks in 13 states, a total of 164 persons were exposed to infected birds. ARI developed in 5 of these persons within 10 days of exposure. H5 influenza virus infection was not identified in any persons with ARI, suggesting a low risk for animal-to-human HPAI H5 virus transmission.

Poultry infections with highly pathogenic avian influenza (HPAI) A H5 viruses have rarely been reported in the United States and have previously occurred as localized events (*1*,*2*). However, during December 15, 2014–March 31, 2015, a total of 60 HPAI H5 outbreaks in wild, captive, and domestic birds were identified in 13 states (*3*).

HPAI H5 viruses emerge sporadically in poultry as a result of interspecies transmission from wild to domestic birds (*4*,*5*). HPAI H5 viruses have caused thousands of outbreaks in poultry worldwide (*6*). Avian influenza viruses have evolved to bind to receptors in birds that differ from those in humans (*7*); therefore, the ability of avian influenza viruses to infect humans is limited. Nonetheless, human infections with avian influenza viruses have occurred. Most human infections have been caused by HPAI subtype H5N1 viruses in several countries and by low pathogenic avian influenza (LPAI) subtype H7N9 virus, primarily in China (*8*,*9*). Often, human infection with avian influenza virus results in severe disease (*10*). For instance, the H5N1 virus found in Asia (referred to here as Eurasian lineage H5N1) has been documented to cause severe disease in humans (*11*). Human infection with Eurasian lineage H5N1 virus was first documented in 1997 in poultry workers in Hong Kong, where the most frequent exposures were touching poultry or poultry parts and butchering poultry (*12*,*13*). In that outbreak, indirect exposures (e.g., feeding poultry, cleaning poultry stalls) were also associated with the presence of H5N1 antibodies in humans (*12*). To date, Eurasian lineage H5N1 virus has caused >800 human infections in Africa, Asia, and Europe, resulting in a 60% case fatality rate. Most of the H5N1 case-patients reported exposure to infected poultry at live bird markets or backyard farms (*14*). The first human cases of LPAI H7N9 virus infection were documented in China in 2013 (*8*); since then, >600 human infections and a case fatality rate of ≈36% have been reported. Like human HPAI H5N1 virus infections, human infections with LPAI H7N9 have been associated with exposure to infected poultry (*8*,*9*).

Of the 3 HPAI H5 subtype viruses recently identified in the United States (H5N1, H5N2, and H5N8) (*15*), only H5N8 virus has been identified previously in birds in Europe and Asia (*16*). One hypothesis for the recent emergence of these viruses in US birds attributes the arrival of H5N8 virus to migratory birds coming from Russia by the Pacific flyway (*16*,*17*). Once in North America, this H5N8 virus purportedly mixed with circulating North American LPAI viruses to generate 2 new reassortant HPAI viruses: subtype H5N2 and H5N1 viruses. In both of these new

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DOI: http://dx.doi.org/10.3201/eid2112.150904

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viruses, the hemagglutinin component originated from the migrant H5N8 virus, and the neuraminidase component originated from circulating LPAI viruses (*18*). Therefore, these 2 new viruses are genetically different from HPAI viruses identified in Asia. Of note, these new H5 viruses found in North American birds so far have not been associated with human infections. To help assess the risk of transmission of these viruses from birds to humans, we describe human exposure to HPAI H5 virus–infected birds during December 2014–March 2015 in the United States. This investigation was conducted as part of a public health response; thus, in accordance with federal human subjects protection regulations, it was not considered to be human subjects research.

Methods

We identified HPAI H5 virus detections (i.e., laboratory-confirmed infections) in US birds by using reports made to the Animal and Plant Health Inspection Service, United States Department of Agriculture (USDA); the US Geological Survey, United States Department of Interior (DOI); the US Fish and Wildlife Service, DOI; and the National Flyway Council. These reports included the following information: county, state, confirmation date, influenza virus subtype, species and quantity of affected birds, and setting (wild, captive wild, backyard poultry, or commercial poultry). For the purpose of this investigation, we grouped detections of HPAI H5 virus–infected birds into outbreaks. We considered all detections in wild birds from specimens collected on the same day and in the same county to be 1 outbreak; those occurring on different dates or in different counties were classified as separate outbreaks. We considered multiple detections in captive wild birds and domestic flocks to be a single outbreak if the same HPAI H5 virus was detected in birds housed at the same location within 5 days of a prior detection.

For each outbreak reported, we contacted state and local public health departments to request information regarding human exposures. This information consisted of the number of persons who reported being exposed to possibly infected birds and the number of persons in whom acute respiratory infection (ARI) or other signs or symptoms compatible with avian influenza developed during a 10-day postexposure monitoring period. ARI was defined as >2 signs or symptoms of respiratory infection (i.e., fever, cough, runny nose or nasal congestion, sore throat, or difficulty breathing). Signs and symptoms considered compatible with avian influenza were eye tearing, irritation or redness, fatigue, muscle or body aches, headache, nausea, vomiting, diarrhea, stomach pain, and joint pain. Monitoring was conducted by state or local health department via direct observation or telephone call. We also requested a narrative description of the nature of exposure to potentially infected birds, when available. Hunters in affected areas were asked to submit their harvested birds for anonymous testing for HPAI H5 virus infection at USDA's National Veterinary Services Laboratories. Because the testing was anonymous, the number of persons exposed to each hunter-harvested bird was largely not available to state and local public health departments; thus, we assumed that 1 person was exposed to an H5 virus-infected bird for each bird identified.

For persons in whom ARI developed during the monitoring period, we asked health departments to collect respiratory specimens for real-time reverse transcription PCR (rRT-PCR) testing at state public health laboratories. Specimens were obtained by using a nasopharyngeal swab or a nasal aspirate or wash or the combination of a nasal or nasopharyngeal swab with an oropharyngeal swab. If respiratory specimens were unavailable within 7 days of illness onset, we evaluated patients with ARI by performing serologic testing at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA).

For rRT-PCR testing, specimens were first screened for universal detection of type A and B influenza viruses and human RNase P gene (InfA, InfB and RP primers, respectively), according to CDC protocol described elsewhere (*19*). Influenza A–positive specimens were tested by using the following primers: H1, H3, pdmInfA (2009 pandemic influenza A), pdmH1 (2009 pandemic H1), and H5. Paired serum samples were tested by microneutralization and hemagglutination inhibition assays, using horse erythrocytes, according to international standards (*20*). The following 2 viruses were used in both assays: A/np/WA/40964/2014 (an H5N2 virus isolated from the index case in birds) and A/gyrfalcon/WA/41088–6/2014 (an H5N8 virus isolated from the index case in birds). The following 3 viruses were used in the microneutralization assay only: A/California/07/2009 (an H1N1 vaccine strain and circulating strain), A/TX/50/2012(H3N2) (an H3N2 vaccine strain), and A/SW9715293/2013 (represents a currently circulating H3N2 strain).

We received information from USDA regarding exposures of persons involved in flock depopulation efforts. We considered these exposures separately because persons involved in depopulation efforts were recommended to wear personal protective equipment (PPE) to decrease the risk of transmission (*21*).

Results

During December 15, 2014–March 31, 2015, a total of 60 outbreaks of HPAI H5 outbreaks were investigated by the Animal and Plant Health Inspection Service, USDA; the National Wildlife Health Center, US Geological Survey, DOI; state agriculture departments; or state natural resources departments. The outbreaks were caused by H5N2

virus (n = 37), H5N8 virus (n = 22), and H5N1 (n = 2) (these numbers total 61, not 60, because 3 outbreaks had a combination of viruses [1 H5N1/H5N2 and 2 H5N2/H5N8] and viruses in 2 outbreaks were not subtyped, but specimens were diagnosed as H5). Of the 60 outbreaks, 38 (63%) occurred in wild birds, 9 (15%) in backyard flocks, 8 (13%) in commercial flocks, and 5 (8%) in captive wild birds (Table). A total of 41 counties in 13 states reported HPAI infections in birds (Figure).

We identified 164 human exposures: 103 (63%) were associated with H5N2 virus, 56 (34%) with H5N8 virus, 3 (2%) with H5N1 virus, and 2 (1%) with H5. Of the 164 exposed persons, 13 (8%) were exposed to captive wild birds, 25 (15%) were exposed to poultry in backyard farms, 62 (38%) were exposed to poultry in commercial flocks, and 64 (39%) were exposed to wild birds (Table).

We received information describing exposures for 60 of the 164 persons; 44 (73%) had exposure to infected birds while not wearing PPE (e.g., while removing dead birds, collecting eggs, cleaning coops, or feeding birds), and 16 (27%) had exposure while wearing recommended PPE or had unclear exposures. ARI developed in 5 (3%) of the 164 exposed persons within 10 days of their last contact with infected birds; 4 of the 5 tested negative for influenza virus by rRT-PCR. The remaining patient with ARI had paired serum samples collected 7 days and 21–28 days after exposure, respectively. This person had serologic evidence of seasonal influenza A(H3N2) virus infection, but had no serologic evidence of infection with an HPAI H5 virus.

An additional 29 persons were involved with depopulation activities of affected flocks while wearing recommended PPE. None of those persons reported ARI within 10 days after last exposure.

Discussion

Within 4 months of the first outbreak of HPAI H5 viruses among birds in the United States, we identified >100 potential human exposures to infected birds. We found no evidence of human infection with these viruses among exposed persons. Our findings suggest that transmission of these HPAI viruses from birds to humans in exposure settings similar to those in this report may be uncommon.

Previous studies have shown that transmission of other H5 viruses from infected birds to humans has rarely occurred in Europe, Asia, and Africa (*10*,*22*,*23*). Exposures in those studies also likely occurred in a different context in many circumstances and may have had higher transmission likelihood than the exposures described in this report. For instance, a study in Egypt found that 12 (86%) of 14 households with an H5N1 virus–infected member lacked

Table. Location and characteristics of highly pathogenic avian influenza A H5 virus outbreaks among birds and minimum number of exposed persons, United States, December 15, 2014–March 31, 2015

Variable	No. (%) outbreaks among birds, n = 60	No. (%) virus-exposed persons,* n = 164
State		
Arizona	1 (2)	2 (1)
California	8 (13)	30 (18)
Idaho	8 (13)	16 (10)
Kansas	2 (3)	5 (3)
Minnesota	3 (5)	17 (10)
Missouri	4 (7)	26 (16)
Montana	1 (2)	2 (1)
New Mexico	1 (2)	1 (1)
Nevada	1 (2)	5 (3)
Oregon	10 (17)	20 (12)
Utah	1 (2)	1 (1)
Washington	19 (32)	37 (23)
Wyoming	1 (2)	2 (1)
Influenza virus subtype†		
H5N1	2 (3)	3 (2)
H5N2	37 (59)	103 (63)
H5N8	22 (35)	56 (34)
H5‡	2 (3)	2 (1)
Outbreak setting		
Wild	38 (63)	64 (39)
Captive	5 (8)	13 (8)
Backyard farm	9 (15)	25 (15)
Commercial farm	8 (13)	62 (38)
No. birds per outbreak		
1–5	42 (70)	71 (43)
6–500	9 (15)	29 (18)
>500	9 (15)	64 (39)

*Excludes persons who participated in culling activities.
†Three outbreaks involved a combination of influenza virus subtypes: H5N1/H5N2 (n = 1) and H5N2/H5N8 (n = 2).
‡No virus was isolated, but specimens were positive by the H5 (intercellular adhesion gene cluster) PCR assay, which targets the Eurasian H5 clade 2.3.4.4 viruses that were detected in the United States in December 2014.

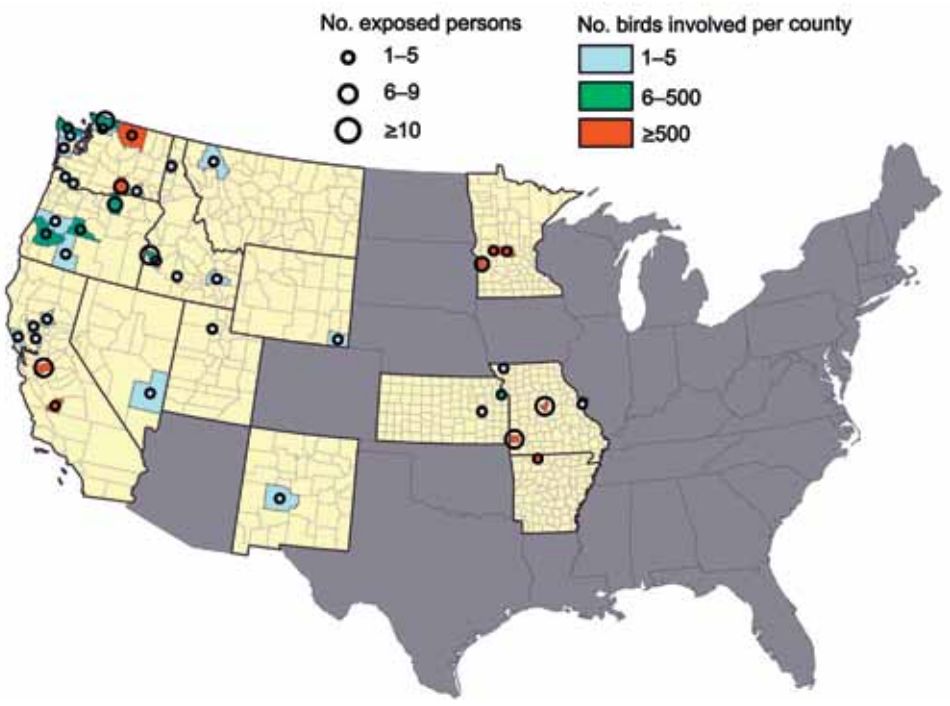

Figure. Number of highly pathogenic avian influenza A H5 virus–infected birds and minimum number of exposed persons by state and county, United States, December 15, 2014–March 31, 2015. Yellow indicates states in which outbreaks occurred.

appropriate disposal of slaughtered poultry waste (e.g., feathers, viscera), and only 1 of 56 households reported using disinfectants when cleaning poultry-contaminated surfaces (*24*). In addition, poultry exposure in these areas frequently involved unprotected and prolonged contact with unconfined poultry in poor sanitary conditions, situations that are infrequently found in the United States (*24*).

We acknowledge the following limitations of this study. First, the number of persons determined to be exposed in this investigation is likely an underestimate because we did not have complete information on human exposures for all outbreaks. Specifically, hunter-harvested birds were in many cases reported anonymously, and our assumption of 1 exposed person per bird may be incorrect. Second, no systematic testing was performed for exposed persons in whom ARI did not develop, and it is possible that we failed to identify instances of bird-to-human HPAI H5 virus transmission that resulted in subclinical infection. However exposed persons were monitored carefully for illness, and previously reported human infections with related HPAI H5 viruses have resulted in severe and prominent symptoms (*25*). Third, we were unable to collect detailed exposure information for all exposed persons; thus, we could not describe the precise nature or duration of exposures we report. Fourth, there have been relatively few HPAI H5 virus exposure events in the United States to date, which limits our ability to provide a reliable quantitative estimate of the zoonotic risk posed by these viruses. Last, because some reporting is delayed, additional outbreaks of H5 in wild birds may be identified retrospectively within the timeframe of this investigation; additional H5 outbreaks in all bird categories will also likely continue after March 31, 2015.

Although this early assessment suggests that the risk of bird-to-human transmission of HPAI H5 viruses in the United States may be low, the CDC recommends vigilance when considering future human exposures to birds that are or may be infected. Similar HPAI H5 viruses, such as Eurasian H5N1 and H5N6 viruses, have caused severe illness and death in humans in Europe, Asia, and Africa (*13*,*26*), and these newly identified US HPAI viruses should be regarded as having the potential to cause severe disease in humans until shown otherwise. The best way to prevent human infection with avian influenza A viruses is to avoid unprotected contact with sick or dead infected poultry. Persons who have been exposed to HPAI-infected birds should be monitored for 10 days after last exposure and be tested for influenza as soon as possible after illness onset if respiratory symptoms develop. Exposed persons may also be offered influenza antiviral chemoprophylaxis. Additional guidance on testing, monitoring, and chemoprophylaxis is available at http://www.cdc.gov/flu/avianflu/guidance-exposed-persons.htm.

HPAI H5 virus outbreaks in US birds will likely continue, and additional reassortment with North American viruses may also occur. Although the risk of virus transmission to humans appears to be low, each exposure incident should be reported immediately and investigated collaboratively by animal and human health partners. A rapid response to any potential human cases of HPAI H5

infection in the United States is critical to prevent further cases, evaluate clinical illness, and assess the ability of these viruses to spread among humans.

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Acknowledgments

We acknowledge the US Geological Survey National Wildlife Health Center for its role in collecting data on HPAI H5 viruses in wild birds. We also acknowledge Magdalena K. Scott, Cathie Bodenhamer, Linda Gladden, Curtis Fritz, and Edward Powers for assisting with the investigation and Feng Liu, Crystal Holiday, Stacie Jefferson, and David Wang for conducting influenza serologic assays.

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High Prevalence of Intermediate *Leptospira* spp. DNA in Febrile Humans from Urban and Rural Ecuador

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Leptospira spp., which comprise 3 clusters (pathogenic, saprophytic, and intermediate) that vary in pathogenicity, infect >1 million persons worldwide each year. The disease burden of the intermediate leptospires is unclear. To increase knowledge of this cluster, we used new molecular approaches to characterize *Leptospira* spp. in 464 samples from febrile patients in rural, semiurban, and urban communities in Ecuador; in 20 samples from nonfebrile persons in the rural community; and in 206 samples from animals in the semiurban community. We observed a higher percentage of leptospiral DNA–positive samples from febrile persons in rural (64%) versus urban (21%) and semiurban (25%) communities; no leptospires were detected in nonfebrile persons. The percentage of intermediate cluster strains in humans (96%) was higher than that of pathogenic cluster strains (4%); strains in animal samples belonged to intermediate (49%) and pathogenic (51%) clusters. Intermediate cluster strains may be causing a substantial amount of fever in coastal Ecuador.

Leptospirosis, caused by spirochetes of the genus *Leptospira*, is a neglected and potentially fatal disease that burdens impoverished communities of developing nations in tropical regions (*1*–*4*). The bacteria cause 1.7 million human cases of severe disease worldwide each year (*1*,*2*); outbreaks frequently occur during the rainy season in cities in the tropics (*4*–*8*). Domestic, peridomestic, and wild mammals harbor diverse *Leptospira* spp. in their kidneys, and their urine contaminates water sources and soil (*6*,*8*).

Leptospira comprises 20 species that are phylogenetically arranged in 3 clusters: pathogenic, saprophytic, and intermediate (*6*,*9*). Nine pathogenic and 5 intermediate species, comprising >200 serovars, have been characterized (*6*,*9*,*10*). Some reports associate intermediate cluster strains with mild (*11*–*14*) to severe (*15*,*16*) leptospirosis; however, this cluster is not well characterized (*3*,*11*,*15*,*16*). Furthermore, the current notion is that human leptospirosis is mainly caused by strains of the pathogenic cluster (*2*,*4*,*6*,*9*,*10*).

Many aspects of leptospirosis epidemiology remain unknown because only limited information exists regarding leptospiral population genetics and the role of environmental factors, including environmental persistence of leptospires, in disease occurrence. These deficiencies in knowledge result from the complexity of the disease (e.g., many animal reservoirs carry 1 of the 14 species of potentially infectious leptospires) and technical difficulties associated with classical diagnostics, such as cumbersome isolation of bacteria from clinical samples, complex standard serologic methods, and a lack of culture techniques to obtain isolates from environmental samples. We present a molecular approach to address some of these shortcomings.

Leptospirosis is common in tropical areas of Ecuador (*17*). The most severe documented outbreak occurred in 1998 in Guayaquil, where 80% of case-patients required hospitalization and 12% died (J. Leake, pers. comm., 2004). During 2010–2012 in Portoviejo, Ecuador, >2,000 serologically confirmed cases of febrile leptospirosis were reported by local health authorities (M. Morales, pers. comm., 2013). We used molecular methods to amplify and sequence the leptospiral 16S *rrs* gene from clinical samples from patients in 3 coastal communities in Ecuador that vary in their levels of urbanization.

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DOI: http://dx.doi.org/10.3201/eid2112.140659

Methods

Human Samples

During February 2011–December 2012, a total of 464 serum and blood spot samples were collected from acute, febrile patients attending hospitals or health posts in rural, semiurban, and urban communities in Ecuador. Samples from Esmeraldas, a rural community, were provided by Hospital de Borbón (Esmeraldas Province) and the Ecuador Ministry of Health (MoH). The hospital provided 108 serum samples from febrile patients; the samples had been tested for dengue virus (IgM ELISA; PanBio, Brisbane, Queensland, Australia) but not *Leptospira* spp.; 33 were positive for dengue virus. During the same time period, the Ecuador MoH collected 102 blood spot samples from febrile patients in Esmeraldas. The samples were collected onto filter paper (Whatman 903 Specimen Collection Paper; Whatman, Springfield Mill, UK), dried at room temperature, and stored at –20°C in plastic zipper bags. Twenty serum samples obtained from nonfebrile persons during March 2012 (rainy season) in the same locality were also provided. Protocols used to obtain human samples from Esmeraldas were approved by the Universidad San Francisco de Quito Bioethics Committee and the University of Michigan Institutional Review Board.

A total of 100 serum samples from febrile patients in Portoviejo, a semiurban community, were provided by the Ecuador MoH; 34 were positive for *Leptospira* spp. (IgM ELISA; PanBio). The other 66 samples were not tested for *Leptospira* spp., but they were tested for dengue virus by IgM ELISA (9 were positive). The samples had been collected during the rainy season, March–June 2012.

A total of 154 serum samples from febrile patients in Guayaquil, an urban community, were provided by the Ecuador MoH. Samples were collected from different medical posts and hospitals around the city during the rainy season, July–October 2011. The samples had been tested for dengue virus by IgM ELISA (all were negative); no samples were tested for *Leptospira* spp. All samples from Portoviejo and Guayaquil were collected by government officials and were deidentified before being sent to our laboratory.

Animal Samples

In Portoviejo, during the dry season in 2009 and the wet season in 2013, we collected urine samples from domestic animals (27 pigs, 30 dogs, and 27 cows in 2009; 30 pigs and 26 cows in 2013) and kidney samples from rats (6 in 2009 and 60 in 2013). We administered 2.5 mg/kg of furosemide (a diuretic) to animals to collect their urine during micturition or by cystocentesis. Rats were captured inside the homes of Portoviejo residents by using traps from Tomahawk Live Trap (Hazelhurst, WI, USA) or snap traps, and as needed, they were euthanized by using chloroform. Urine samples collected in 2009 from cattle, pigs, and dogs were obtained from residential areas. Cattle and pig urine samples collected in 2013 were obtained from a local slaughterhouse that processed animals from the same location.

Overview of the Molecular Analyses

Our overall analytic goal was to ensure detection of leptospires of the intermediate and pathogenic clusters. To this end, we adapted a previously used protocol to amplify the *rrs* genes from pathogenic and intermediate clusters. We then sequenced the *rrs* gene to detect leptospiral species and anomalous amplification products. A sample was considered positive when its amplicon comprised sequences for leptospira bacteria.

DNA Extraction

Frozen animal urine samples (10 mL) were thawed on ice and pelleted by centrifugation at 3,287 × *g* for 15 min. DNA was extracted from the pellets by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) and stored at –80°C. Frozen serum samples were thawed on ice, and 200 mL was used for DNA extraction (QIAamp DNA Mini Kit); the DNA was stored at –80°C. Eight punches (6-mm diameter) from blood spots were placed in a 1.5-mL microcentrifuge tube and incubated in 180 mL of ATL buffer (QIAGEN) for 10 min at 85°C, and the supernatant was transferred into a new 1.5-mL microcentrifuge tube and processed for DNA extraction.

A 2-mm^3 section of rat kidney was cut and washed 3 times with 1 mL of PBS. DNA was extracted by dissolving the kidney tissue in 700 μL of CTAB extraction buffer, followed by incubation (with shaking every 15 min) for 2 h at 65°C. The tubes were cooled to room temperature, and 700 μL of a chloroform–isoamyl alcohol (24:1) mixture was added to each tube. Contents were mixed and then centrifuged at 6,000 × *g* for 5 min, and the aqueous phase was transferred to another tube. DNA was precipitated with a 3 M sodium acetate (pH 5) solution and ethanol, and the pellet was washed with 70% ethanol, dried, and dissolved in 50 mL of Tris-EDTA buffer.

Amplification of Leptospiral *rrs* Gene

Leptospiral DNA from samples was detected by using 1 of the following primer sets (AB or CD), both of which amplify the same small fragment target of 16S *rrs* gene specific to leptospiral species: forward A 5′-GGCGGCGCGTCTITAAACATG-3′, reverse B 5′-TTCCCCCCATTGAGCAAGATT-3′, forward C 5′-CAAGTCAAGCGGAGTAGCAA-3′, reverse D 5′-CTTAACCTGCTGCCTCCCGTA-3′ (*18*). Amplicon sizes were 332 bp for primers AB and 290 bp for primers CD. We adapted this protocol for real-time PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR master mix included iQ SYBR Green Supermix (Bio-Rad), 0.5 mM each

primer and molecular biology–grade water, and 1 mL of DNA template for a final reaction volume of 10 mL. Our amplification protocol used an initial enzyme activation step at 95°C for 3 min, followed by 45 amplification cycles (30 s at 95°C, 30 s at 62.5°C, 30 s at 72°C). To detect the presence of amplified *rrs* gene amplicon, we performed a melting curve analysis (65°C to 85°C, with a ramp of 0.5°C/5 s). To increase the concentration of the *rrs* gene amplicon, we subjected the PCR products from samples positive for *rrs* gene amplification to a second round of PCR amplification by using the conventional PCR protocol (*18*). Using the same AB or CD primer pairs and 0.5 mL of *rrs* gene amplicons from the first PCR amplification, we initiated the amplification protocol for the second PCR with denaturation at 94°C for 3 min, followed by 29 cycles of 94°C for 1 min, 63°C for 1.5 min, and 72°C 2 min, and then ended with a final 10-min elongation at 72°C. To rule out accidental contamination of PCR reagents, we included negative controls in all reactions. In addition, all reagents used in our analyses performed during 2012–2013 were subjected DNA amplification by using primer sets AB and CD (real-time and conventional PCR).

Sequence Analysis of Leptospiral *rrs* Gene

Concentrated *rrs* gene amplicons of 277 samples were sequenced at Functional Biosciences (Madison, WI, USA) by using primers AB or CD. To analyze DNA sequences, we used MEGA 5.08 (http://www.megasoftware.net) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For sequencing, we selected 19 amplicons from samples collected in 2009 from different animals in Portoviejo. Overall, 11 *Leptospira* spp. sequences were submitted to GenBank under accession nos. JN377490 and JN377491 (*L. inadai* from animals in Portoviejo, 2009); JN377492 (*L. borgpetersenii* from an animal in Portoviejo, 2009); KF303505 (*L. wolffii* from a human in Esmeraldas, 2012); KF285460 (*L. wolffii* from a human in Portoviejo, 2012); KF285460 (*L. wolffii* from a human in Guayaquil, 2011); KM259910 (*L. wolffii* from an animal in Portoviejo, 2013); KF303504 (*L. noguchii* from a human in Esmeraldas, 2012); KF303503 (*L. borgpetersenii* from a human in Guayaquil, 2011); KJ573104 (*L. noguchii* from an animal in Portoviejo, 2013); and KJ573105 (*L. borgpetersenii* from an animal in Portoviejo, 2013).

Design of Intermediate *Leptospira* spp.–Specific Assay

We designed a *Leptospira* spp. assay to target only intermediate *Leptospira* spp. We used Primer3 (*19*) to design a reverse primer (R Inter: 5′-TCTTTACCTATCARATCYTGTGATCCA-3′) to be used with A or C forward primers; amplicon sizes were 160 bp for A and 143 bp for C. The specificity of this assay was validated with 17 leptospiral DNA samples from reference strains of pathogenic, saprophytic, and intermediate leptospiral species obtained from the Royal Tropical Institute (Table 1). We tested for the intermediate leptospiral genotype in 75 human serum samples from Portoviejo that were real-time PCR positive for *Leptospira* spp. The real-time PCR amplicons were subjected to a second PCR amplification using R-Inter reverse primer. The total reaction volume of the intermediate-specific real-time PCR assay, using GoTaq Flexi Polymerase (Promega, Madison, WI, USA), was 20 mL; 0.5 mL of the real-time PCR amplicon was used as template for the conventional PCR.

Results

Human Samples

Leptospiral DNA was detected in 73 (68%) of 108 serum samples and 59 (57%) of 102 blood spots from febrile patients in the rural study site (Esmeraldas) (Table 2; Figure). All *Leptospira* spp.–positive amplicons from blood spots and 70 (96%) of the 73 *Leptospira* spp.–positive amplicons from serum samples showed 100% DNA sequence identity with *L. wolffii* (intermediate cluster). The remaining 3 positive amplicons from serum samples showed 99% identity with *L. noguchii* (pathogenic cluster). Of the 108 serum samples, 31 (29%) were positive for *Leptospira* spp. (PCR) and dengue virus (IgM ELISA), 4 (3.7%) were positive for dengue virus only (IgM ELISA), and 42 (39%) were positive for *Leptospira* spp. only (PCR). DNA sequences of 6 (4%) of 135 amplicons showed anomalous amplification products. All serum samples from nonfebrile patients were either PCR negative for *Leptospira rrs* gene (n = 15) or determined to be negative for *Leptospira* spp. because of anomalous amplification products (n = 5).

Table 1. PCR results by using primer Inter-R combined with primers A and C*

Leptospira spp.	Strain	PCR result
Intermediate		
L. broomii	5399	+
L. fainei	BUT 6	+
L.inadai	10	+
L. wolffii	Korat-H2T	+
L. licerasiae	VAR010	+
Pathogenic		
L. interrogans	Pomona	–
L. kirschneri	Kambale	–
L. borgpetersenii	MUS 127	–
L. noguchii	M7	–
L. alexanderi	A85	–
L. santarosai	CZ 390	–
L. weilii	Sarmin	–
Saprophytic		
L. vanthielii	WazHolland	–
L. biflexa	Patoc I	–
L.meyeri	ICF	–
L. wolbachii	CDC	–
L. kmetyi	Bejo-Iso9T	–

*Only intermediate *Leptospira* species were amplified by using Inter-R specific primer. +, positive; –, negative.

Table 2. *Leptospira* spp.–positive samples from febrile patients in 3 communities along the coast of Ecuador, 2011–2012*

Location, year	No. samples analyzed	*Leptospira* spp.–positive samples				No. (%) spurious PCR products†
		Pathogenic cluster		Intermediate cluster		
		No. (%)	Species	No. (%)	Species	
Esmeraldas, 2011–2012‡	108§	3 (2.7)	*L. noguchii*	73 (68)	*L. wolffii*	6 (4)
	102¶	0	–	59 (58)	*L. wolffii*	0
Portoviejo, 2012#	100	0	–	24 (24)	*L. wolffii*	0
				1 (1)	*L. inadai*	15 (32)
Guayaquil 2011**	154	3 (1.9)	*L. borgpetersenii*	28 (18)	*L. wolffii*	9 (21)
		1 (0.6)	*L. kirschneri*/*L. interrogans*††	–	–	0

*The 3 communities were in rural (Esmeraldas), semiurban (Portoviejo), and urban (Guayaquil) locations. Leptospiral DNA in patient samples was detected by PCR. Molecular methods were used to amplify and sequence the leptospiral *rrs* gene from DNA. –, not applicable/no value.
†The spurious products represent serum samples that produced amplicons of the correct size but with DNA sequences different from *Leptospira* (for the pathogenic and intermediate cluster).
‡Of samples from Esmeraldas, 27% were positive for dengue virus (IgM ELISA) and *Leptospira* sp. (PCR).
§Serum samples.
¶Blood spot samples.
#Sixty-six samples were tested for dengue virus by IgM ELISA (57 negative, 9 positive) but were not tested for *Leptospira* sp.; 34 samples were IgM ELISA–positive for *Leptospira* sp. but were not tested for dengue virus.
**Samples tested negative for dengue virus by IgM ELISA.
††The amplicon showed the same degree of identity to both species.

Leptospiral DNA was detected in 25 (25%) of 100 serum samples from the semiurban study site (Portoviejo): 7 of these were also IgM positive for *Leptospira* spp. (dengue IgM unknown), 16 were IgM ELISA negative for dengue virus (*Leptospira* spp. IgM unknown), and 2 were IgM ELISA positive for dengue virus (*Leptospira* IgM unknown). Twenty-four *Leptospira* spp.–positive amplicons showed 100% DNA sequence identity to *L. wolffii*, 1 amplicon showed 98% identity to *L. inadai*, and 15 amplicons (of the expected size) were anomalous amplification products.

Leptospiral DNA was detected in 32 (21%) of 154 serum samples from the urban study site (Guayaquil) (Table 2). As with samples from Portoviejo and Esmeraldas, most samples from Guayaquil had amplicon sequences that shared 100% identity with *L. wolffii* (intermediate cluster). Only 3 (2%) samples shared amplicon sequence identity with pathogenic *Leptospira* spp. and 2 with *L. borgpetersenii* (99% identity); 1 could not be differentiated as *L. kirschneri* or *L. interrogans* (both 99% identity) (Table 2). Of 43 amplicons displaying the expected size, 9 were anomalous amplification products.

Animal Samples

Of the 90 animal samples collected from Portoviejo during the 2009 dry season, 65 (72%) were PCR positive for *Leptospira* spp.: 21 (70%) of 30 samples from dogs, 18 (67%) of 27 from pigs, 20 (74%) of 27 from cattle, and all 6 rat kidney samples. However, we sequenced only 19 amplicons from these samples (3 from dogs, 3 from pigs, 7 from cattle, and 6 from rats). BLAST analysis of amplicon sequences from these 19 samples showed that 14 (74%) had 100% sequence identity to *L. inadai* (intermediate cluster), whereas amplicons from 5 animals (3 cows, 1 pig, and 1 rat) had 100% identity to *L. borgpetersenii* (pathogenic cluster) (Figure; Table 3). During 2009–2013, the dominant species

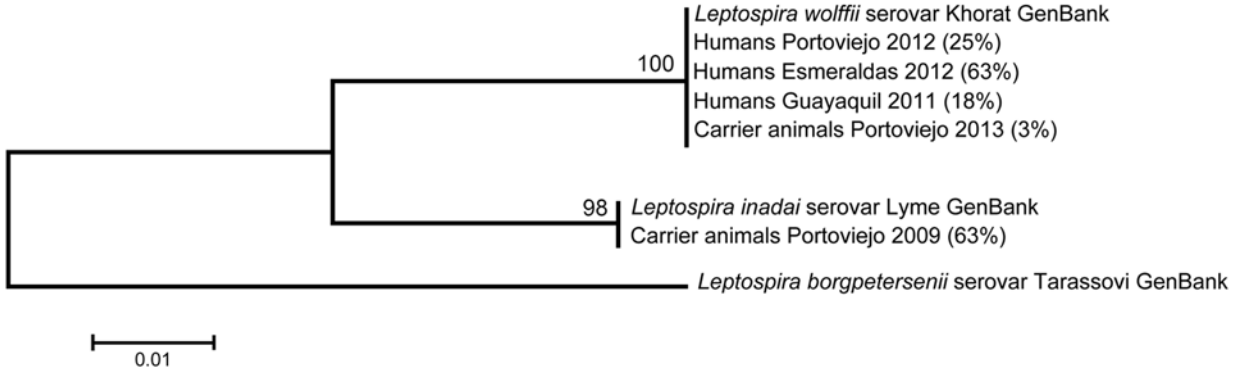

Figure. Maximum-likelihood tree for DNA sequences of the *Leptospira* spp. *rrs* gene recovered from serum samples from febrile humans and from urine and kidney samples from animal carriers in Ecuador. Esmeraldas, Portoviejo, and Guayaquil are 3 rural, semiurban, and urban communities, respectively, along the coast of Ecuador. Pathogenic *L. borgpetersenii* was used as an outgroup. Numbers in parentheses indicate the percentage of samples per community that contained DNA signatures highly similar to GenBank reference strains *L. wolffii* (NR_044042), *L. inadai* (accession no. JQ988844.1), and *L. borgpetersenii* (accession no. JQ988861.1). Scale bar indicates the degree of nucleotide substitutions.

of leptospires shifted from *L. inadai* (intermediate cluster) to *L. borgpetersenii* (pathogenic cluster) (Table 3). In addition, among intermediate types, we observed a population shift from *L. inadai* to *L. wolffii* (Table 3; Figure); the identity for *L. wolffii* sequences was 99%.

Verification of the Intermediate *Leptospira* spp.–Specific Assay

The R-Inter primers amplified only intermediate *Leptospira* sequences when tested against 17 leptospiral DNA from reference strains (Table 1). Of the 75 human serum samples with a supportive real-time PCR melting curve, 12 were positive for leptospiral sequence when primer pair AB was used, but 23 were positive when primer R-Inter was used. Of the 12 PCR reaction products that were positive by primer pair AB, 10 were also positive when using primer pairs A/R-Inter or C/R-Inter, and 2 DNA samples positive for leptospiral sequence using primers AB were negative when using R-Inter primer. The amplified sequences showed 100% identity to *L. wolffii.* We did not run this test with *L. inadai*–positive samples collected from animals in Portoviejo in 2009 because the samples were unavailable for this analysis. Nevertheless, in silico testing showed that the nucleotide sequence of R-Inter primer was identical to *L. inadai* sequences.

Discussion

Our findings show that leptospiral DNA was present in various proportions in febrile patients living in 3 communities in Ecuador; the DNA was present in 63% of samples from persons at a rural site and in 25% and 21% of samples from persons at semiurban and urban sites, respectively. The use of leptospiral *rrs* DNA amplification and subsequent sequencing enabled us to detect leptospiral DNA (pathogenic and intermediate clusters) and rule out false-positive reactions. Of note, 96% of leptospiral DNA from human serum showed identity with intermediate rather than pathogenic clade strains. This finding is in contrast with the current notion that human leptospirosis is mainly caused by pathogenic cluster strains (*2*,*4*,*6*,*9*,*10*). One reason our findings contrast with those of prior studies is that we sequenced the amplified *rrs* gene to identify false-positive reactions and to identify intermediate cluster *Leptospira* spp.

Although our study lacked serologic data to determine acute leptospirosis (seroconversion using paired serum samples), the presence of leptospiral DNA in febrile persons combined with no evidence of dengue infection (a major cause of fever in coastal Ecuador) makes it plausible that the fever was caused by leptospirosis. The finding that none of the serum samples from asymptomatic persons contained leptospiral DNA also supports this finding.

The presence of intermediate leptospiral DNA and the absence of more serious symptoms of leptospirosis (jaundice, hemorrhages, renal failure) in our study are consistent with reports of mild disease linked to intermediate *Leptospira* spp., such as *L. licerasiae* in Peru (*11*), *L. wolffii* in Thailand (*14*), and *L. inadai* (*12*). Severe leptospirosis symptoms have been associated only with the intermediate cluster species *L. broomii* (*15*).

Febrile symptoms could be caused by many infectious agents (*17*), as evidenced by our finding that in Guayaquil and Portoviejo, 80% and 57% of the febrile population, respectively, did not show evidence of leptospirosis or dengue infection. In addition, environmental factors in these communities may facilitate exposure of inhabitants to multiple infectious agents; thus, febrile symptoms may be due to co-infections. We found evidence of concurrent dengue virus (IgM ELISA) and *Leptospira* spp. (PCR) infection in 27% of serum samples from Esmeraldas and 22% from Portoviejo. However, concomitant positive diagnostic outcomes for leptospirosis and dengue might be due to persistent presence of antibodies. Detection of IgM antibodies to dengue virus starts 4–5 days after the onset of symptoms

Table 3. Species and cluster of leptospiral DNA sequences recovered from animals in 2009 and 2013, Portoviejo, Ecuador*

Location, year, animal, no analyzed samples	*Leptospira* spp.–positive samples				No. (%) spurious PCR products†
	Pathogenic cluster		Intermediate cluster		
	No. (%)	Species	No. (%)	Species	
Portoviejo, 2009‡					
Cattle, n = 7	3 (43)	*L. borgpetersenii*	4 (57)	*L. inadai*	0
Rats, n = 6	1 (17)	*L. borgpetersenii*	5 (83)	*L. inadai*	0
Dogs, n = 3	0	–	3 (100)	*L. inadai*	0
Pigs, n = 3	1 (33)	*L. borgpetersenii*	2 (67)	*L. inadai*	0
Portoviejo, 2013§					
Cattle, n = 26	5 (19)	*L. borgpetersenii*	1 (4)	*L.wolffii*	3 (27)
	1 (4)	*L. kirschneri*	–	–	
Rats, n = 60	3 (5)	*L. borgpeterseni*	1 (1.7)	*L.wolffii*	3 (21)
	2 (3.3)	*L. kirschner*¶	–	–	
Pigs, n = 30	2 (6.7)	*L. borgpetersenii*	1 (3.3)	*L.wolffii*	5 (50)

*–, not applicable/no value.
†Percentage of amplicons (obtained from samples of each animal species) which showed expected size but the DNA sequences were different from *Leptospira* spp.
‡Dry season.
§Rainy season.
¶One amplicon also showed the same degree of identity to *L. interrogans*.

and extends for up to 5 months after infection (*20*), whereas PCR for *Leptospira* spp. on blood samples mainly detects acute infection (*21*) and, as reported by others (*22*,*23*), is unsuitable for detecting asymptomatic renal colonization. Thus, while co-infection cannot be ruled out, it is conceivable that most, if not all, of these co-infected febrile patients had acute leptospirosis.

We also found high carriage rates of intermediate leptospires (*L. inadai* and *L. wolffii*) among domestic and peridomestic animals in Portoviejo in 2009 and 2013 (Figure; Table 3). This finding concurs with those in published reports showing intermediate leptospires carried by domestic and peridomestic animals (*11*,*13*,*24*). The meaning of the relative proportion of intermediate cluster strains observed in this animal study must be considered with caution as we cannot exclude the possibility of selection bias, given the fact that animals were not randomly sampled.

We present molecular evidence of the presence of a similar intermediate *Leptospira* spp. (*L. wolffii*) among animal populations and humans in the same locality (Portoviejo). However, because the sampling was conducted at different times, we were unable to directly link *Leptospira* spp. carriage among animals and humans. This linkage is further complicated by a difference in prevalence rates of *L. wolffii* DNA among humans in 2012 and animals in 2013 (24% and 2.6%, respectively) (Figure). The difference in distribution of *Leptospira* spp. in humans and animals may be caused by human lifestyle, which can reduce direct or indirect exposure to the animals, or by different environmental survival capacities of pathogenic and intermediate *Leptospira* spp. (*25*). Nevertheless, we showed presence of the same intermediate DNA species of *Leptospira* in humans and animals, which is consistent with findings in other studies that suggest a link between human disease caused by intermediate leptospiral species (*L. licerasiae*) from rats and water sources in Peru (*3*,*11*).

The presence of leptospiral species in animals appears to be temporally dynamic. In Portoviejo, we observed that the dominant leptospiral species shifted from *L. inadai* in 2009 to *L. borgpetersenii* and *L. wolffii* in 2013 (Table 3). Temporal changes in leptospiral sequence types have been previously reported (*26*). It is possible that environmental conditions (e.g., humidity, intensity of rainy season, abundance of some animal species, chemical changes in natural water sources) may favor colonization of reservoir animals with a given type of leptospires. These temporal dynamics may explain the apparent sporadic nature of leptospirosis outbreaks; the circulation of pathogenic *Leptospira* spp. may cause typical and easily recognizable disease, whereas the circulation of intermediate species may cause a generally milder disease with a broad spectrum of symptoms, which makes the disease prone to misdiagnosis. These results highlight the need to conduct longitudinal surveys of leptospiral populations.

Differences in sanitary infrastructure may explain the higher prevalence of leptospiral infection in the rural community as compared with the more urban communities (Table 2). Communities in Esmeraldas tend to rely more on rivers for fresh water and transportation, increasing the probability for leptospiral exposure. Because both urban and rural communities co-exist with animals that carry leptospires, the difference in prevalence we observed likely reflects the efficiency of leptospiral dispersal by water. Unfortunately, we were unable to obtain information about water exposure or occupation of the febrile patients.

Our study also lacked clinical data for febrile patients, which prevented the investigation of presumptive leptospirosis–dengue co-infections or of the difference between infections with pathogenic or intermediate leptospiral species. Although these limitations prevent us from drawing stronger conclusions, our study clearly showed compelling evidence of the abundant presence of intermediate *Leptospira* spp. in humans and animals. This finding warrants further investigation of the effect of these species on the disease burden observed in veterinary and human public health.

Other limitations in our study were the low number of DNA sequences obtained in 2009 from animals in Portoviejo and the lack of leptospiral isolates belonging to the intermediate cluster. A year later, we attempted without success to amplify leptospiral sequences from positive samples. Also, despite many attempts, we failed to isolate intermediate *Leptospira* spp. from febrile humans and domestic animals, although we isolated *L. santarosai* (pathogenic cluster) from a dog urine sample collected in Portoviejo in 2009 (data not shown). It is possible that intermediate species circulate at lower numbers than pathogenic counterparts or that some of these species may be more fastidious than pathogenic species.

Intermediate leptospires are rarely detected in humans, probably because many PCR protocols amplify genes that are present only in pathogenic species (*21*). Genetic characterization of *Leptospira* spp. makes it possible to understand disease transmission patterns and to obtain new insights by reinterpreting serologic and clinical epidemiologic data within a genetic context. Correct identification of the etiologic agent is critical for disease management in regions where dengue, malaria, leptospirosis, and, more recently, chikungunya are present (*27*,*28*). Our finding of a high number of false-positive reactions reveals the risks of using the 16S PCR (without amplicon sequencing) for diagnosis of leptospirosis.

Acknowledgments

We thank Ministerio de Salud Pública–Ecuador, Celia Riera, Erika Keim, Daisy Parrales, María Inés Baquero, Nadia López, Iván Haro, and Soraya Reyes for their contributions to this investigation; William Cevallos for providing samples from

nonfebrile patients in Esmeraldas; and Josefina Coloma for her contribution to the dengue virus detection in Esmeraldas.

This work was funded by the National Institute of Allergy and Infectious Disease, National Institutes of Health (grants AI050038 and AI101913); Organización Panamericana de la Salud cede Ecuador; Universidad San Francisco de Quito Ecuador; and Center for Microbial Genetics and Genomics, Northern Arizona University.

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Biological Warfare Plan in the 17th Century—the Siege of Candia, 1648–1669

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A little-known effort to conduct biological warfare occurred during the 17th century. The incident transpired during the Venetian–Ottoman War, when the city of Candia (now Heraklion, Greece) was under siege by the Ottomans (1648–1669). The data we describe, obtained from the Archives of the Venetian State, are related to an operation organized by the Venetian Intelligence Services, which aimed at lifting the siege by infecting the Ottoman soldiers with plague by attacking them with a liquid made from the spleens and buboes of plague victims. Although the plan was perfectly organized, and the deadly mixture was ready to use, the attack was ultimately never carried out. The conception and the detailed cynical planning of the attack on Candia illustrate a dangerous way of thinking about the use of biological weapons and the absence of reservations when potential users, within their religious framework, cast their enemies as undeserving of humanitarian consideration.

In the course of history, plague, caused by *Yersinia pestis*, has been responsible for at least 3 widespread pandemics with high mortality rates. The first, the "Justinian plague," spread around the Mediterranean Sea and Western Europe in the 6th century; the second, the so-called Black Death, struck Europe in the 14th century; and the third began in China during the middle of the 19th century and spread throughout the world (*1*–*3*). The Black Death decimated Medieval Europe and had a major effect on the continent's socioeconomic development, culture, art, religion, and politics (*4*,*5*).

In 1346, plague was deliberately used as a biological weapon. During the siege of Caffa, a Genoese possession in Crimea (now Feodosia, Ukraine), the attacking Mongol forces experienced an epidemic of plague (*6*–*8*). The Mongols, however, converted their misfortune into an opportunity by hurling the cadavers of their deceased into the city, and this action perhaps initiated the ensuing plague epidemic. In 1710, during the Swedish–Russian War, in the siege of Reval (now Tallin, Estonia), the Russians were said to have hurled corpses of plague victims into the besieged city (*9*). During World War II, Japan conducted biological weapons research at facilities in China. Prisoners of war were infected with several pathogens, including *Y. pestis;* >10,000 died as a result of experimental infection or execution after experimentation. At least 11 Chinese cities were attacked with biological agents sprayed from aircraft or introduced into water supplies or food products. *Y. pestis*–infected fleas were released from aircraft over Chinese cities to initiate plague epidemics (*10*). We describe a plan—ultimately abandoned—to use plague as a biological weapon during the Venetian–Ottoman War in the 17th century.

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DOI: http://dx.doi.org/10.3201/eid2112.130822

Archival Sources

Our research has been based on material from the Archives of the Venetian State (*11*). The sources are the communications between the Inquisitors of the State, the Council of Ten, and the commander of Dalmatia. The letters cover the period from February 5, 1649, through August 3, 1651 (i.e., February 5 and 22, 1649; April 1, 21, and 29, 1649; May 9, 1649; December 16, 1650; and August 3, 1651). Although the letters were included in the collections of Venetian documents in works by Lamasky (*12*) and Brown (*13*), they have escaped the attention of medico-historical researchers.

Historical Background of Venetian–Ottoman Wars

The siege of Candia, 1648–1669, is the longest in the military history. The city of Candia (now Heraklion, Greece) was the capital of the Kingdom of Candia (Crete) (*Regno di Candia*), which had been a Venetian possession since the fall of Constantinople during the Fourth Crusade in 1204. During the Venetian–Ottoman Wars, the island was the key for the supremacy of the eastern Mediterranean (*14*).

After the fall of Constantinople (1453) and the fall of Rhodes, the possession of the Order of the Knights of Saint John (1522), to the Ottomans, the Republic of Venice was the ultimate protector of Christianity in the eastern Mediterranean. Until the siege of Candia, the 2 superpowers of that era, Venice and the Ottoman Empire, many times crossed swords for the supremacy of the Mediterranean Sea: in 1463–1479, 1499–1502, 1537, and in 1571 in the naval

battle of Lepanto (*14*). In addition to the geostrategic and economic reasons, the conflicting religions were another cause of war between Venice and Ottoman Empire. At that time, under the cover of a Holy War, atrocities against the "unbelievers" were a common phenomenon. After so many years of Holy Wars in the area, religious fanaticism was the first instinctive feeling in the mind of the rival armies.

During the first year of a new war (1644), the Ottoman forces landed in Crete, and the Venetians asked for help from the Pope and other European nations. However, Europe was in the flames of the Thirty Years War (Germany, Austria, and Spain versus Denmark, Sweden, and France). Because England and Holland had commercial agreements with the Ottoman Empire, their leaders refused to help Venice (*15*). In fact, the European efforts to help Candia were disastrous: for example, the expeditions of King Louis XIV of France in 1668 and 1669. The last *provveditore* (governor) of Candia, Francesco Morosini, surrendered the city to the Turks on September 6, 1669, after a blockade of 21 years.

Inquisitori di Stato and the Idea of Biological Warfare

The *Inquisitori di Stato di Venezia* (Inquisitors of the State of Venice) had been established during 1539, after the decision of the *Consiglio dei Dieci* (Council of Ten) to protect the state (*16*). This intelligence service was one of the most effective and deadly in the history of espionage. Venice had an impressive network of spies, and its dark history was connected with political plots, torture, and assassination attempts too numerous to count (*17*).

Venetian Plan

On February 5, 1649, the heads of the *Inquisitori*, Piero Morosini, Piero Querini, and Geronimo Giustiniani, received a top-secret letter from Zara (now Zadar, Croatia), a Venetian possession on the Dalmatian Coast. In it, the *Provveditore Generale di Dalmazia et Albania*, Lunardo Foscolo, proposed a plan to end the siege of Candia by infecting the Ottoman forces with a poisonous liquid that he described as "the quintessence of the plague." The plan was likely to be undetected and successful because plague outbreaks occurred frequently on the island. In fact, since the Black Death pandemic began devastating Europe, 20 outbreaks had occurred on Crete from 1348 through 1645 (*18*,*19*).

Because an outbreak that occurred only in the Ottoman camp around the city of Candia would be suspicious, Foscolo proposed an alternative "perfect plan"—a massive plague attack against all the Ottoman camps in different places of the island, which would look like a real epidemic of large scale. The plan is detailed as follows (in a typical, Venetian-style letter with long sentences) (*11–13*):

To the most Illustrious and most Honored Lords my Masters

My incessant occupation in the discharge of this most laborious service never makes me forget my intent and desire to procure advantage to my country. I then, considering the perilous state of the kingdom of Candia, first treacherously invaded, and now openly occupied by the Turks, the pre-eminence of their forces, the copiousness of their soldiery, the opulence of the Turkish treasury, which will enable them to maintain the war for many years, and also being well aware that, although the public spirit of Venice yields to none in courage and magnanimity, the Republic has neither forces, men, nor money, wherewith to resist much longer the attacks of its foes, and reflecting on the impossibility to meet such a heavy expenditure, have applied myself to a study of the methods whereby the Turkish power might be overcome without risk of men or burden to the exchequer, and how the kingdom of Candia might be recovered; for, after God, our hope to reacquire it is small indeed.

Now there is here a good subject of Venice, lately appointed doctor, who besides his skill in healing is also a famous distiller. His name is Michiel Angelo Salamon. He is desirous to prove himself, what he is in fact, a faithful servant of your Excellencies. I explained my wishes to him, and he availed himself of the presence here of the plague to distil a liquid expressed from the spleen, the buboes, and carbuncles of the plague stricken; and this, when mixed with other ingredients, will have the power wherever it is scattered to slay any number of persons, for it is the quintessence of plague I considered that if this quintessence of plague were sown in the enemies' camps at Retimo, Cannea, and San Todero, and if it operates as Dr. Michiel assures me it will, this would greatly assist us to recover the kingdom of Candia.

I accordingly determined not to lose the opportunity to have a vase of the poison prepared, and this jar shall be kept, with all due precautions, for the service of your Excellencies. I believe, however, that some ruse must be adopted to entice the Turks into the trap, and would suggest that we should make use of the Albanian fez, or some other cloth goods, which the Turks are accustomed to buy, so that the poison may pass through as many hands in as short a time as possible. The cloth should be made up in parcels as if for sale, after

> having been painted over with the quintessence, and then placed in separate boxes destined for the various places where we desire to sow the poison. The quintessence, well secured in several cases for the greater safety of those who have to handle and transport it, should be sent to the commander-in-chief that he may take the necessary steps for causing it to pass into the enemies' hands.
>
> This may be done either by lading several vessels with the cloth, which vessels are to be abandoned by their crews when the enemy comes in sight; or else by means of peddlers who shall hawk the cloth about the country; so that the enemy, hoping to make booty, may gain the plague and find death. The affair must be managed with all circumspection, and the operator must be induced to his work by hopes of gain and by promises, for it will be a dangerous undertaking, and when the operation is over he must go through a rigorous quarantine. While handling the quintessence, it will be of use to the operator to stuff his nose and mouth with sponges soaked in vinegar; and while poisoning the cloth, he may fasten the brush to an iron rod, and when finished, he must put brush and rod into the fire. Having given the Turk the plague, every care must be taken to prevent our people coming in contact with them.
>
> The proposition is a virtuous one, and worthy of the composer of the quintessence. It is, however, a violent course, unusual, and perhaps not admitted by public morality. But desperate cases call for violent remedies, and in the case of the Turks, enemies by faith, treacherous by nature, who have always betrayed your Excellencies, in my humble opinion, the ordinary considerations have no weight."

In the next letters, the Council of Ten and the *Inquisitori* thank the *provveditore* for his plan and agree that Dr. Salamon, who invented the mixture, should be appointed to carry the poison to the commander-in-chief of the fleet. The commander-in-chief must be warned of the great risk to his own troops from the deadly mixture. However, although Dr. Salamon showed great unwillingness to participate in this operation, the Council of Ten insisted on his presence.

To protect the town of Zara from a possible plague outbreak, the *Inquisitori* further insisted that the cloth goods must be infected on the ship. If, during the voyage, the jar was broken, the crew must empty the contents into the sea. Foscolo succeeded in overcoming Dr. Salamon's objections, and the appropriate doctor and his jar of quintessence reached the fleet in 1649. Dr. Salamon found Foscolo moving his ships into naval bases to protect them from winter, and he was unable to make use of the mixture at once. Moreover, the commander failed to keep the jar beside him during through the winter. For this reason, Dr. Salamon and "the quintessence of the plague" were once more shipped on board and returned to Zara, likely in late 1649. In Dalmatia, the *Inquisitori,* for safety reasons, put the sample and the doctor in quarantine in a prison. During 1650, Foscolo prepared the Venetian Armada for his attack on Crete and immediately demanded that Dr. Salamon to be sent to him in Candia. The doctor and his jar were liberated, but not before 200 ducats had been exacted from him (as security money for the public property) and given to the Republic of Venice. After Foscolo's letter about Dr. Salamon's liberation, on August 3, 1651 (*11*), the story suddenly ended. No further information exists, and probably no further details about this attempt will be known.

Discussion

Even without knowledge of plague's microbial nature, the militants of that era understood the "value" of the contamination of an enemy army. The deadly liquid was expressed from the spleens, the buboes, and carbuncles of the victims of a plague outbreak in Dalmatia *(…un liquore scatturito da fieli, bubone et carboni d'appestati con altri ingredient, che averà forza et virtù, dove sarà sparso, essendo la quinta essenze della peste…*). The instructions to the men offer the view of the miasmatic air theory, a theory about the nature of disease that existed as late as the 19th century (*11*,*20*). According to the theories of the era, an infectious disease was the result of the inhalation of miasma (poisonous vapor) of decomposing animal matter, either in the form of aerial emanations or of local pollution of the drinking water by infiltration of such substances. Fundamentally, 2 medical schools of thought existed: those who believed in person-to-person infection and those who believed the existence of a poisonous miasmatic cloud (*21*). Because of the danger of miasma, Foscolo's men were required to keep their faces covered by sponges soaked in vinegar: "*…l'otturarsi le narici et la bocca con sponga bagnata in aceto….*"

In terms of beliefs regarding the way the infection could spread, the choice of hats and clothes reflect another issue of that era, that is, the understanding of contaminated objects. Until the 19th century and before the revolution of microbiology, 3 theoretical positions may be distinguished: 1) the miasmatic theory that proposes that contamination is caused by the state of the atmosphere, 2) a modified miasmatic theory that proposes that poor sanitary conditions affect the atmospheric disturbances, and 3) a theory about the combination of miasma/contagion, which may be called contingent contagionism (e.g., that a disease was not contagious in a so-called healthy atmosphere, but might be contagious in an impure atmosphere) (*20*).

After the end of the operation, the authorities had the duty to isolate the peddlers in a pesthouse. This requirement seems logical for the inventors of the quarantine. After the Venetians became a military power, the Venetian Republic soon realized the demographic, economic, and military importance of infectious diseases. The Venetians showed a great interest in preventive medicine and the protection of public health in their possessions (*22*). The Venetian possessions each had their own *proveditore alla sanità* (governor of health), *magistrato alla sanità* (health magistrate), and a *lazaretto* (pesthouse) with its *priore* (director), *dottori* (physicians), and sanitation guards (*23*).

Quarantine of 40 days (from the Italian "quaranta," meaning 40) was adopted as an obligatory means of isolating persons, animals, and goods that may have been exposed to a contagious disease (*24*). Since the 14th century, quarantine has been the major 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 (*25*). Also, if the duration of the quarantine is compared with the incubation periods of infectious diseases (e.g., cholera, plague, yellow fever, smallpox), the isolation period overlapped their incubation period (*26*,*27*). During epidemics, the urban health authorities adopted social interventions and traditional health tools, such as quarantine of travelers who had contact with infected persons or who came from a place where the disease was endemic or epidemic (*28*).

The tactics of the so-called dirty war were known to the Venetian army. In Venetian military history, some cases of attacks with chemical agents are recorded (*17*). We suppose that for this reason, Foscolo thought that the Turks would understand the Venetian trick. In the history of the biological war, some cases were complex and results were mixed. A biological attack by the Japanese in 1941 in Changde, China, against the Chinese army and civilians led to 10,000 deaths from cholera in the Chinese population (caused by ingestion of *Vibrio cholera*e–contaminated food and water) but also 1,700 deaths among the unprepared Japanese troops (*29*,*30*).

The Venetian operation would likely have been possible (and not detected) was because of the history of 20 previous plague outbreaks on the island; for this reason, Foscolo proposed the massive attack against the entire island. According to Dr. Salamon and Foscolo, the liquid would have been quite effective; however, this view was not based on the results of an experimental study but on empirical surveillance of the disease's death rates.

The main question of the operation, however, was the final efficacy of the mixture. On the basis of current knowledge of *Y. pestis*—the viability of the bacterium outside its normal hosts and its modes of transmission—we do not believe that the Venetian plan would have been effective. How long the bacterium survives outside the host depends greatly on the nature of the material in which it is found. Recent studies evaluating viability of *Y. pestis* on manufactured surfaces (e.g., steel, polyethylene, glass) have shown that survival is typically <72 hours (*31*). Also, the persistence of *Y. pestis* in soil has been suggested as a possible mechanism of interepizootic persistence and epizootic spread and as a factor in defining plague foci (*32*). The studies on plague bacterium survival under natural exposure conditions have shown that *Y. pestis* can survive for at least 24 days in contaminated soil (*33*). In the case described here, the bacteria were unlikely to have remained viable at ambient temperatures for long periods in a distilled solution made from dead host tissues.

The reason the operation was first postponed illustrates another medical theory of that period. Plague was considered a disease of the hot summer "miasmatic" months and was not believed to appear during the winter.

Unfortunately, we have no information after 1651, but the long interval between the last letters (1650–1651) raises the suspicion that the authorities lost interest in the operation, possibly because of the military and political events during the siege. The Venetian victories in the Aegean Sea (1649, 1651) isolated the Ottoman fleet in Istanbul and left the army in Crete without supplies. Also, the failure provoked angry reactions, and Sultan Mehmed IV changed the leaders of the expedition in Crete. Under those circumstances, the Venetians probably believed that the Ottoman retreat was only a matter of time and postponed the operation.

Concerning the ethics involved in such a plan, Foscolo himself states that this act was violent, unusual, beyond the war rules, and in contrast to the public morality (*...è però violente, insolito et forse non più dalla pieta publica pratticato…*). Even so, the Venetian authorities easily adopted the plan of a massive spreading of the disease. This act could be explained mainly by the religious fanaticism during the cruel Venetian–Ottoman Wars. Also, we must take into consideration the vanity of some leaders who would sacrifice everything to achieve their objectives. As Karl von Klausewitz states in his famous treatise *On War*, "As War is no act of blind passion, but is dominated by the political object, therefore the value of that object determines the measure of the sacrifices by which it is to be purchased" (*34*).

The initial response to deliberate release of infective agents targeted against armies or civilian populations is largely a local responsibility in many parts of the world. To prepare for biological attack, the authorities concerned should be encouraged to make maximum use of existing emergency-response resources. A biological agent attack will generally have the characteristics of an infectious disease

outbreak. The respond to a biological incident depends on preparedness (i.e., threat analysis, preparing to response, preparing public information and communication packages, validation of response capabilities) and response (*35*).

Some guidelines have been developed during a crisis and in the absence of experimental data or investigations, such as in the case of the letters filled with a powder containing anthrax (*Bacillus anthracis*) spores in the United States in 2001 (*36*) or the order for smallpox vaccination in Israel during the preparation for the Second Gulf War (*37*). A concept of a modern system of preparedness could be that the risks are not located in the present or in the future but in a shared temporal space and thus can be seen to exist simultaneously (*37*).

In addition, the experience of preparing for bioterrorist attacks can be useful in control of other resurgent infectious diseases and nonbioterrorism emergencies (*38*). For resurgent infectious diseases and microbes classified as agents of biological terrorism, such as *Mycobacterium tuberculosis*, the health care community should have an infection control plan as a part of an overall control program (*39*). In the case of tuberculosis, detecting and curing infective case-patients are the most effective methods of preventing transmission and of controlling the disease in the community (*40*).

Conclusions

The use of biological agents as weapons has a long history. In the incident described here, the Venetian authorities in the 17th century adopted a plan for a massive plague attack in Crete to save their possession from Ottoman forces. As we know from the sources, a detailed plan was made for the operation, and the presumably deadly extract from plague victims in Dalmatia was ready for use. The approach of the winter months was an obstacle to operation's success. According medical thought of that time, plague was a disease of the hot summer months. The Venetians took into consideration the safety of their possessions and, for this reason, they adopted prophylactic measures against plague in their territories. Finally, after 2 years of preparations, they postponed the operation for unknown reasons.

Obviously, according to modern data on the nature of *Y. pestis*, the Venetian plan would not have been effective. In any case, the core of the story is not whether "the quintessence of the plague" would have been effective but the concept of mass destruction through biologic agents. The Venetian plan is another example in the history of biological warfare. In particular, it raises the problem that biological weapons can be used in the name of religious faith, motivated by a deep fanaticism. Unfortunately, throughout history, those driven by this impetus have caused numerous crimes against innocent persons. Such examples prove the need for control and preparedness to ensure national and international safety.

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Influenza A(H6N1) Virus in Dogs, Taiwan

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We determined the prevalence of influenza A virus in dogs in Taiwan and isolated A/canine/Taiwan/E01/2014. Molecular analysis indicated that this isolate was closely related to influenza A(H6N1) viruses circulating in Taiwan and harbored the E627K substitution in the polymerase basic 2 protein, which indicated its ability to replicate in mammalian species.

Infections with influenza viruses are rare in dogs. However, interspecies transmission of an equine influenza A(H3N8) virus to dogs was identified during a respiratory disease outbreak in Florida, USA, in 2004 (*1*). Influenza A(H6N1) virus is the most common naturally occurring avian influenza virus in Taiwan (*2*). Therefore, to determine to the prevalence of influenza A virus infection in dogs in Taiwan, we performed serologic analysis, 1-step reverse transcription PCR (RT-PCR) screening, and virus isolation.

The Study

A total 474 serum specimens were collected in Taiwan during October 2012–October 2013. Two hundred eighty-one specimens were collected from household (owned) dogs at the National Taiwan University Veterinary Hospital in Taipei. The remaining 193 serum specimens were obtained from free-roaming dogs in rural areas.

All serum specimens were tested for antibodies against influenza A virus by using a species-independent blocking ELISA (Influenza A Virus Antibody Test Kit; Idexx, Westbrook, ME, USA). All antibody-positive serum specimens were further tested by using a hemagglutination inhibition (HI) assay. HI was determined according to procedures recommended by the World Organisation for Animal Health. Chicken erythrocytes (1%) were used. Serum samples were treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) before conducting the assay to destroy nonspecific inhibitors (*3*). A/chicken/Taiwan/2838V/2000 (H6N1) and A/chicken/Taiwan/1209/03 (H5N2) viruses were used as antigens.

Nasal swab specimens were collected from dogs with respiratory signs, such as nasal discharge, sneezing, coughing, at the National Taiwan University Veterinary Hospital during November 2012–February 2014. Specimens were suspended in viral transportation medium (Creative, Taipei, Taiwan), and RNA was extracted by using a commercial kit (Viral RNA Mini Kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A 1-step RT-PCR was then performed by using the One-Step RT-PCR Kit (QIAGEN). A primer set (M52C/M253R) specific for a highly conserved region of matrix (M) gene was used for detection of influenza A virus nucleotides (*4*). The remaining nasal swab suspension solutions from dogs positive by RT-PCR were used for virus isolation from 10-day-old specific pathogen–free chicken eggs (Animal Health Research Institute, Taipei, Taiwan).

Phylogenetic trees were constructed with complete nucleotide sequences obtained from the Global Initiative on Sharing All Influenza Data (http://platform.gisaid.org/epi3/frontend#185d95) and GenBank. Multiple sequence alignments and phylogenetic analyses were performed by using MEGA6 software (*5*). Sequences were aligned by using the ClustalW method (http://www.genome.jp/tools/clustalw/). Trees were constructed by using the maximum-likelihood method and analysis with 1,000 bootstrap replications.

A total of 3/281 (1.1%) household dogs and 6/193 (3.1%) free-roaming dogs were positive by ELISA for influenza A virus. The HI assay showed that 1 of the 9 virus-positive dogs had antibodies against influenza A(H6N1) virus (titer = 20). This dog was from Yunlin, Taiwan, a rural area that is a major site for poultry production. No serum samples had antibodies against influenza A(H5N2) virus.

Nasal swab specimens from 4/185 (2.1%) dogs were positive by RT-PCR for influenza virus M gene. All 4 RT-PCR positive dogs had nasal discharges or coughing. Three of these dogs were <6 months of age and adopted from an animal shelter (n = 1) or rescued from the streets (n = 2). The fourth dog was a 15-year-old household pet.

Influenza A virus was isolated from a 4-month-old dog co-infected with canine distemper virus. The virus influenza isolate was designated A/canine/Taiwan/E01/2014 (GenBank accession nos. KM20333–KM203344). This dog was rescued from the streets and had severe purulent nasal discharge, cough, and fever. Chest radiographs showed a severe bilateral air bronchogram in the lung field. Serum specimens (collected on days 1, 7, 14, and 19 after hospitalization) from this dog were assessed by using ELISA and HI assay, but no seroconversion was observed.

Sequence homology of 8 influenza virus gene segments from A/canine/Taiwan/E01/2014 (H6N1) was compared with segments in the Global Initiative on Sharing All Influenza Data (Table 1). Hemagglutinin (HA) and neuraminidase (NA) genes of this virus had the highest nucleotide sequence similarity (99%) with A/chicken/Taiwan/1843/2012 (H6N1) and A/chicken/Taiwan/2084/2012

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DOI: http://dx.doi.org/10.3201/eid2112.141229

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Table 1. Homology of nucleotide sequences of A/canine/Taiwan/E01/2014 (H6N1) influenza virus isolated from dogs in Taiwan compared with related sequences from the Global Initiative on Sharing All Influenza Data*†

Gene segment	Virus with highest identity	% Identity	Accession no.
PB2	A/chicken/Taiwan/1843/2012 (H6N1)	98	EPI510830
PB1	A/chicken/Taiwan/A2837/2013 (H6N1)	97	EPI459872
PA	A/chicken/Taiwan/2593/2012 (H5N2)	99	EPI510622
HA	A/chicken/Taiwan/1843/2012 (H6N1)	99	EPI519832
NP	A/chicken/Taiwan/67/2013 (H6N1)	98	EPI510875
NA	A/chicken/Taiwan/2084/2012 (H6N1)	99	EPI510837
M	A/chicken/Taiwan/2593/2012 (H5N2)	99	EPI510660
NS	A/chicken/Taiwan/67/2013 (H6N1)	97	EPI510878

*All viruses were from avian sources. PB, polymerase basic; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural protein.
†http://platform.gisaid.org/epi3/frontend#185d95

(H6N1), respectively. Polymerase basic 2 (PB2), PB1, nucleoprotein (NP), and nonstructural protein (NS) genes were closely related to those of H6N1 subtype virus isolates from chickens in Taiwan (similarity range 97%–99%). Polymerase acidic (PA) and M genes had the highest nucleotide sequence similarity (99%) to A/chicken/Taiwan/2593/2012 (H5N2). On the basis of HA and NA sequence analysis results, A/canine/Taiwan/E01/2014 was designated an H6N1 subtype influenza virus.

Only 1 basic amino acid (PQIATR*G) was found at the HA cleavage site of A/canine/Taiwan/E01/2014. G228S substitution (H3 numbering) on the receptor binding site for HA was also observed for this virus, which indicated increased virus binding ability for the α2–6 sialic acid receptor (*6,7*). In NA, a 14-aa deletion in the NA stalk region was observed at aa positions 42–53 and 68–69, which is associated with virus circulation in domestic poultry. The amino acid H275Y substitution (oseltamivir resistance marker) in NA was not found in this virus. In the M2 protein, A/canine/Taiwan/E01/2014 had an S31N substitution, which suggested resistance to admantanes (*8,9*).

Other major signatures associated with replication ability in a mammalian host or pathogenicity were also observed, including E627K in the PB2 and the PDZ ligand domain at the C-terminal region of NS1 of this virus. Additional molecular comparisons with H6N1subtype virus (A/Taiwan/2/2013) isolated from humans (*7,10,11*) and from dogs experimentally infected H6N1 subtype virus (A/mallard/San-Jiang/275/2007) (*12*) were made (Table 2).

Phylogenetic analysis of HA and NA gene segments indicated that A/canine/Taiwan/E01/2014 belongs to the H6N1 lineage that has been circulating in chickens in Taiwan since 1997 (Figure, panels A, B). Although the lineage of internal gene segments (PB2, PB1, PA, NP, M, and NS) is composed mainly of H6N1 subtype viruses isolated in Taiwan, some H5N2 subtype isolates in the H6N1 lineage were observed

Table 2. Molecular characterization of A/canine/Taiwan/E01/2014 (H6N1) influenza virus and 2 other influenza viruses, Taiwan*

Gene, amino acid substitution	Virus			Function
	A/canine/Taiwan/E01/2014	A/Taiwan/2/2013	A/mallard/San-Jiang/275/2007	
PB2				
E627K	K	E	E	Replication ability in mammalian host
D701Q	D	D	D	Nuclear import
PB1-F2				
N66S	N	Truncated form	S	Induction of apoptosis
HA				
Cleavage site	Single basic amino acid (PQIATR†G)	Single basic amino acid (PQIATR†G)	Single basic amino acid (PQIETR†G)	HA cleavage
Q226L	Q	Q	Q	Increased virus binding ability of α2–6 sialic acid receptor
G228S	S	S	G	
NA				
H275Y	H	H	H	Oseltamivir resistance
41–52 and 68–69 deletions	Deleted	Deleted	Complete	Adaptation hallmark of waterfowl viruses to terrestrial poultry
M2				
S31N	N	N	S	Adamantane resistance
NS1				
D92E	D	D	D	Unknown
EPEV sequence (C-terminus)	EPEV	EPEV	ESEV	PDZ ligand domain

*PB, polymerase basic; HA, hemagglutinin; NA, neuraminidase; M, matrix; NS, nonstructural protein.
†HA cleavage site.

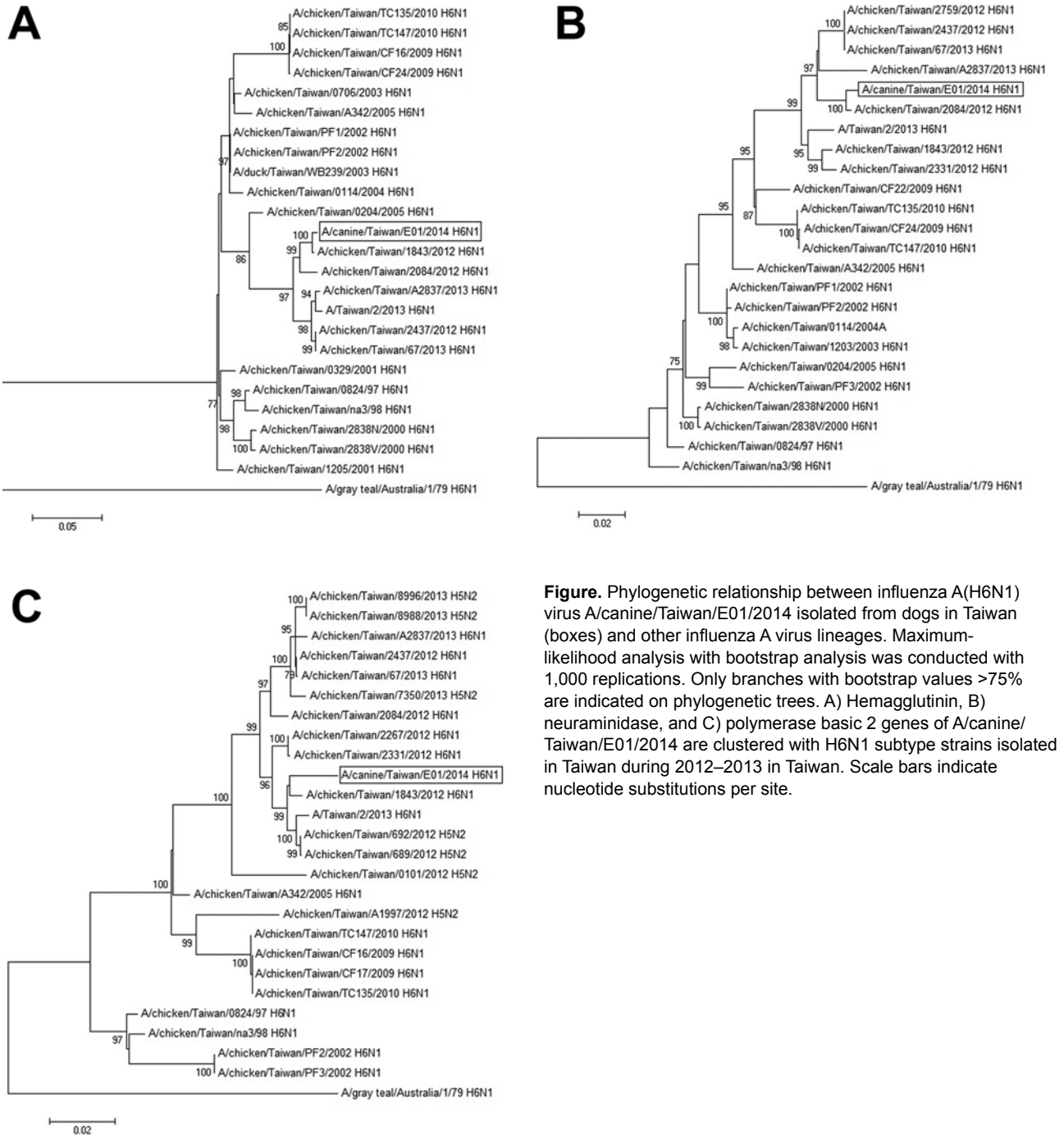

Figure. Phylogenetic relationship between influenza A(H6N1) virus A/canine/Taiwan/E01/2014 isolated from dogs in Taiwan (boxes) and other influenza A virus lineages. Maximum-likelihood analysis with bootstrap analysis was conducted with 1,000 replications. Only branches with bootstrap values >75% are indicated on phylogenetic trees. A) Hemagglutinin, B) neuraminidase, and C) polymerase basic 2 genes of A/canine/Taiwan/E01/2014 are clustered with H6N1 subtype strains isolated in Taiwan during 2012–2013 in Taiwan. Scale bars indicate nucleotide substitutions per site.

(Figure, panel C; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/12/14-1229-Techapp1.pdf).

Conclusions

Avian influenza A(H6N1) viruses have been widespread in chickens in Taiwan since 1972 (*13*–*15*). These viruses are clustered in a unique lineage that differs from viruses circulating in Hong Kong and southeastern China since 1997 (*13*). Unlike avian species, H6 subtype virus infections are rare in mammals.

In this study, 9 of 474 dog serum specimens were positive for influenza A virus by ELISA, and 4/185 (2.1%) dogs had RT-PCR–positive results for this virus. A/canine/Taiwan/E01/2014 was isolated from 1 dog that was co-infected with canine distemper virus. On the basis of molecular analysis of A/canine/Taiwan/E01/2014, HA,

NA, PB1, PB2, NP, and NS genes showed high homology (>97% nucleotide identity) with avian H6N1 subtype virus isolates that are currently prevalent in Taiwan. PA and M genes of A/canine/Taiwan/E01/2014 showed 99% nucleotide identity with A/chicken/Taiwan/2593/2013 (H5N2).

Phylogenetic analysis showed that 8 eight virus genes were derived from H6N1 subtype viruses isolated in Taiwan. All 8 influenza virus genes found in the dog probably originated from avian sources. We speculate that a complete avian influenza virus had infected this dog. However, additional analysis is required to verify this hypothesis.

Acknowledgments

We thank the staff of the Heart of Taiwan Animal Care and National Taiwan University Veterinary Hospital for their assistance in sample collection.

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Methicillin-Resistant *Staphylococcus aureus* Prevalence among Captive Chimpanzees, Texas, USA, 2012[1]

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infection in humans and animals is concerning. In 2012, our evaluation of a captive chimpanzee colony in Texas revealed MRSA prevalence of 69%. Animal care staff should be aware of possible zoonotic MRSA transmission resulting from high prevalence among captive chimpanzees.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a threat among humans; ≈80,000 infections and 11,000 deaths occur each year (*1*). MRSA has been also identified in animals of various species, leading to concerns about animal health and zoonotic transmission. Studies have found animal-origin MRSA in humans and human-origin MRSA in animals. Strains of MRSA isolated from companion animals tend to be the same as the strains isolated from humans in the same geographic areas (*2*), and these isolates cluster together even according to highly discriminatory whole-genome sequencing (*3*). In contrast, livestock-associated MRSA strains, which are sequence type (ST) 398, can be found in humans, and animal contact is a well-characterized risk factor for human infection or colonization (*4*).

Although MRSA colonization in companion animals has been established, a paucity of literature exists on MRSA in laboratory animals, specifically those most closely related phylogenetically to humans: chimpanzees (*Pan troglodytes*). Recent studies have demonstrated possible transmission of *Staphylococcus* spp. with no methicillin resistance between sanctuary workers and chimpanzees in Africa (*5*). An additional report from Africa identified human-associated strains of *S. aureus* in captive and wild chimpanzees; some antimicrobial drug–resistant isolates were identified, but oxacillin (methicillin) resistance was not found (*6*).

At the University of Texas MD Anderson Cancer Center Michale E. Keeling Center for Comparative Medicine and Research in Bastrop, Texas, USA, convenience sampling during physical examinations of chimpanzees revealed increased coagulase-positive *Staphylococcus* spp. resistant to methicillin. On the basis of this information, we prospectively evaluated MRSA prevalence among chimpanzees in this facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

The Study

At the time of the study, 167 chimpanzees at the facility were housed in male and female groups and had daily indoor/outdoor access. All animals were part of an approved Institutional Animal Care and Use Committee protocol and were managed in accordance with the US Department of Agriculture Animal Welfare Regulations and the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/12910/guide-for-the-care-and-use-of-laboratory-animals-eighth). Chimpanzees were observed at least 3 times daily by licensed veterinarians or experienced caretakers, and each year, chimpanzees were sedated and physically examined.

All chimpanzees were enrolled in a comprehensive training and enrichment program. Positive reinforcement training techniques facilitated voluntary cooperation with daily husbandry or veterinary procedures (*7*). The chimpanzees had an extensive repertoire of trained behaviors including, but not limited to, presenting body parts for inspection and medical treatment, voluntarily presenting legs or arms for intramuscular anesthetic injections, and voluntary submitting to venipuncture (*8*). During this study, the chimpanzees voluntarily presented their faces so that trainers could swab the internal nares. Nasal swab samples were collected for culture from any animals for whom a veterinary examination was scheduled during the collection period; no animals were sedated solely for this study. We tested animals from 18 social groups (4–12 animals/group) that had no between-group physical contact. We excluded 9 chimpanzees that had a history of experimental exposure to hepatitis C virus or HIV.

Nasal samples for culture were taken with a polyurethane foam swab (CultureSwab EZ Collection and Transport System; Becton, Dickinson and Company, Sparks,

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DOI: http://dx.doi.org/10.3201/eid2112.142004

[1]Preliminary results from this study were presented at the 3rd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications; November 4–7, 2013; Copenhagen, Denmark.

MD, USA, and Franklin Lakes, NJ, USA). The swabs were placed in 2 mL of enrichment broth containing 10 g/L tryptone T, 75 g/L sodium chloride, 10 g/L mannitol, and 2.5 g/L yeast extract and were incubated for 24 h at 35°C. Aliquots of 100 µL were streaked onto MRSA chromogenic agar (BBL CHROMagar; Becton, Dickinson and Company) and incubated at 35°C for 48 h. Tube coagulase-positive isolates were identified as *S. aureus* by latex agglutination test (Pastorex Staph Plus; Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Methicillin resistance was confirmed by presence of penicillin-binding protein 2a antigen detected by use of a latex-agglutination test (Oxoid Ltd., Basingstoke, UK). MRSA isolates were characterized by *spa* typing; types were characterized by using the Ridom SpaServer (http://SpaServer.ridom.de) (*9*). Real-time PCR was used for detection of the *luk*F-PV gene encoding Panton-Valentine leukocidin (*10*).

During a 1-month period, samples were collected from 125 chimpanzees and MRSA was isolated from 86 (69%; 95% CI 61%–77%). Three chimpanzees were sampled twice, and results were positive on both occasions, for a total of 89 positive samples. A total of 57 of the 86 MRSA isolates (66%; 95% CI 58%–74%) were positive for Panton-Valentine leucocidin t008, consistent with the ST8 USA300 clone. Most of the remaining isolates corresponded to 6 spa types related to t008: t818 (19 [22%]); t024 (4 [4.7%]); t197 (2 [2.3%]); t2030 (2 [2.3%]); and (1 [1.2%] each t9141, t682, and t6172) (Table). Single isolates of t116 and t1754, related to each other but distinct from ST8, were also found. Of the 3 chimpanzees that were sampled twice, the same strains (t008, t818) were identified in both cultures for 2, and 2 different, but related, strains (t024, t818) were identified in each culture for 1.

Conclusions

On the basis of the presence of MRSA in clinical specimens, along with the close contact between animals in the facility, we hypothesized that the prevalence of nasal carriage of MRSA in the chimpanzee colony would be similar to that in high-risk human populations, such as hospitalized patients in long-term care facilities (58%–67%) (*11*). Our finding of nasal carriage of MRSA in 69% (95% CI 61%–77%) of chimpanzees was consistent with that estimate but remarkable and concerning. Limited corresponding data from other facilities that house nonhuman primate species hampers our ability to compare rates. However, the paucity of published data does not indicate that nasal carriage of MRSA in nonhuman primates is rare; anecdotal information suggests that MRSA is widespread in these colonies. The lack of data may result from reluctance to publicize infections, given the sensitivities regarding management of research animals or from not using culture methods to identify MRSA (*12*).

Most isolates were characterized as the USA300/ST8 strain, which is considered a community-associated strain (*13*). The predominance of human epidemic clones of MRSA was not surprising because human strains are found in animals (*14*). However, the predominance of USA300-related strains was noteworthy because in the United States, this strain is most often found in community-associated MRSA infections and the USA100 strain is commonly

Table. *spa* types of methicillin-resistant *Staphylococcus aureus* cultured from the nasal cavity of captive chimpanzees that were separated according to sex, University of Texas MD Anderson Cancer Center, Bastrop, Texas, USA, 2012

	Chimpanzee sex		
spa type	M	F	Total
t008			
No.	30	27	57
% Within type	52.6	47.4	100
% Within sex	71.4	57.4	64.0
% Of total	33.7	30.3	64.0
T818			
No.	9	11	20
% Within type	45.0	55.0	100
% Within sex	21.4	23.4	22.5
% Of total	10.1	12.4	22.5
t024			
No.	1	3	4
% Within type	25.0	75.0	100
% Within sex	2.4	6.4	4.5
% Of total	1.1	3.4	4.5
T197			
No.	0	2	2
% Within type	0	100	100
% Within sex	0	4.3	2.2
% Of total	0	2.2	2.2
T2030			
No.	1	1	2
% Within type	50.0	50.0	100
% Within sex	2.4	2.1	2.2
% Of total	1.1	1.1	2.2
T9141			
No.	0	1	1
% Within type	0	100	100
% Within sex	0	2.1	1.1
% Of total	0	1.1	1.1
T682			
No,	0	1	1
% Within type	0	100	100
% Within sex	0	2.1	1.1
% Of total	0	1.1	1.1
T6172			
No.	1	0	1
% Within type	100	.0	100
% Within sex	2.4	.0	1.1
% Of total	1.1	.0	1.1
T1754			
No.	0	1	1
% Within type	0	100	100
% Within sex	0	2.1	1.1
% Of total	0	1.1	1.1
Total			
No.	42	47	89
% Within type	47.2	52.8	100
% Within sex	100	100	100
% Of total	47.2	52.8	100

found in human carriers. Our finding could be the result of a chance entry of those strains into the facility. It is unclear why no USA100 MRSA clones were found. It is possible that USA300 strains are more adept at colonizing chimpanzees. The variety of related strains could reflect long-standing presence of MRSA in the population and gradual genetic variation or repeated introduction of strains. Host tropism of different MRSA strains in chimpanzees warrants further attention.

This level of MRSA positivity is cause for high concern for possible transmission to animal care staff. Among veterinary personnel, rates of MRSA colonization are high (*15*) and exceed rates among their animal patients. On the basis of personal protective equipment use at this facility, we would expect low or absent carriage rates among the animal care staff. Further study of the dynamics of MRSA in nonhuman primate colonies and interspecies transmission is warranted.

Acknowledgments

We thank Erica Thiele, Rachel Maller, and Becky Jones for their technical support in obtaining cultures; the animal technicians for their animal care; and veterinarians Stephanie Buchl, Elizabeth Magden, and Bruce Bernacky for their support.

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Novel *Waddlia* Intracellular Bacterium in *Artibeus intermedius* Fruit Bats, Mexico

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An intracellular bacterium was isolated from fruit bats (*Artibeus intermedius*) in Cocoyoc, Mexico. The bacterium caused severe lesions in the lungs and spleens of bats and intracytoplasmic vacuoles in cell cultures. Sequence analyses showed it is related to *Waddlia* spp. (order Chlamydiales). We propose to call this bacterium *Waddlia cocoyoc*.

Because animals and humans have shared health risks from changing environments, it is logical to expand the perspective of public health beyond a single species. Bats are unique among mammals in their ability to fly and inhabit diverse ecologic niches. These characteristics together with their regularly large colonial populations highlight their potential as hosts of pathogens (*1*). Their role in disease epidemiology is supported by their susceptibility to different microorganisms such as bacteria, fungi, parasites, and viruses, as illustrated by the recent Ebola outbreak in West Africa (*2*). Previous and ongoing research is predominantly focused on viral agents, and the prevalence and effects of pathogenic bacteria in bats have been neglected (*3*).

Artibeus intermedius (the great fruit-eating bat) is a common frugivorous bat in the tropical Americas. Several pathogens of interest have been isolated from or detected in *Artibeus* spp. bats, including *Histoplasma capsulatum*, *Trypanosoma cruzi*, and eastern equine encephalitis, Mucambo, Jurona, Catu, Itaporanga, and Tacaiuma viruses (*4–6*), but their pathogenicity in bats is not known. In this study, a novel *Chlamydia*-like pathogenic bacterium was isolated from *A. intermedius* bats that were collected to characterize rabies virulence in a frugivorous bat species.

The Study

Adult *A. intermedius* bats (n = 38) were captured in the municipality of Cocoyoc in the state of Morelos, Mexico, in May 2012. Animals were kept in captivity by following the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (*7*). Bats were observed for 2 months to ensure that existing infectious diseases did not develop. No animals had rabies antibodies detectable through rapid fluorescent focus inhibition test. Animals were inoculated intramuscularly with rabies virus (vampire bat variant 5020, $1\times10^{5.34}$ Fluorescent Focus). After 5 days, an adult male exhibited emaciation, restlessness, and depression. On day 20, the animal could not fly and remained on the floor of the cage. Areas of pallor appeared on its wings (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0002-Techapp1.pdf). The animal died on day 28. Testing showed negative results for rabies virus by direct immunofluorescence of brain tissue smears and by PCR of nervous tissue. Skin biopsies were taken from the wing lesions for histopathologic analyses and isolation.

Vero cells inoculated with supernatant from homogenates of white spot lesion biopsies showed cytopathic effect (CPE) within 72 to 96 h postinoculation. CPE consisted of lytic plaque formation. Acidophilic inclusions visible by using Diff-Quick (VWR International, Briare, France) staining were detected within 48 and 72 h postinoculation (Figure 1). Similar inclusions could be seen after inoculation of BHK21 cells. The microorganism could not be cultured on blood or chocolate agar, aerobically or anaerobically, when incubated for up to 7 days.

Experimental inoculation was then established. Three bats that were seronegative for the isolated microorganism were inoculated intraperitoneally. The 3 animals were euthanized on days 5, 10, and 15. The bats euthanized on days 5 and 10 postinoculation showed signs of severe multifocal interstitial pneumonia (online Technical Appendix Figure 2) and severe diffuse lymphoid hyperplasia in the spleen. On euthanization, the third bat showed signs of mild multifocal interstitial pneumonia and mild diffuse lymphoid hyperplasia in the spleen.

Two additional bats were inoculated subcutaneously; areas of pallor developed in the wing skin similar to those observed in the originally infected bat (online Technical Appendix Figure 3). Mononuclear cells infected with bacteria were localized in skin (online Technical Appendix Figure 4) and lung lesions of experimentally inoculated animals by immunofluorescence.

Histopathological findings in the areas of pallor through hematoxylin and eosin staining revealed the

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DOI: http://dx.doi.org/10.3201/eid2112.150002

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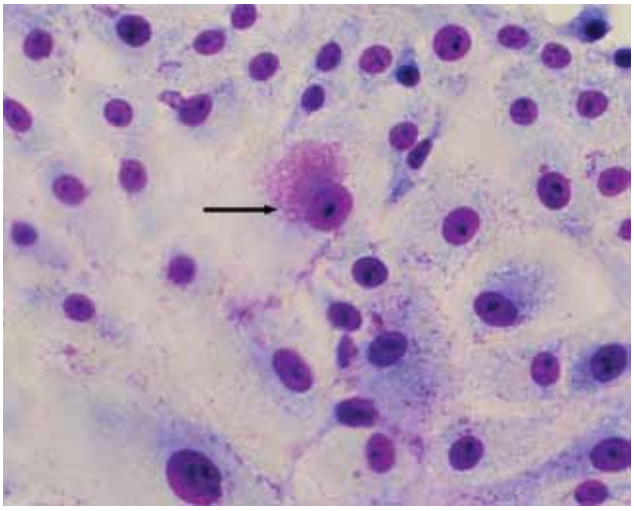

Figure 1. Bacterial cytoplasmic inclusions (arrow) in Vero cell cultures 72 h postinoculation with supernatant from homogenates of white spot lesion biopsies of adult *A. intermedius* bats in Mexico by using Diff–Quick stain (VWR International, Briare, France). Original magnification ×700.

presence of mononuclear cell infiltrates in all subjects. Because of the resemblance of the wing lesions to those typically seen in white nose syndrome infection, which is caused by the fungus *Pseudogymnoascus destructans,* we applied periodic acid–Schiff staining to rule out fungal infection. No hyphae were identified.

Hyperimmune serum samples raised against the isolated bacteria neutralized the CPE of the bacteria up to a 1:719 dilution. Five (13%) of the 38 serum samples taken during captivity neutralized the CPE of the bacteria in dilutions ranging from 1:9 to 1:81. This result suggested circulation of this bacterium within the sampled population. All experimentally inoculated animals seroconverted. Serum samples from both animals that were inoculated subcutaneously neutralized the CPE of the bacteria up to a 1:27 dilution at day 28 postinoculation. Animals inoculated intraperitoneally and euthanized at 5, 10, and 15 days postinoculation neutralized CPE up to dilutions of 1:27, 1:27, and 1:81, respectively. No serum samples from the researchers who handled the bats showed seroneutralization activity.

DNA from skin biopsy samples and Vero and BHK 21 cells experimentally infected by using primers directed against domain I of the 23S gene of the family *Waddliaceae* yielded PCR products of the expected size (627 bp) (*8*). Vero cell culture was used to amplify the infection of the bacterial agent and DNA extracts were subjected to high throughput sequencing (SRA: PRJNA268154). Analysis of assembled contigs by using blastx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that sequences close to *Waddlia* spp. were abundant (43%) and were only surpassed by 2 sequences of primate origin (online Technical Appendix Figure 5). A neighbor-joining phylogenetic tree with 10,000 replicates was built with 16S sequences from assorted members of the order Chlamydiales and the cultured microorganism. The Chlamydiales have evolved from a single genus to a diverse order including new families such as *Candidatus* Parichlamydiaceae and *Rhabdochlamiaceae* (*9*). Phylogenetic analyses revealed that the newly identified *Waddlia* sp. segregates with known *Waddlia* spp. Although the new *Waddlia* sp. fell in the same taxonomic unit, it is found in its own branch (Figure 2). This finding was confirmed by a maximum-likelihood phylogeny with approximate likelihood ratio test (online Technical Appendix Figure 6).

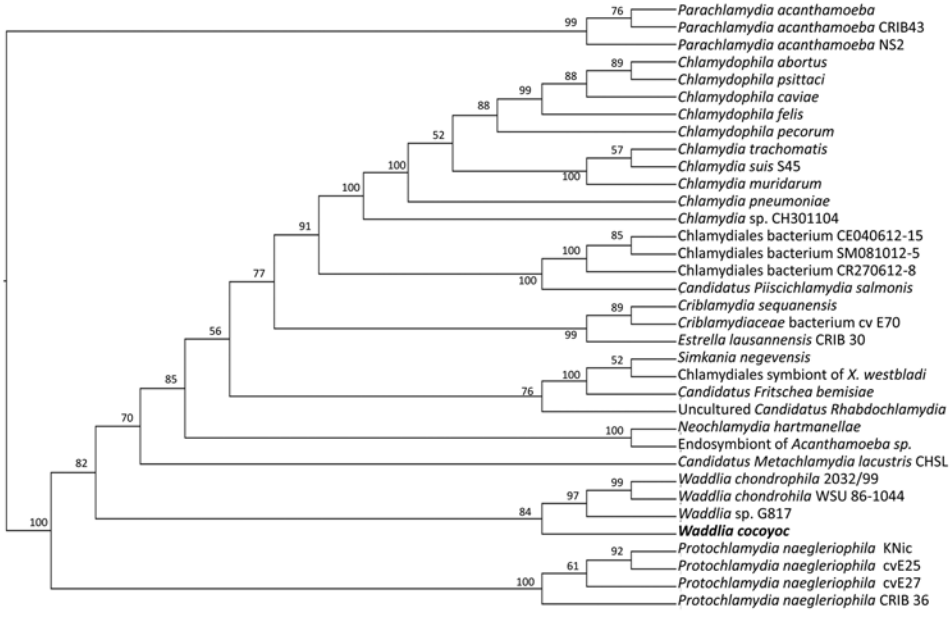

Figure 2. Phylogenetic relationships of bacterium newly identified in *Artibeus intermedius* fruit bats in Mexico (*Waddlia cocoyoc*, bold text), to other Chlamydiales. 16S sequences were used to infer relationships. *X. westbladi, Xenoturbella westbladi*.

Conclusions

We report the isolation of a newly identified bacterial pathogen of *A. intermedius* bats and propose naming it *Waddlia cocoyoc*. The isolated bacterium was successfully grown in cell culture but not in inert bacterial growth media, suggesting dependence on host cells. Staining of inoculated cells revealed lysis and large intracytoplasmic vacuoles. Infected bats showed areas of pallor on the wings and had severe lesions in the lungs and the spleen. Histopathological analyses on the areas of pallor revealed mononuclear cell infiltrates in infected bats. Detection of the bacterium in lesion sites by immunofluorescence and PCR strongly suggests that it caused the observed pathogenesis. Phylogenetic analyses indicate that the pathogen is closely related to organisms in the family *Waddliaceae*.

Diversity in *Waddliaceae* increases as reports of new species surface. *Waddlia* spp. have been previously associated with Malaysian fruit bats (*10*). *W. chondrophila* has been isolated from aborted cattle fetuses in the United States (*11*), and was detected in a potoroo (*Potorous* spp.), a threatened marsupial native to Australia (*12*). Serologic evidence showed a substantive association between high titers of *W. chondrophila* antibodies and bovine abortion (*13*). In addition, *W. chondrophila* seroprevalence was found to be high in women who have had recurrent and sporadic miscarriages (*14*). *W. chondrophila* was also found in patients with community-acquired pneumonia (*15*). The host range and zoonotic potential of *Waddlia* spp. open multiple research avenues for this newly identified organism.

Acknowledgments

We thank António González Origel and Edgar A. Cuevas Domínguez from Wildlife Health, SEMARNAT (Ministry of Environment, Mexico) for his assistance in capturing and experimentation in wild animals.

This work was partially supported by IMSS (Mexico) grant FIS/IMSS/PROT/828.

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Tembusu-Related Flavivirus in Ducks, Thailand

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Since 2013, outbreaks of disease caused by duck Tembusu virus (DTMUV) have been observed in layer and broiler duck farms in Thailand. The virus is closely related to Chinese DTMUVs and belongs to the Ntaya group of mosquito-borne flaviviruses. These findings represent the emergence of DTMUV in ducks in Thailand.

In 2010, a severe contagious disease emerged in layer and breeder duck farms in China (*1*). The infected ducks typically exhibited a dramatic reduction in egg production and severe neurologic disorders. The causative agent of this emerging disease was identified as the new duck Tembusu virus (DTMUV), a member of the Ntaya virus group in the genus *Flavivirus* (*1*,*2*). In addition to China, new DTMUV was recently detected among ducks in Malaysia (*3*). In Thailand, a severe contagious disease affecting ducks has newly emerged since 2013. The disease rapidly spread through duck farms in high-density duck-producing areas, causing economic losses for both traditional and agro-industrial duck businesses. This study reports the emergence of DTMUV infection among domestic ducks in Thailand.

The Study

Since 2013, several layer and broiler duck farms located in high-density duck-producing areas of Thailand have had an emerging, contagious disease characterized by severe neurologic dysfunction and dramatically decreased egg production among domestic ducks. Outbreaks have been reported on farms in the northeastern (Nakhon Ratchasima), eastern (Prachinburi and Chonburi), and central (Suphanburi) provinces of Thailand. At least 7 duck farms were affected, and outbreaks occurred throughout the year (August 2013–September 2014). However, the disease occurred more frequently during the rainy season (July–December). We estimated the mean prevalence of the outbreaks at 17.19% (online Technical Appendix Figure 1, panels A, B, http://wwwnc.cdc.gov/EID/article/21/12/15-0600 Techapp1.pdf). Clinical signs were usually observable in broiler ducks >3 weeks of age and in layer ducks during their production period. Infected ducks typically exhibited neurologic signs, including ataxia, reluctance to walk, and progressive paralysis (Figure 1, panels A, B). A remarkable drop in egg production was usually observed among layer ducks. The main pathologic changes were ovaritis, ovarian hemorrhage, and ovarian atrophy (Figure 1, panel C). Splenic enlargement was observed in some ducks. Histopathologic analysis showed moderate multifocal gliosis and perivascular cuffing in the brain (cerebellum) and spinal cord of most sick ducks (Figure 1, panels D, E). Rates of illness and death ranged from 20% to 50% and 10% to 30%, respectively, correlating positively with secondary bacterial infection.

We identified 22 DTMUVs through reverse transcription PCR using E gene–specific primers (*1*) (online Technical Appendix Table 1). One virus (DK/TH/CU-1) was inoculated into embryonated chicken eggs. The embryos died within 3–5 days after inoculation, with severe cutaneous hemorrhages (Figure 1, panel F). The allantoic fluid tested negative through hemagglutination test and PCR for common duck viruses, including avian influenza virus, Newcastle disease virus and duck herpesvirus 1. In addition, 5 representative viruses (DK/TH/CU-2, DK/TH/CU-3, DK/TH/CU-4, DK/TH/CU-5, DK/TH/CU-6) from duck farms located in the northeastern (3 farms) and the eastern (2 farms) provinces were selected for partial E gene sequencing (Table). The nucleotide sequences of the Thai DMTUVs used in this study were submitted to GenBank under accession nos. KR061333–KR061338.

To characterize Thai DTMUV, DK/TH/CU-1 was subjected to whole-genome sequencing. The whole-genome length of DK/TH/CU-1 is 10,278 nt, encoding 3,426 aa. BLAST analysis (http://www.ncbi.nlm.nih.gov/blast) showed that the polyprotein gene sequences of DK/TH/CU-1 shared very high identity (98.3%) with GX2013E, a Chinese DTMUV strain isolated in 2013. Phylogenetic analysis of the polyprotein gene sequence using the neighbor-joining and maximum-likelihood algorithms showed that DK/TH/CU-1 is grouped into the major cluster with mosquito-borne flaviviruses (65.2%–77% nt identity with viruses in the Ntaya group) and is most closely related to Chinese DTMUVs (97.3%-98.3% nt identity). DK/TH/CU-1 shared only 90.3% and 89.4% nt identity with MM1775 strain and Sitiawan virus, which are Tembusu viruses isolated from mosquitos and chickens, respectively (Figure 2, panel A; online Technical Appendix Table 2).

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DOI: http://dx.doi.org/10.3201/eid2112.150600

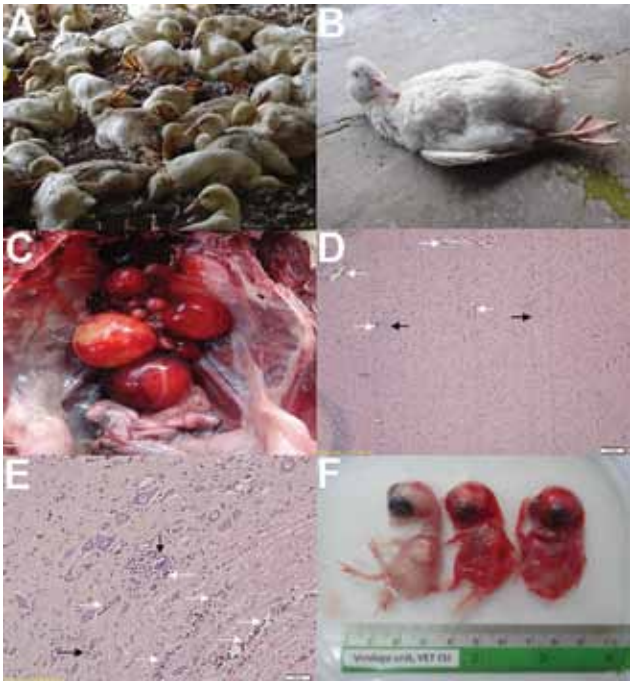

Figure 1. Clinical signs and pathologic lesions of duck Tembusu virus (DTMUV)–infected ducks, Thailand. A, B) Clinical signs; DMTUV-infected ducks showed neurologic signs, including inability to stand, ataxia, and paralysis. C) Gross lesion; severe hemorrhage and regression of ovarian follicles. D, E) Histopathologic lesion; moderate multifocal gliosis (black arrows) and perivascular cuffing (white arrows) in cerebellum (D) and spinal cord (E). Scale bars indicate 100 μm (D) and 50 μm (E). F) Chicken embryos infected with DTMUV strain DK/TH/CU-1. Normal embryo is shown at left; infected embryos at right died 3–5 days after inoculation, with severe cutaneous hemorrhage.

Analysis of the partial E gene sequences of the 5 Thai DTMUVs (DK/TH/CU-2 to 6) showed that the viruses are grouped with DK/TH/CU-1 and Chinese DTMUVs (Figure 2, panel B). The partial E gene sequences of Thai DTMUVs shared 97.5%–99.7% and 96.7%–98.9% nt identity with each other and with the Chinese DTMUVs, respectively. However, the nucleotide identities were lower (88.6%–90.6%) than Malaysian DTMUVs. The E gene sequence of a DK/TH/CU-1 shares only 89.1% and 90.9% nt identity with TMUV strains isolated in 2002 from mosquitos and healthy ducks in Thailand, respectively (*6*). Analysis of partial NS5 gene showed similar findings with those of polyprotein and E genes (Figure 2, panel C). DK/TH/CU-1 shared 96.4%–98.1% and 92.7%–93% nt identity with Chinese DTMUVs and Malaysian DTMUVs, respectively.

Conclusions

Since 2013, outbreaks of a severe contagious disease among domestic ducks have been occurring and spreading in the high-density duck-producing areas of Thailand, causing substantial economic losses in the agricultural sector. On the basis of pathologic examinations, virus isolation, virus identification and genetic characterization, we found an association with the new DTMUV.

Despite lack of the experimental pathogenicity testing of the virus isolates, our observations on clinical signs and pathologic findings were consistent with previously reported findings of DTMUV infections in China and Malaysia (*1*,*3*). Therefore, the isolated DTMUVs can be considered as the causative agent. Because DTMUV is a mosquito-borne flavivirus, it can be transmitted to ducks from mosquitos. Our data indicated that the disease caused by DTMUV occurred most frequently during rainy season, when mosquito activity in Thailand is highest. A previous study detected TMUV in *Culex* mosquitos in Thailand in 2002. The *Culex* mosquito has also proven to be a vector for transmitting TMUV to chickens (*6*). DTMUV transmission through the fecal–oral route also has been reported (*2*,*7*,*8*). However, the pathogenicity and transmission routes of Thai DTMUV were not determined in this study. Further studies on the Thai DTMUVs should be conducted.

Genetic analyses of polyprotein sequences of the Thai DTMUVs showed higher nucleotide identity with DTMUVs reported from China (97.9%) than with those reported from Malaysia (90.3%), indicating that Chinese DTMUVs are possible ancestors of Thai DTMUVs. Phylogenetic analyses based on polyprotein, E gene and NS5 gene using 2 algorithms (neighbor-joining and maximum-likelihood) have displayed similar results that the Thai isolates were grouped with the Chinese DTMUV with high bootstraps value. The Malaysian DTMUVs were grouped into a subcluster apart from Thai and Chinese DTMUV. Although TMUV strains were isolated from Thai mosquitos and healthy ducks

Table. Detailed description of DTMUVs characterized in study of DTMUV in ducks, Thailand*

Virus name	Study designation	Time of collection	Duck age	Duck type	Location in Thailand	Genome sequencing	GenBank accession no.
DTMUV strain DK/TH/CU-1	DK/TH/CU-1	2013 Nov	39 d	Broiler	Nakhon Ratchasima	WG	KR061333
DTMUV strain DK/TH/CU-2	DK/TH/CU-2	2014 Aug	38 wk	Layer	Chonburi	Partial E	KR061334
DTMUV strain DK/TH/CU-3	DK/TH/CU-3	2014 Aug	35 d	Broiler	Nakhon Ratchasima	Partial E	KR061335
DTMUV strain DK/TH/CU-4	DK/TH/CU-4	2014 Aug	42 d	Broiler	Nakhon Ratchasima	Partial E	KR061336
DTMUV strain DK/TH/CU-5	DK/TH/CU-5	2013 Sep	24 d	Broiler	Nakhon Ratchasima	Partial E	KR061337
DTMUV strain DK/TH/CU-6	DK/TH/CU-6	2013 Oct	35 d	Broiler	Prachinburi	Partial E	KR061338

*All samples were pooled organs (i.e., brain, spinal cord, spleen, lung, kidney, proventiculus, and intestine). DTMUV, duck Tembusu virus; partial E, partial E gene sequence; WG, whole-genome.

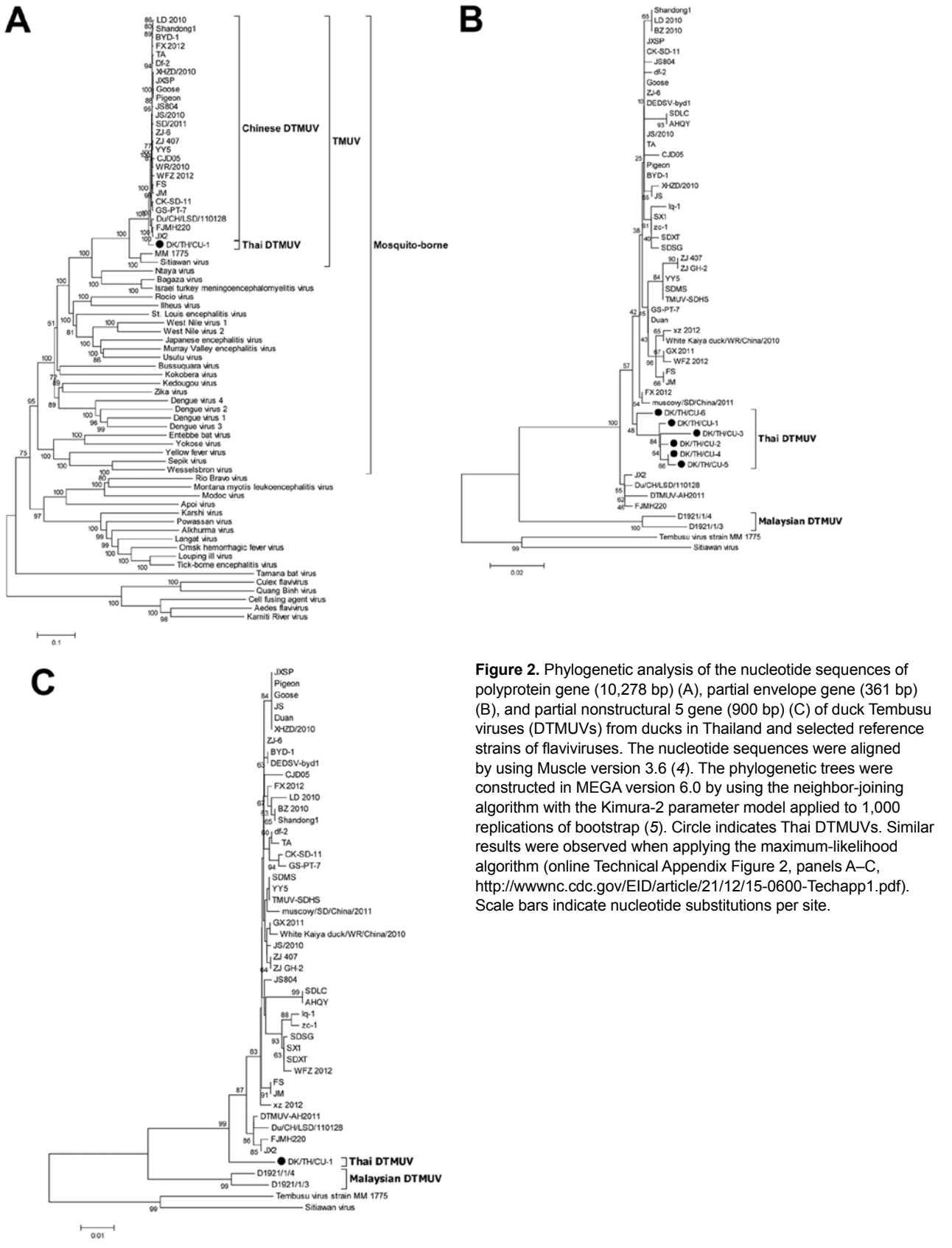

Figure 2. Phylogenetic analysis of the nucleotide sequences of polyprotein gene (10,278 bp) (A), partial envelope gene (361 bp) (B), and partial nonstructural 5 gene (900 bp) (C) of duck Tembusu viruses (DTMUVs) from ducks in Thailand and selected reference strains of flaviviruses. The nucleotide sequences were aligned by using Muscle version 3.6 (*4*). The phylogenetic trees were constructed in MEGA version 6.0 by using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap (*5*). Circle indicates Thai DTMUVs. Similar results were observed when applying the maximum-likelihood algorithm (online Technical Appendix Figure 2, panels A–C, http://wwwnc.cdc.gov/EID/article/21/12/15-0600-Techapp1.pdf). Scale bars indicate nucleotide substitutions per site.

in 2002, the nucleotide sequences of those viruses were less similar to Thai DTMUVs than those of Chinese DTMUVs. Nevertheless, the source of the novel DTMUV emergence in Thailand remains unknown and requires further investigation.

As a member of the *Flavivirus* genus, DTMUV has a high potential to become a zoonotic pathogen that threatens public health. Thus far, DTMUV has not been reported to cause illness in humans. However, DTMUV-specific antibodies and DTMUV RNA were detected in duck farm workers in China (*9*). Therefore, a novel DTMUV that can cause disease in humans possibly could emerge. Previous studies have reported that DTMUV can infect a wide variety of avian species, including geese, chickens, pigeons, and house sparrows, indicating the continued expansion of its host range (*7*,*10*–*12*). Thus, the continued monitoring of DTMUV in animals and humans is essential to preventing economic losses in animal production as well as zoonotic potential in humans. In summary, our data collectively demonstrate that a newly emerged, contagious disease among ducks in Thailand is caused by DTMUV. Our findings highlight the necessity of systemic surveillance of DTMUVs in animals and in humans for early detection and prevention.

Acknowledgments

We thank the staff of the Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University; Rachod Tantilertcharoen for his contribution in histopathologic analysis; and Joshua A. Felber for reviewing the manuscript.

This research was supported by the Ratchadaphiseksomphot Endowment Fund 2013 of Chulalongkorn University (CU-56-642-HR and WCU-005-HR-57). The authors also thank the Thailand Research Fund (TRG5780010) and Grants for Development of New Faculty Staff, Rachadapiseksompoch Endowment Fund, Chulalongkorn University. Chulalongkorn University provided financial support to the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals, and Thailand Research Fund provided financial support to the TRF Senior Scholar to A.A. (RTA5780006).

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Japanese Macaques (*Macaca fuscata*) as Natural Reservoir of *Bartonella quintana*

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Bartonella quintana bacteremia was detected in 6 (13.3%) of 45 wild-caught Japanese macaques (*Macaca fuscata*). Multilocus sequence typing of the isolates revealed that Japanese macaques were infected with a new and specific *B. quintana* sequence type. Free-ranging Japanese macaques thus represent another natural reservoir of *B. quintana*.

Bartonella quintana is the causative agent of trench fever, which is characterized in humans by headache, recurrent fever, and pretibial pain. Major epidemics of the disease occurred among soldiers in Europe during World Wars I and II. More recently, trench fever has occurred sporadically in urban areas, mainly among homeless persons, drug-addicted persons, and HIV-positive patients in Europe and the United States (*1*). Body lice have been recognized as the only competent vector for *B. quintana* in humans, and poor hygienic conditions are strongly related to the occurrence of trench fever. Thus, *B. quintana* is considered a notable agent of a reemerging infectious disease.

Humans were thought to be the unique natural reservoir for *B. quintana* (*2*). However, this bacterium has also been isolated from cynomolgus macaques (*Macaca fascicularis*) bred in captivity in the United States (*3,4*) and from captive cynomolgus and rhesus macaques (*M. mulatta*) in China (*5,6*). These findings suggest that macaques may be another natural reservoir for *B. quintana*.

The number of wild Japanese macaques (*M. fuscata*) has recently increased throughout Japan, and these primates have become a serious nuisance by damaging crops, invading human residential areas, and biting persons (*7*). Because of this increasing human contact, if these primates become infected with *B. quintana*, they could transmit this bacterium to humans. However, no epidemiologic studies have been conducted to evaluate *B. quintana* in Japanese macaques and their role as a potential source of human *B. quintana* infection. Our goal was to investigate the prevalence of *B. quintana* in wild, free-ranging Japanese macaques and clarify the genetic characteristics of the strains by multilocus sequence typing (MLST).

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DOI: http://dx.doi.org/10.3201/eid2112.150632

The Study

During July 2011–April 2014, a total of 45 blood samples were collected in EDTA-containing collection tubes from wild Japanese macaques in Aomori (n = 25), Yamagata (n = 5), and Wakayama (n = 15) Prefectures in Japan. The animals were captured by licensed trappers, in accordance with the Wildlife Protection and Proper Hunting Act, by using large hand-made cage traps and commercial cage traps (no. AM-181; Fujita Shoji Corp., Hiroshima, Japan). The physical conditions of each animal were recorded before they were euthanized, according to the guidelines of the Japanese Veterinary Medical Association. Freeze-thawed blood samples were spread onto chocolate agar plates (*8*) for isolation of *Bartonella* spp. and incubated at 35°C under 5% CO_2 for up to 4 weeks. Then, CFUs per milliliter of blood were calculated. Five colonies from each culture-positive macaque sample were submitted for further characterization.

Bartonella-specific PCRs that targeted the *gltA* (*9*) and *rpoB* (*10*) genes and the 16S–23S rDNA intergenic transcribed spacer (ITS) regions (*11*) were used for identification of *Bartonella* isolates; genomic DNA of *B. alsatica* strain IBS 382^T and nuclease-free distilled water were used as positive and negative controls for the PCRs, respectively.

Bartonella isolates were obtained from 6 (13.3%) of 45 Japanese macaques; 1 (4.0%) of 25 macaques in Aomori, 1 (20.0%) of 5 in Yamagata, and 4 (26.7%) of 15 in Wakayama prefectures. No clinical signs were observed in the macaques with culture-positive samples. The bacteremia levels in the macaques ranged from 5.0×10^1 to 3.7×10^4 CFU/mL.

The DNA sequences of all 30 isolates were identical in the *gltA* (338 bp), *rpoB* (825 bp), and ITS (1,297 bp) regions; the sequences were registered in GenBank, European Nucleotide Archive in EMBL, and DDBJ under accession nos. LC031777 (*gltA*), LC031778 (*rpoB*), and LC031779 (ITS). BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that the DNA sequences of the isolates had the highest degree of similarity (100% for *gltA* and *rpoB*, 99.5% for ITS) with those of *B. quintana* RM11 strain from rhesus macaques. Subsequently, MLST analysis with 9 loci (*12*) revealed that 6 representative strains (MF1–1, MF3–1, MF10–1, MF11–1, MF19–1, and

MF34–1 strains) from each culture-positive macaque were identical and belonged to a new sequence type (ST), ST22. The allelic profiles of ST22 and other STs are shown in the online Technical Appendix Table (http://wwwnc.cdc.gov/EID/article/21/12/15-0632-Techapp1.pdf).

A phylogenetic relationship between ST22 and other known STs was analyzed by using eBURST version 3 (http://eburst.mlst.net/default.asp) in combination with the MLST data. A clonal complex was defined as the group of STs that had identical alleles at 8 of 9 loci, and the lineage was defined as the group of STs that had identical alleles at 7 of the 9 loci. As previously reported (*6*,*12*), STs 1–4, STs 6 and 7, STs 8–10 and 14, and STs 15–21 formed clonal complexes 1, 2, 3, and 4, respectively, whereas ST22 remained a singleton (Figure 1). In terms of lineage classification, all STs, except ST22, were divided into 3 individual lineages by the host animal species: STs 1–7 for human strains, STs 8–14 for cynomolgus macaque strains, and STs 15–21 for rhesus macaque strains. In contrast, ST22 from Japanese macaque strains belonged to a singleton lineage.

We constructed a phylogenetic tree with the concatenated sequences (4,270 bp) of the 9 loci in each ST using the maximum-likelihood method in MEGA6 (*13*). The STs 1–7 from human strains, STs 8–14 from cynomolgus macaque strains, and STs 15–21 from rhesus macaque strains were classified into groups 1, 2, and 3, respectively, as with the lineage classification by eBURST analysis. All strains of ST22 formed a monophyletic clade defined as group 4 (Figure 2).

Figure 1. Phylogenetic relationship among 1 to 22 sequence types (STs) of *Bartonella quintana* strains based on eBURST analysis (http://eburst.mlst.net/default.asp). Black dots indicate ST numbers of *B. quintana* strains. A clonal complex was defined as a group of STs that had 8 identical alleles. Clonal complexes 1, 2, 3, and 4 consist of STs 1–4, STs 6–7, STs 8–10 and 14, and STs 15–21, respectively. A lineage was defined as a group of STs that had ³7 identical alleles. Color circles show 4 lineages classified by host species.

Conclusions

Our study shows that natural infection with *B. quintana* can occur in free-ranging nonhuman primates in Japan. The Japanese macaques harboring *B. quintana* showed no clinical abnormalities, although bacteremia levels were considerably high (>10^3 CFU/mL) in 3 animals that tested positive. These data suggest that Japanese macaques are one of the natural reservoirs of *B. quintana*.

All isolates from Japanese macaques were more closely related to rhesus macaque strains than to human strains in the *gltA*, *rpoB*, and ITS regions. By MLST analysis with 9 loci, all 6 representative strains from Japanese macaques were identified as ST22 (*6*). Thus, ST22 is likely a new genotype of *B. quintana* specific to Japanese macaques. Because wild-caught Japanese macaques from only 3 prefectures were examined for *B. quintana* bacteremia, a large-scale surveillance study would help elucidate the genetic diversity of Japanese macaque strains.

According to eBURST analysis, human, cynomolgus macaque, and rhesus macaque lineages were formed by 4 clonal complexes. As reported previously (*6*,*12*), the primary founders in clonal complexes 1, 3, and 4 were reconfirmed as STs 2, 9, and 15, respectively. However, ST22 from wild-caught Japanese macaque strains was not found in any other

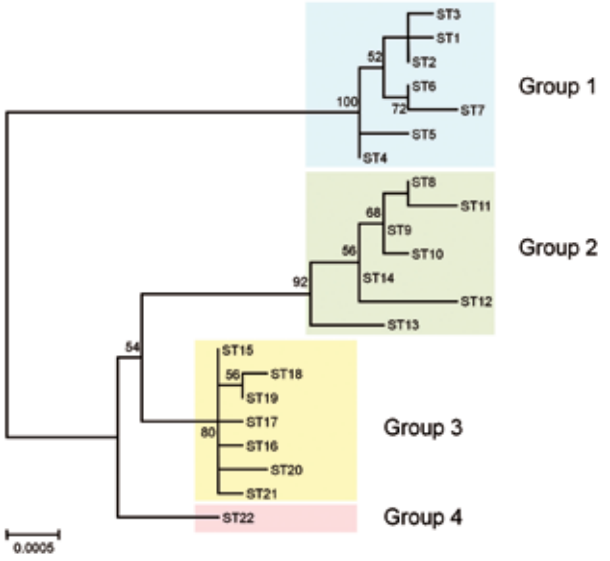

Figure 2. Phylogenetic tree showing the genetic relationship among *Bartonella quintana* strains from humans and macaques. The tree was constructed from the concatenated sequences (4,270 bp) of the 9 loci used for multilocus sequence typing by using the maximum-likelihood method based on the Tamura 3-parameter model in MEGA6 (*13*). The 22 sequence types (STs) of *B. quintana* strains from humans (STs 1–7), cynomolgus macaques (STs 8–4), rhesus macaques (STs 15–21), and Japanese macaques (ST22) were included in the tree. Colored rectangles show 4 groups classified by host species. The scale bar indicates estimated evolutionary distance. Bootstrap values were obtained with 1,000 replicates. Only bootstrap replicates >50% are noted.

clonal complex and formed an independent lineage. Through phylogenetic analysis with concatenated MLST sequences, Li et al. (*6*) showed that 3 groups were formed by each host species; this finding was confirmed in our study. However, ST22 from Japanese macaques formed another independent group (group 4). *Bartonella* spp. are known to have an adaptive strategy of causing asymptomatic and prolonged bacteremia in their specific reservoirs (*14*). Our data support the idea that *B. quintana* may have separately co-evolved with the macaque species and humans.

Notably, *Bartonella* DNA was recently detected in gorillas from West Africa, suggesting that nonhuman primates, including apes, could be naturally infected with *Bartonella* spp. (*15*). Further studies are necessary to clarify the prevalence of *B. quintana* and the vector of the organism in other nonhuman primates, and the potential of these primates to serve as a source of infection to humans.

Acknowledgments

We thank Hideo Higashi and Shiro Matsuoka for their help with sample collection.

This work was supported in part by the Japan Society for the Promotion of Science Grant-in-Aid for Young Scientists (B) grant no. 26850188, and the Strategic Research Base Development Program, International Research on Epidemiology of Zoonoses and Training for Young Researchers grant no. S1491007 by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Increased Number of Human Cases of Influenza Virus A(H5N1) Infection, Egypt, 2014–15

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During November 2014–April 2015, a total of 165 case-patients with influenza virus A(H5N1) infection, including 6 clusters and 51 deaths, were identified in Egypt. Among infected persons, 99% reported poultry exposure: 19% to ill poultry and 35% to dead poultry. Only 1 person reported wearing personal protective equipment while working with poultry.

Highly pathogenic avian influenza virus A(H5N1) has been detected among poultry in >60 countries, with sporadic transmission to humans that results in a large number of deaths (*1*). Of 842 persons with H5N1 virus infection reported as of June 23, 2015, worldwide, 447 (53%) died (*2*,*3*). During November 2014–February 2015, the Egyptian Ministry of Health (MoH) surveillance systems identified an unprecedented number of persons with severe respiratory illness caused by infection with H5N1 virus. These illnesses occurred during months when seasonal influenza is typically epidemic in Egypt (*4*). In response, the MoH initiated an investigation into potential causes of the increased number of cases.

The Study

Since 2006, MoH mandates that clinicians refer all persons with influenza-like illness (ILI) and ≤2-week history of poultry contact to 1 of ≈83 Chest and Fever hospitals (i.e., a category of referral hospitals) throughout Egypt (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/12/15-0885-Techapp.pdf). Persons meeting the ILI case definition have fever ≥38°C and ≥1 of the following: cough, dyspnea, sore throat, myalgia, and body aches. Persons meeting the ILI case definition are admitted, and respiratory samples are collected for influenza testing.

Technicians at 8 sentinel sites also collected daily respiratory samples from 2 patients meeting the ILI case definition and from all patients admitted with severe acute respiratory infection, defined as hospitalization occurring within 2 weeks of onset of fever and cough. Nasal and oropharyngeal swabs were transported to Egypt's National Influenza Center for testing by reverse transcription PCR (*2*). For patients testing positive for H5N1, MoH staff visited their households; administered a standardized questionnaire to obtain demographic, exposure, clinical, and treatment information; and searched among patient contacts for additional case-patients.

We obtained surveillance data collected during 2006–2015 and compared case-patients with H5N1 virus infection for November 2014–April 2015 to those reported in previous years. We obtained the average epidemic period by calculating the proportion of samples testing positive for H5N1 each month throughout the period (*4*). An epidemic was defined as consecutive months having a proportion of H5N1-positive samples that exceeded the annual July–June median.

During March 20, 2006–April 20, 2015, a total of 342 persons with H5N1 virus infection were identified in Egypt (Figure). Annual epidemics typically occurred during November–April, when 299 (87%; 95% CI 84%–91%) of the 342 H5N1 illnesses occurred, resulting in a median of 23 case-patients (interquartile range [IQR] 13–31) per epidemic. Of the total 342 case-patients, 165 were identified during 2014–15, including 6 clusters of 2–3 case-patients. Although this season had a higher number of case-patients than previous years, other seasons had higher proportions of H5N1 detections or clusters among samples tested. The median percentage (5.4%) of monthly H5N1 detections among humans sampled during November 2014–April 2015 was statistically similar to the median percentage (2.9%) for March 2006–October 2014 (Kruskal-Wallis test; $p = 0.5$). The proportion of clustered case-patients was also similar for the 2 periods: 12 (7.3%; 95% CI 3.3%–11.2%) of 165 case-patients during 2014–15 and 12 (6.8%; 95% CI 3.1–10.5) of 177 case-patients during 2006–2014. The number of human H5N1 case-patients identified each month was highly correlated with the number of H5N1 poultry outbreaks identified each month during 2006–2015 by the Ministry of Agriculture in the same communities ($R = 0.3$, $p = 0.002$).

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DOI: http://dx.doi.org/10.3201/eid2112.150885

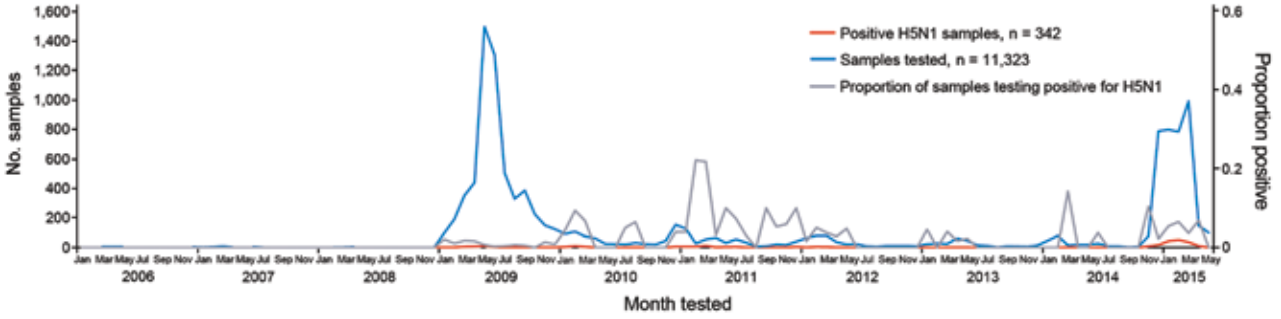

Figure. Number of human respiratory samples tested, number of samples testing positive for influenza virus A(H5N1), and proportion of positive samples, Egypt, March 2006–May 2015.

The case-patients identified during 2014–15 were characteristically similar to those from previous seasons. Similar percentages were found to be women (100/165 [61%] for 2014–15; 105/177 [59%] for 2006–2014), homemakers (50/164 [30%] for 2014–15; 46/177 [26%] for 2006–2014), and poultry farm workers (2/165 [1%] for 2014–15; 0 for 2006–2014). Case fatality rates were also similar: 51 (31%) deaths among 165 case-patients during 2014–15 versus 64 deaths (36%) among 177 case-patients for March 2006–October 2014 (p = 0.3).

Almost all (163/165) case-patients during 2014–15 had exposure to domestic poultry 1–2 weeks before symptom onset; 58% were involved in breeding, 24% in slaughtering, and 21% in preparing poultry. Most (115/165 [70%]) were exposed at home; 4% recalled exposure in shops, 3% at live bird markets, and 1% at farms. Although 35% recalled exposure to dead birds and 19% to ill birds, 35% recalled exposure only to birds that appeared healthy. One case-patient reported wearing personal protective equipment when working with poultry.

Case-patients identified in 2014–15 were admitted and received oseltamivir treatment a median of 4 (IQR 2–7) days after symptom onset. Sixteen percent had preexisting medical conditions: 8 (5%; 95% CI 2%–8%) had chronic chest illnesses; 5 (3%; 95% CI 0.3%–6%) had cardiovascular disease; 5 (3%; 95% CI 0.3%–6%) had diabetes; 2 (1%; 95% CI 0%–3%) had renal failure; 2 (0.1%; 95% CI 0%–3%) had liver failure; and 1 (0.6%; 95% CI 0%–2%) was obese. In addition, 9 (10%; 95% CI 4%–16%) of 92 women 15–49 years of age were pregnant. Most case-patients had cough (87%) and dyspnea (72%) during their illness.

As of July 2015, of the 165 case-patients, 114 (69%) had survived and 51 (31%) died. Survivors were younger than decedents (median age 16 [IQR 3–36] vs. 33 [IQR 20–43] years; p = 0.0001). Oseltamivir treatment was begun within a median 4 (IQR 2–6) days of symptom onset for survivors, compared with a median 5 (IQR 3–7) days for decedents (2-sided Wilcoxon rank-sum test; p = 0.07).

Conclusions

Our analyses suggest that H5N1 infections have recurred annually in Egypt during November–April. Although MoH identified an unprecedented number of H5N1 case-patients during 2014–15, the proportion of persons testing positive was similar to proportions of previous epidemic seasons. During 2006–2015, the Ministry of Agriculture identified 3,273 outbreaks among poultry, primarily during Egypt's November–April winter months (*1*). One study found that ≈2% of Egyptians exposed to poultry were seropositive for H5N1 virus (*5*). The large number of H5N1 case-patients identified during 2014–15 could result in part from increased respiratory sampling in communities with poultry outbreaks, rather than from marked changes in the virus's transmission characteristics.

The H5N1 case-patients during the 2014–15 season had similar characteristics to those of previous seasons (*6*). Nearly all had recent exposure to domestic poultry (*7*). Active surveillance from 2010–2012 suggests that 8% of healthy-appearing poultry in Egypt were infected with H5N1 clade 2.2.1 (*8*), yet only 1 case-patient in 2014–15 reported using personal protective equipment.

Human H5N1 infections have been shown to occur during poultry outbreaks, overlapping with October–December influenza epidemics (*4*). Egypt currently recommends seasonal influenza vaccination among health care workers, pregnant women, persons with chronic diseases, and Hajj and Umrah travelers. Countries where seasonal influenza overlaps with H5N1 circulation in poultry might explore the feasibility of vaccinating persons at high risk for influenza co-infections and complications (*9*).

After identification of case-patients in Egypt, officials investigated contacts. This strategy perhaps enriched the number of H5N1 case-patients identified during peak epidemic months, compared with randomly selecting persons meeting case definitions for respiratory illnesses. Although we did not find increased rates of persons testing positive for H5N1, all H5N1 case-patients are unlikely to have the

same probability of being identified (i.e., contacts vs. randomly selected persons).

Egypt continues to have substantial H5N1 circulation among poultry. Although the characteristics of case-patients during 2014–15 were similar to those of previous seasons and do not suggest increased efficiency of H5N1 transmission between humans, MOH would be warranted in examining H5N1 virus circulating in Egypt for genomic markers of mammalian adaptation (*10*), which have been identified since 2010 (*11*), and in using a cross-sectoral approach to evaluate interventions to prevent H5N1 infections.

Acknowledgments

We thank Ann Moen, Joseph Bresee, and Margaret McCarron for their assistance through the Ministry of Health–Centers for Disease Control and Prevention cooperative agreement; Emad Mohareb, Mary Younan, Marc Wooster, and Patrick Blair for their assistance with outbreak response; and Mark Thompson and Jerome Tokars for their review of the manuscript. We also thank Egypt's Epidemiology and Surveillance Unit staff (Ibrahim Saeed, Mahmoud Hassan, Ahmed Helmy, Fatma Shahat, El-Sabah Ali, and Mohamed Kamal); the Field Epidemiology Training Program staff and fellows (Sahar Samy, Eman Hamed, Reham Abdel-Mohaymen, and Mahmoud Taha); and the Communicable Disease Control Department staff (Doaa Zahran, Mohamed Shabaan, Mohamed Attia, and Mohamed Daif).

The surveillance systems that yielded data for this investigation were partially funded through the Centers for Disease Control and Prevention cooperative agreement CDC-RFA-IP09-90204CONT12.

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Replication Capacity of Avian Influenza A(H9N2) Virus in Pet Birds and Mammals, Bangladesh

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Avian influenza A(H9N2) is an agricultural and public health threat. We characterized an H9N2 virus from a pet market in Bangladesh and demonstrated replication in samples from pet birds, swine tissues, human airway and ocular cells, and ferrets. Results implicated pet birds in the potential dissemination and zoonotic transmission of this virus.

Avian influenza A(H9N2) virus is endemic among poultry throughout Eurasia (*1*–*3*). In Bangladesh, subtype H9N2 viruses are unique reassortants, containing genes from highly pathogenic avian influenza A(H7N3) viruses. The H9N2 virus poses a substantial infection risk to poultry (*2*) and has infected pigs and humans (*4*,*5*). Its evolution is continually monitored by the World Health Organization (http://www.who.int/influenza/vaccines/virus/201502_zoonotic_vaccinevirusupdate.pdf?ua = 1).

Ongoing influenza surveillance in Bangladesh found H9N2 virus primarily in poultry (*5*,*6*); we also surveyed a pet market that sold avian pets (parrots, finches, pigeons) and poultry (quail, turkey, chickens) and obtained isolates from nonpoultry terrestrial birds (*6*). This mixture of birds and mammals, some for which little associated influenza pathogenesis data exists, provided a unique opportunity to study the ecology, host range, and transmission potential of H9N2 virus.

The Study

We obtained H9N2 virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) from a fecal sample collected from a parrot cage. Phylogenic data are available for other H9N2 viruses isolated in Bangladesh (*5*), but little phenotypic data exists for this lineage, which represents most H9N2 strains isolated in Bangladesh during 2010–2012. This strain clusters with isolates from Pakistan and India and has mammalian adaptations (*2*,*5*). We examined the pathogenicity of Env/9306 in birds commonly found at pet markets and assessed its capacity to replicate in and transmit among mammals by using ex vivo and in vivo models.

To examine H9N2 replication in bird species, we inoculated 5 finches, 5 parakeets, and 6 chickens oculonasally with 10^5 $\log_{10}$ 50% egg infectious doses ($\log_{10}$ EID_{50}) of Env/9306 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/12/15-1152-Techapp1.pdf). Oropharyngeal and cloacal swab samples were collected every 2 days postinoculation (dpi) and titrated in eggs. Measurement of donor and contact animal virus shedding is based on the inoculation date of donors; donor and contact animals were kept in the same cage. Inoculated pet birds shed virus oropharyngeally (Figure 1) for 6 days, but not cloacally (data not shown). Chickens, a control H9N2 virus host, shed 2–3 logs more than did pet birds, and for a significantly longer time by area under the curve analysis (up to 10 dpi; $p \leq 0.001$). Finches remained asymptomatic; parakeets and chickens showed sporadic clinical signs (lethargy, hunched posture, labored breathing) at 5–10 dpi. No birds died.

Tissue samples were collected at 3 dpi (Table 1). Virus was isolated from the respiratory tract of 1 parakeet, 2 finches, and all 3 chickens, 2 of which had virus in the gastrointestinal tract. Virus was also isolated from the brain (2 finches, 1 chicken) and eye (1 finch, 1 chicken) (Table 1).

Naive contacts of inoculated pet birds were not infected, but naive chicken contacts became infected and shed virus as early as 2 dpi (Figure 1). All birds were tested for seroconversion at 16 dpi by hemagglutination inhibition (HI) assay (*7*; online Technical Appendix). Among finches, 1 of 5 donors and no contacts seroconverted. Among parakeets, 4 of 5 donors and 1 of 5 contacts seroconverted. All chickens seroconverted, and titers exceeded those of pet birds (Table 1).

To determine environmental shedding, we collected swab samples of drinking water, feces, and cages on 1–6 dpi. Virus was detected in water for finches (4 time points) and parakeets (1 time point) but not in fecal or cage swab

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DOI: http://dx.doi.org/10.3201/eid2112.151152

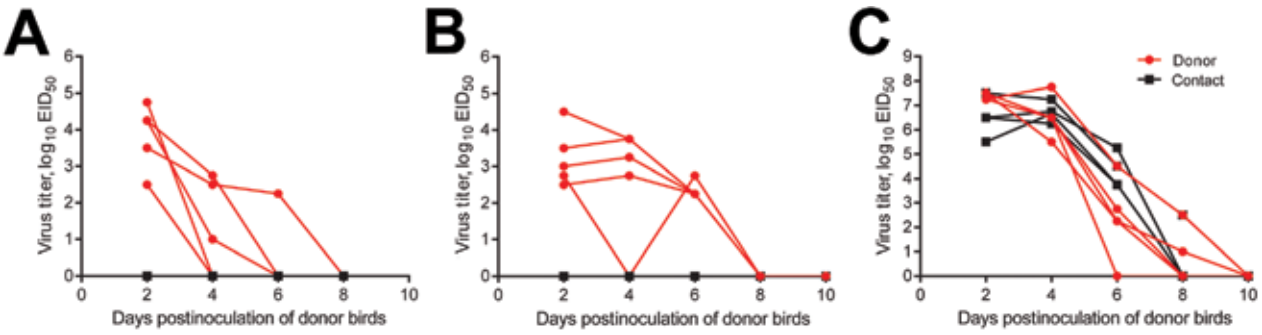

Figure 1. Oropharyngeal shedding of influenza A(H9N2) virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) by pet birds and chickens, Bangladesh. Measurement of donor and contact bird virus shedding is based on the inoculation date of donors; donor and contact birds were kept in the same cage or enclosed environment. A) Donor finches (n = 5), B) parakeets (n = 5), or C) chickens (n = 6; red lines) were inoculated with 10^5 $\log_{10}$ 50% egg infectious doses (EID_{50}) units of Env/9306 and paired with naive birds of the same species (n = 4 or 5; black lines) in the same cage. Birds were swabbed every 2 dpi and virus titer ($\log_{10}$ EID_{50}/mL) was determined in eggs. Individual shedding curves for each animal are provided.

samples, consistent with oropharyngeal shedding patterns (*8*). All chicken environmental samples contained virus for at least 4 of 6 time points (Table 2).

The H9N2 virus strain Env/9306 contains mammalian-like mutations in genes, including HAQ226L (H3 numbering) (*5*), which increase H9N2 virus transmissibility to and among mammals (*9*). We modeled replication in humans (respiratory and ocular routes) by inoculating differentiated normal human bronchial epithelial cells (NHBEs) or primary human ocular cells (cornea and trabecular meshwork) with Env/9306 (multiplicity of infection 0.01) (online Technical Appendix). The Env/9306 strain replicated in NHBEs to ≥7 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) per mL and exceeded titers of control human pandemic virus A/California/04/2009 (pH1N1) beyond 48 hours postinoculation ($p<0.0001$; Figure 2, panel A). Despite the higher titers, Env/9306 did not induce noticeable cytopathology in NHBEs, but pH1N1 did. In corneal and trabecular meshwork cells, Env/9306 replicated to similar titers as did control virus H7N3, a subtype previously shown to replicate in ocular cells (Figure 2, panels B, C) (*10*).

To assess replication in swine, we inoculated tracheal or lung tissue explants (online Technical Appendix) from 1–2 week old piglets, with 10^5 EID_{50}/explant. Virus replicated ≥6 $\log_{10}$ $TCID_{50}$/mL, comparable to a control swine H2N3 virus (Figure 2, panel D).

We modeled replication and transmission in vivo by inoculating 3 donor ferrets with strain Env/9306 (10^6 EID_{50} units); each was co-housed with a separate naive, direct contact. Donors shed 4 $\log_{10}$ $TCID_{50}$/mL in nasal washes for 6 dpi; 2 of 3 donors displayed lethargy, swollen sinuses, sneezing, or a combination of these during this period. No virus was shed by naïve direct contacts. One donor ferret displayed lethargy (4–8 dpi) and 1 sneezing (10–12 dpi) (Figure 2, panel F, data not shown). To examine whether the lack of transmission correlated with virus tropism, ferret tracheal and lung explants (online Technical Appendix) were inoculated with 10^5 EID_{50}/explant of Env/9306 or pH1N1. Env/9306 replicated in ferret tracheal explants to titers >5 $\log_{10}$ EID_{50}/mL (72 hours postinoculation), statistically lower than the rate for pH1N1 (Figure 2, panel E). No replication of either virus was observed in lung explants.

Conclusions

We demonstrated replication of a nonpoultry avian influenza A(H9N2) virus in finches and parakeets with limited environmental shedding (water), but no transmission to cage mates. Shedding routes were more limited, virus titers lower, and clinical signs less frequent in pet birds than in chickens. Nevertheless, the potential for pet birds to act as vectors of the virus should not be underestimated. We recently showed that novel influenza A(H7N9) virus

Table 1. Replication of avian influenza A(H9N2) virus in organs and seroconversion of inoculated birds, Bangladesh*

	Organ titer†						HI titer‡	
Bird	Brain	Eye	Trachea	Lung	Small intestine	Large intestine	Donor	Contact
Finch	2.9 (2/3)	3.5 (1/3)	3 (2/3)	3.5 (1/3)	–	–	4.3 (1/5)	– (0/5)
Parakeet	ND	ND	3.5 (1/3)	–	–	–	6.1 (4/5)	5.3 (1/5)
Chicken	5.5 (1/1)	4.25 (1/1)	4.5 (3/3)	5.1 (3/3)	3 (2/3)	4.5 (1/3)	10.8 (6/6)	10.9 (4/4)

*HI, hemagglutination inhibition; –, below the limit of detection (<0.75 50% egg infectious doses [EID_{50}]/mL or serum dilution <1:20); ND, not determined.
†Tissues were harvested 3 days postinoculation and titrated in embryonated chicken eggs, and were reported as $\log_{10}$ EID_{50}/mL. Data are the means of positive samples (>0.75 EID_{50}/mL) (no. birds shedding/total birds sampled at given time point).
‡Mean reciprocal values ($\log_2$/50μL) of the highest titer that inhibited 4 hemagglutinating units of homologous virus (no. seropositive animals/total no. sampled).

Table 2. Detection of avian influenza A(H9N2) virus in swab samples from environment of inoculated birds, Bangladesh*

Bird	Sample	Swab titer†					
		1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi
Finch	Water	3.25	2.5	2.5	1	–	–
	Cage	–	–	–	–	–	–
	Feces	–	–	–	–	–	–
Parakeet	Water	–	–	–	–	1	–
	Cage	–	–	–	–	–	–
	Feces	–	–	–	–	–	–
Chicken	Water	3.25	2.25	4.5	3.5	5.5	–
	Cage	3.5	4.5	4.5	4.5	3.25	2.5
	Feces	3	<	3.25	3.75	2.5	–

*dpi, days postinoculation; –, below the limit of detection (<0.75 50% egg infectious doses [EID_{50}]/mL).
†Samples were titrated in embryonated chicken eggs and reported as $\log_{10}$ EID_{50}/mL.

transmits between passerines, which include finches, and poultry by water despite a lack of intraspecies transmission (*8*); H9N2 virus has also been isolated from wild, finch-like birds in China (*11*).

Interspecies transmission of the Env/9306 strain remains a risk to mammals because of adaptation mutations (*5*,*9*) and is supported in this study by replication in ferrets and in human and swine tissues. Physical contact between pet birds and their owners, as well as shedding of virus into the environment (water), could be transmission sources.

Live bird markets are crucial to zoonotic spread of avian influenza viruses (AIVs) (*12*). However, our data suggest transmission potential in pet markets and vendor sites other than poultry markets; these sites may house birds infected with AIVs and should be included in future surveillance. Our results may also inform surveillance sample collection. Oropharyngeal samples were collected from pet birds; collecting environmental swabs alone may yield lower isolation rates or fail to detect this virus. H9N2 virus replication in pet birds also has implications for viral spread. Poultry are a major source of dissemination, but our data show domesticated or pet birds can harbor H9N2. Pet trading can extend across international borders and greatly expand the range of AIVs, as when H9N2 virus was repeatedly imported into Japan in infected parakeets (*13*). Finally, the unique influenza varieties among pet birds may provide more opportunities for H9N2 virus to gain novel genetic elements; this subtype has had remarkable levels of reassortment activity with influenza A(H7N9) and highly pathogenic avian influenza A(H5N1) viruses (*14*,*15*).

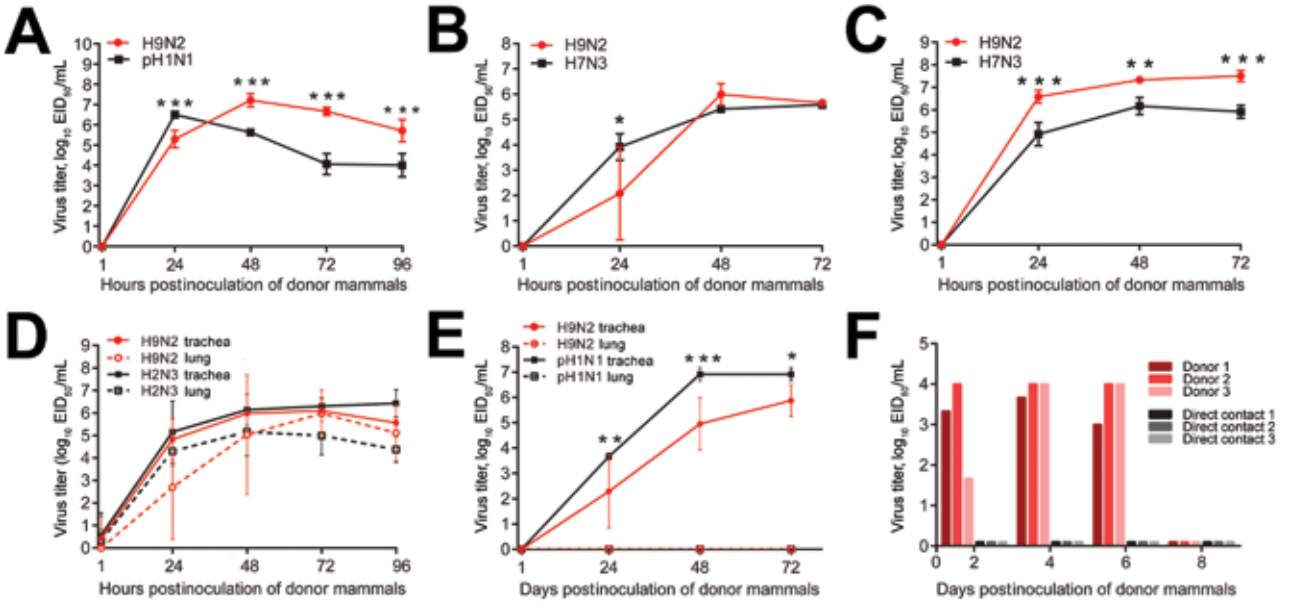

Figure 2. Pathogenesis of influenza A(H9N2) virus isolate A/environment/Bangladesh/9306/2010 (Env/9306) in ex vivo and in vivo mammalian models, Bangladesh. Replication kinetics of Env/9306 or a virus control are shown in A) primary normal human bronchial epithelial cells, B) primary human corneal epithelial cells, C) primary human trabecular meshwork cells, D) swine respiratory tissue explants, and E) ferret respiratory tissue explants. Error bars indicate mean ± SD of the combined results of 2 individual experiments of n = 3 inserts, wells, or tissue explants per virus group. Env/9036 replication is indicated in red, and control virus replication in black. F) Replication of Env/9306 in ferrets (n = 3; red bars) and transmission to naïve, direct contact ferrets (n = 3; black bars) housed in the same cage. Statistical significance of replication between virus groups at a given time point was determined by performing a 2-way analysis of variance. *p≤0.05; **p≤0.01; ***p≤0.0001. EID_{50}, 50% egg infectious doses.

H9N2 virus will remain a threat in the foreseeable future. Efforts are needed to identify its presence in poultry and nonpoultry avian species. Phenotypic properties of these viruses, including replication ex vivo and in vivo, are a valuable supplement to existing genotypic data and further inform the risk for spread within avian and human populations.

Acknowledgments

We thank Angela Danner, Chelsi Stultz and Sharon Lokey for experimental support and James Knowles, Kimberly Friedman, and Jennifer Debeauchamp for administrative assistance.

This work was supported by contract no. HHSN272201400006C from the US National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, and by the American Lebanese Syrian Associated Charities.

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Pyrethroid and DDT Resistance and Organophosphate Susceptibility among *Anopheles* spp. Mosquitoes, Western Kenya

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We conducted standard insecticide susceptibility testing across western Kenya and found that the *Anopheles gambiae* mosquito has acquired high resistance to pyrethroids and DDT, patchy resistance to carbamates, but no resistance to organophosphates. Use of non–pyrethroid-based vector control tools may be preferable for malaria prevention in this region.

During the past decade, a massive scale-up of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) of insecticides in malaria-endemic areas worldwide have led to a substantial reduction in mosquitoes and, paired with the use of artemisinin combination treatments, in overall malaria prevalence and incidence (*1*). However, although most studied sites showed sustained low-level transmission, other sites had stable or resurging malaria cases and vector populations (*2*–*5*). It is generally believed that the recent resurgence in malaria was caused in part by increased vector resistance to pyrethroid insecticides related to the intensive use of ITNs and IRS (*6*–*8*). Insecticide resistance is among the most critical challenges in malaria control. Although several new insecticides have been tested as alternatives to pyrethroids for IRS, there is strong debate among decision makers at the national level on whether to implement IRS and which insecticides should be used. Comprehensive evaluation of insecticide resistance across different malaria-endemic areas will provide critically needed data on use of new IRS strategies as alternative malaria control tools for further reducing malaria incidence in Africa.

The Study

During April 2012–July 2013, we conducted this study in 7 sentinel sites across different malaria-endemic zones in western Kenya (Figure 1). Malaria vector dynamics and parasite prevalence have been studied in 3 sites (*9*), and ITN coverage was generally >80% (*10*). Bungoma, Emutete, Iguhu, and Emakakha are in the highland-fringe malaria epidemic area; Chulaimbo, Ahero, and Kisian are in the malaria-endemic basin region of Lake Victoria (lowland). All sample sites were in rural or suburban areas.

Agricultural and public health use of insecticides in each study site was surveyed by using questionnaire surveys in 30 randomly selected households per site. Mosquito larvae were collected from each study site, fed with TetraMin fish food (Spectrum Brands, Inc., Blacksburg, VA, USA), and raised to adults in the insectary at the Kenya Medical Research Institute in Kisumu. The insectary was not regulated for temperature and humidity; ambient temperature (average ≈24°C) and humidity (≈75% relative humidity) were used for the study. Emerged adults were fed with 10% sucrose solution, and 2- to 5-day-old females were used to determine insecticide susceptibility by using the standard World Health Organization (WHO) insecticide susceptibility tube test http://www.who.int/malaria/publications/atoz/9789241505154/en/. Four classes of insecticides were tested, including pyrethroids lambdacyhalothrin (diagnostic dose 0.05%), deltamethrin (4%), and permethrin (0.75%); organochlorine DDT (0.05%); organophosphate malathion (5%); and carbamate bendiocarb (0.1%) (*11*). The WHO-designated, pyrethroid-susceptible *An. gambiae* mosquito in Kisumu was used as a control.

Mosquitoes were exposed to each insecticide for 1 h and then maintained in holding tubes with 10% sucrose solution for 24 h. Mortality rates were scored after the 24-h recovery period; the susceptibility status of the mosquito populations was graded according to WHO criteria (*11*). Knockdown time (time required to render an adult mosquito unable to fly) was recorded every 10 minutes. Tests were done at 26°C ± 2°C and 80% ± 10% relative humidity during the 1-h exposure period and the subsequent 24-h period during which the mosquito would die or recover, with a 12D:12N photoperiod. We tested 200 mosquitoes per site per insecticide; that is, 8 replicates of exposure and 2 replicates of control, with 20 mosquitoes per replicate. A total of 8,400 (200 per site × 7 sites × 6 insecticides) female mosquitoes were tested. Knockdown rates are provided in the online Technical Appendix Figure (http://wwwnc.cdc.gov/EID/article/21/12/15-0814-Techapp1.pdf).

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DOI: http://dx.doi.org/10.3201/eid2112.150814

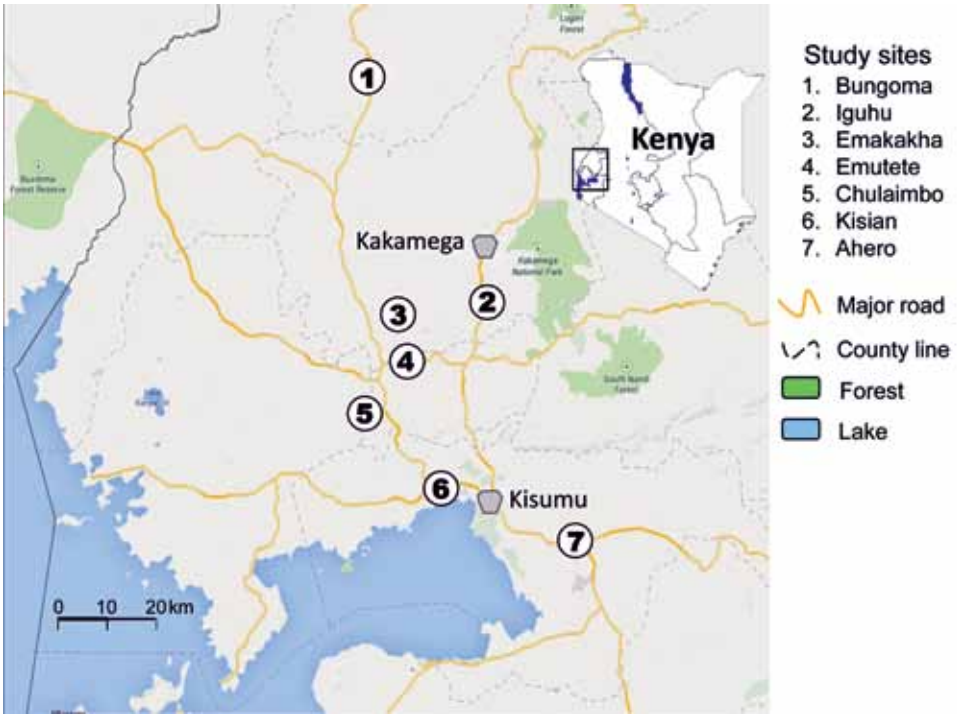

Figure 1. Study sites (circles) for discerning the presence of pyrethroid and DDT resistance and organophosphate susceptibility among *Anopheles* spp. mosquitoes, western Kenya, 2012–2013.

We identified species of a subset of randomly selected susceptible and resistant mosquitoes from the bioassay by using 16s rDNA PCR (*12*). A total of 1,002 specimens were molecularly identified. The real-time TaqMan assay was used to detect knockdown resistance (*kdr*) gene mutations and genotypes at amino acid position L1014 of the voltage-gated sodium channel gene (*12*). A total of 579 mosquitoes were examined for *kdr* mutation.

Results of WHO susceptibility bioassays showed a 100% mortality rate in the susceptible Kisumu *An. gambiae* reference strain after exposure to all insecticides tested and 50.4%–87.2% in the 7-field *An. gambiae* sensu lato populations resulting from exposure to all pyrethroids and DDT. These field populations were highly resistant to pyrethroids, demonstrated by an observed mortality rate that was considerably less than the WHO 90% threshold for resistance. The Bungoma population was the most resistant, exhibiting only a 50% mortality rate against permethrin. The WHO susceptibility bioassay also indicated that *An. gambiae* sensu lato was highly resistant to DDT: mortality rates ranged 50.4%–73.2% at all sites (Figure 2). Five of the 7 study populations were susceptible to bendiocarb, but 2 populations (Iguhu and Bungoma) were resistant (Figure 2). A 100% mortality rate was observed in mosquito populations exposed to malathion at all sites (Figure 2).

PCR analysis found that *An. gambiae* sensu stricto (s.s.) was the predominant species in Chulaimbo (72.0%), Iguhu (88.0%), Bungoma (90.0%), Emakakha (93.4%), and Emutete (94.0%), whereas *An. arabiensis* was predominant in Kisian (64.4%) and Ahero (89.3%) (Table 1). The L1014F mutation was not detected in *An. gambiae* s.s or *An. arabiensis* mosquitoes at any sites. Frequency of L1014S point mutation was high for *An. gambiae* s.s. (85.8%–92.9%) except in the Kisian population (33.0%) (Table 2). For *An. arabiensis*, L1014S mutation frequency was lower (1.2%–39.1%). Homozygosity of L1014S genotype was high in *An. gambiae* s.s (30.0%–89.3%), but low in *An. arabiensis* (0–39.1%) (Table 2).

We found through a survey that pyrethroids were the most frequently used insecticide for mosquito control (online Technical Appendix Table). Pyrethroids were also frequently used for control of livestock disease vectors and agricultural pests. Most (73.3%–96.6%) surveyed households used pyrethroids for malaria vector control in the form of ITNs and IRS (online Technical Appendix Table). Carbamate was mainly used for livestock disease vector control, and organophosphate was used

Table 1. *Anopheles* mosquitoes observed for insecticide resistance in 7 study sites, Western Kenya, 2012–2013

Study site	No. collected	% *An. arabiensis*	% *An. gambiae* subsp.	% Not amplified
Ahero	56	89.3	5.4	5.4
Kisian	225	64.4	32.9	2.4
Chulaimbo	100	24.0	72.0	4.0
Emutete	200	3.5	94.0	2.5
Emakakha	61	3.3	93.4	3.3
Iguhu	300	8.0	88.0	4.0
Bungoma	60	3.3	90.0	6.7

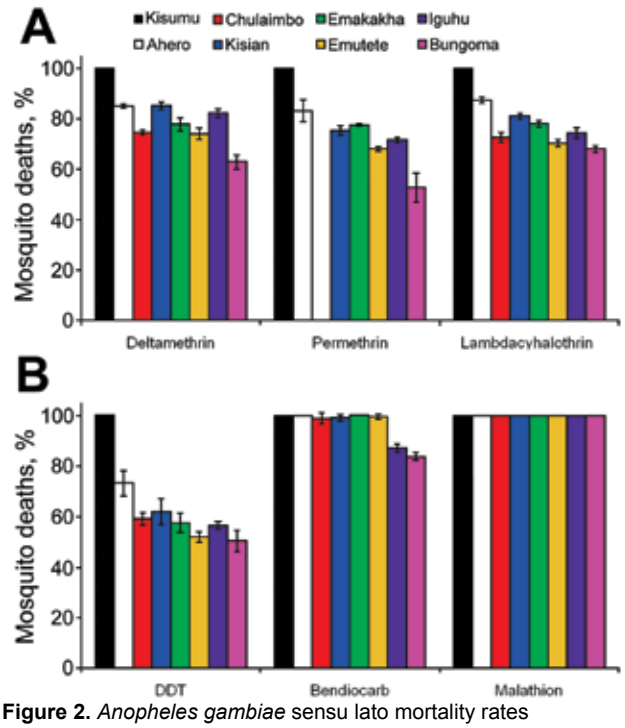

Figure 2. *Anopheles gambiae* sensu lato mortality rates associated with various insecticides and study sites, western Kenya. A) Mortality rates associated with pyrethroid insecticides deltamethrin, permethrin, and lambdacyhalothrin. In Chulaimbo, permethrin was not tested because of a lack of mosquitoes. B) Mortality rates associated with DDT (organochlorine), bendiocarb (carbamate), and malathion (organophosphate). The susceptible Kisumu strain at Kenya Medical Research Institute was used as a control. Error bars indicate 95% CIs.

seasonally for crop pest control among a small proportion of households.

Conclusion

This study found high resistance to pyrethroid insecticide in all 7 study populations, no resistance to organophosphate, and patchy distribution of resistance to carbamate insecticide in *An. gambiae* and *An. arabiensis* mosquitoes in western Kenya. This finding has critical implications in guiding malaria vector control in Kenya. In Kenya, current policy on IRS use of insecticides is limited to pyrethroids and DDT (*13*). Considering widespread pyrethroid resistance, non–pyrethroid-based vector control tools may be preferable. There is a growing debate among government decision makers on whether to use organophosphates (such as malathion or chlorpyrifos methyl) and carbamates (such as bendiocarb) for IRS in Kenya. Our finding on the complete susceptibility to organophosphates in malaria vectors suggests that organophosphates are a potentially effective insecticide for IRS. The patchy distribution of resistance to carbamates calls for careful resistance baseline monitoring if carbamates are considered for IRS.

Although we detected widespread and strong phenotypic resistance to pyrethroids in *An. gambiae* mosquitoes, whether this resistance could result in operational ITN or IRS malaria control failure in the field is unknown. A report from Côte d'Ivoire showed that ITNs remained effective in reducing entomological inoculation rate in an area of higher *kdr* frequency in *An. gambiae* mosquitoes (*14*). Similarly, a cohort study in Malawi found that the use of ITNs reduced the incidence of cases of malaria by 30% in children in an area that has documented moderate levels of pyrethroid resistance and considerable malaria transmission (*15*). Cost-effectiveness is another consideration. A thorough assessment of the effect of resistance to pyrethroids on the efficacy and cost-effectiveness of LLINs and IRS for malarial disease and transmission will clarify the need to consider a shift from pyrethroids to alternative carbamate or organophosphate insecticides or to other integrated strategies to control malaria.

Acknowledgment

We thank the staff of the Climate and Human Health Research Unit, Centre for Global Health Research, Kenya Medical Research Institute, Kenya, for field assistance. We thank 2 anonymous reviewers for comments and suggestions that greatly improved the manuscript.

This paper was published with permission from the director of Kenya Medical Research Institute. This study was supported by grants from the National Institutes of Health (R01 A1050243 and D43 TW001505).

Table 2. Distribution of knockdown resistance genotypes and mutation frequencies by *Anopheles* mosquito species and study sites of pyrethroid- and DDT-resistant, organophosphate-susceptible *Anopheles* mosquitoes, Western Kenya, 2012–2013*

	An. gambiae					*An. arabiensis*				
Study site	No.	LL	LS	SS	Frequency, %	No.	LL	LS	SS	Frequency, %
Ahero	ND	ND	ND	ND	ND	50	46	4	0	4.0
Kisan	50	32	3	15	33.0	42	41	1	0	1.2
Chulaimbo	56	2	4	50	92.9	23	14	0	9	39.1
Emutete	87	7	4	76	89.7	ND	ND	ND	ND	ND
Emakakha	57	1	7	49	92.1	ND	ND	ND	ND	ND
Iguhu	108	10	7	91	87.5	16	15	1	0	3.1
Bungoma	53	5	5	43	85.8	ND	ND	ND	ND	ND

*LL, wild genotype at L1014 codon; SS, homozygous genotype for L1014S mutation; LS, heterozygous genotype; Frequency, allele frequency of L1014S mutation. ND, not done because of insufficient number of specimens.

Ms. Wanjala is a PhD candidate at Kenyatta University and a trainee supported by a research training grant from the National Institutes of Health. Her research interests include vector ecology and malaria epidemiology.

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Hendra Virus Infection in Dog, Australia, 2013

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Hendra virus occasionally causes severe disease in horses and humans. In Australia in 2013, infection was detected in a dog that had been in contact with an infected horse. Abnormalities and viral RNA were found in the dog's kidney, brain, lymph nodes, spleen, and liver. Dogs should be kept away from infected horses.

Hendra virus (HeV) is a paramyxovirus (genus *Henipavirus*) (*1*) that causes respiratory and neurologic disease in horses and humans; the case-fatality rate is >60%. Fruit bats are the reservoir hosts (*2*,*3*) and excrete virus in urine (*4*). Disease outbreaks among horses occur sporadically along the eastern coast of Queensland and New South Wales, Australia. Infection of veterinarians after close contact with infected horses presents a serious occupational hazard. After confirmation of Hendra infection in horses, an affected farm is quarantined by animal health authorities. All horses, cats, and dogs determined to be at risk for infection are monitored for clinical signs and tested for virus until they are no longer considered to be potentially infected. Cats and dogs are included in this surveillance because they have been shown to be susceptible to experimental infection with HeV (*5*; D. Middleton, unpub. data). In July 2013, during investigation of HeV infection in a horse near Macksville, New South Wales, Australia, infection was also detected in a dog on the same farm.

The Study

The infected horse was a 6-year-old Australian stock horse gelding. HeV RNA was detected by quantitative reverse transcription PCR (qRT-PCR) in EDTA-treated blood (cycle threshold [C_t] 26.82), serum (C_t 30.87), and nasal swab samples (C_t 34.56) collected on July 4, 2013. Later that day, the horse was killed by shooting. During follow-up investigations on July 6, negative HeV results (qRT-PCR and ELISA) were obtained from whole blood, serum, and nasal swab samples collected from 2 additional horses; whole blood and oral swab samples collected from 2 dogs; and oral swab samples collected from a third dog. These dogs were from the same farm as the HeV-positive horse.

Twelve days later, additional blood samples were collected (placed in EDTA or allowed to clot) from the 3 dogs, and oral swab samples were collected from 1 of these dogs (a 6-year-old cross-bred female fox terrier). HeV RNA was detected in the EDTA-treated blood (C_t 31.48) and serum (C_t 34.01), but not from the oral swab samples, from this dog. Results from all samples from the other dogs were negative by qRT-PCR and ELISA. Serum from the dog with positive results by qRT-PCR gave a weak positive result by ELISA and a virus neutralization titer of 8. The dog showed no signs of ill health, although it had winced several times, suggesting discomfort or pain. Because the transmission risk posed by the dog was uncertain, it was euthanized 14 days after collection of the first samples. Blood (placed in EDTA or allowed to clot); oral, nasal, rectal, and vaginal swab samples; and urine were collected immediately thereafter. The cadaver was immediately transported to the laboratory, and a postmortem examination was conducted later that day.

No external gross abnormalities were detected. Internal examination revealed diffuse marked reddening of all lung lobes and overlying dark patchy discoloration of dependent lobes; abundant frothy tracheal and bronchial fluid; enlargement and diffuse reddening of bronchial, tracheobronchial, and mandibular lymph nodes; prominent and diffuse reddening of both tonsils; and prominent white streaks at the corticomedullary junction of both kidneys. The spleen and liver were enlarged with rounded edges, and the liver had a mild cobblestone pattern (Table 1). Histopathology findings closely aligned with gross findings; lesions in the brain were also histologically detected. The predominant lesion, found in decreasing severity in kidney, brain, lymph nodes, spleen, liver, intestine, and lung, was fibrinoid necrosis of vessels with marked segmental to diffuse vasculitis, disruption of subendothelial tunica intima, and expansion with thick bands of deeply eosinophilic hyaline to fibrinoid material admixed with karyorrhectic debris and degenerate neutrophils (Figure 1). Surrounding inflammatory infiltrates (plasma cells, lymphocytes, and karyorrhectic debris) often effaced and replaced surrounding normal structures. Cerebral and cerebellar meninges were moderately expanded with lymphocytes, plasma cells, and macrophages (Figure

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DOI: http://dx.doi.org/10.3201/eid2112.151324

Table 1. Gross and histopathologic findings in tissues of Hendra virus–infected dog, Australia, 2013*

Sample	Gross pathology	Histopathology
Pharynx	ND	NSF
Soft palate	ND	NSF
Tonsil	Moderate	NA
Lymph node		
Submandibular	ND	NSF
Mandibular	Mild	NSF
Bronchial	Moderate	Moderate
Tracheobronchial	Moderate	Moderate
Axillary	ND	NSF
Inguinal	ND	NSF
Lung	Moderate	Mild
Myocardium	Mild	Mild
Spleen	Mild	Mild
Liver	Mild	Mild
Kidney	Moderate	Marked
Adrenal gland	ND	NSF
Bladder	ND	NSF
Intestine		
Small	ND	NSF
Large	ND	Mild
Brain		
Olfactory	ND	Moderate
Occipital	ND	Moderate
Cerebellum	ND	Moderate
Brain stem	ND	Moderate
Meninges	ND	Moderate
Spinal cord	ND	NA
Turbinate	ND	NSF
Trigeminal ganglion	ND	NA
Brachial nerve	ND	NA

*NA, tissue not available; ND, not detected; NSF, no significant changes found.

2), and cerebral vasculitis was associated with surrounding malacia. Pulmonary alveoli were flooded with lightly eosinophilic fluid (edema) containing scattered erythrocytes, plasma cells, and macrophages. Hepatocytes were diffusely expanded, and floccular vacuolation was compressing adjacent sinusoids. Small amounts of viral antigen were detected in a necrotic glomerulus and within the media of a renal arteriole by immunoperoxidase staining.

An extensive range of fresh tissues and swab samples were collected for testing by qRT-PCR, and HeV RNA was found in many of the tissues (Table 2). No virus was isolated from any of the tissues in cell culture. Serum collected at the time of euthanasia was positive by ELISA; virus neutralization titer was 128. All other animals on the farm remained seronegative when sampled 4 weeks after the infected dog had been euthanized. Laboratory methods are described in

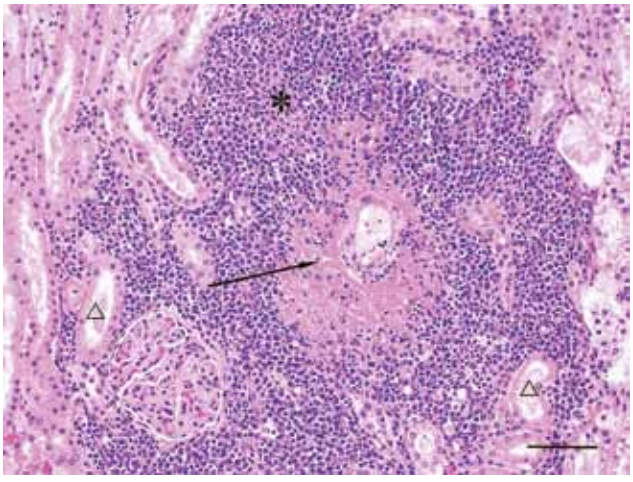

Figure 1. Kidney of dog infected with Hendra virus, showing marked vasculitis (arrow) and inflammatory infiltrates (*) effacing renal tubules (△). Scale bar indicates 75 μm.

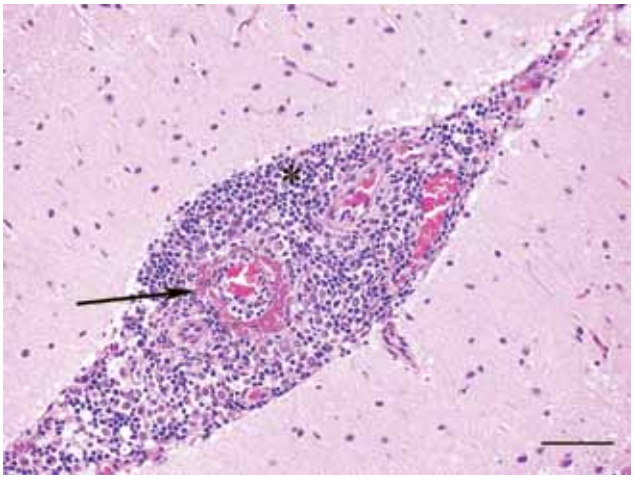

Figure 2. Cerebellum of dog infected with Hendra virus, showing expansion of the meninges with inflammatory infiltrates (*) and marked vasculitis (arrow). Scale bar indicates 75 μm.

Table 2. Hendra virus RNA levels in tissues and blood of Hendra virus–infected dog, Australia, 2013*

Sample	RNA level†
Blood (in EDTA)	33.36
Pharynx	36.52
Soft palate	35.08
Tonsil	36.12
Lymph node	
Submandibular	ND
Mandibular	33.91
Bronchial	28.32
Tracheobronchial	28.26
Axillary	32.87
Inguinal	33.91
Spleen	29.64
Lung	35.00
Myocardium	28.62
Liver	27.65
Kidney	29.03
Adrenal	34.06
Bladder	33.68
Intestine	
Small	35.01
Large	ND
Spinal cord	28.67
Brain	
Olfactory	34.84
Occipital	34.38
Cerebellum	ND
Brain stem	ND
Meninges	ND
Turbinate	ND
Trigeminal ganglion	ND
Brachial nerve	ND

*ND, not detected.
†Cycle threshold.

the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/12/15-1324-Techapp1.pdf).

Conclusions

Dogs and cats have been infected with HeV under experimental conditions. Previously, a dog located on the same property as 3 infected horses in Queensland, Australia, was found to be seropositive (*6*) without having shown clinical signs. The dog reported in this article, which also remained clinically healthy, was naturally infected and was identified during the acute stages of infection. Viral RNA was detected in this animal 12 days after euthanasia of the clinically affected horse. The dog was known to have been in close contact with the live infected horse and is suspected of having been exposed to its blood after the horse was euthanized. The epidemiologic and laboratory evidence supports transmission of HeV from horse to dog. In horses naturally infected with HeV, the development of neutralizing antibodies is associated with virus clearance from the infected animal. The detection of seroconversion and rising neutralizing antibody titers in canine serum collected ≈14 and then 16 days after putative virus exposure is consistent with the early stages of HeV infection and aligns with the low viral RNA levels in blood and a wide range of tissues (the highest levels were found in liver, bronchial lymph node, kidney, and myocardium). Failure to isolate virus in cell culture was probably the result of increasing antibody levels. It is difficult to establish from the qRT-PCR results whether virus replication occurred in tissues such as kidney, liver, myocardium, and spinal cord or whether this finding represents residual RNA from blood. However, the levels in these sites were 10–100-fold higher than that in blood, suggesting either local replication or accumulation of viral RNA. Very low levels of viral RNA were detected in the soft palate, pharynx, and tonsil, although virus was not detected in nasal, oral, rectal, or vaginal swab samples and urine. The risk for transmission of HeV from infected dogs to other susceptible species—including humans—remains unknown.

The histopathologic finding of widespread necrotizing vasculitis supports the current understanding of the pathogenesis of HeV infection, during which virus binds to the endothelial ephrin-B2 transmembrane protein receptor (*7*) and localizes in vessel walls, leading to endothelial cell damage. The most severe vascular lesions were found in kidney, brain, and lymph nodes; the lungs were relatively spared, and fulminant pulmonary edema and interstitial pneumonia were not significant findings in this case.

The route of infection for the dog reported here is unknown, but the dog was in close contact with the infected horse and is suspected to have had contact with its blood. Because viral loads in acutely infected horses are usually very high, dogs can be readily infected and should be kept away from infected horses, which seem to be efficient amplifying hosts.

Acknowledgments

The technical assistance of the staff of the histology and virology laboratories at the Elizabeth Macarthur Agriculture Institute is greatly appreciated. From the Australian Animal Health Laboratory, Geelong, we recognize the technical expertise of Jean Payne and Jenni Harper for conducting the immunohistochemical staining and the members of the diagnostic virology group for their contributions to the virus neutralization testing and virus isolation.

Dr. Kirkland is a veterinary virologist with special interests in the diagnosis and investigation of emergency animal diseases and vectorborne viral infections of animals and in the development and application of molecular-based rapid diagnostic assays for viral infections.

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Kinetics of Serologic Responses to MERS Coronavirus Infection in Humans, South Korea

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We investigated the kinetics of serologic responses to Middle East respiratory syndrome coronavirus (MERS-CoV) infection by using virus neutralization and MERS-CoV S1 IgG ELISA tests. In most patients, robust antibody responses developed by the third week of illness. Delayed antibody responses with the neutralization test were associated with more severe disease.

Knowledge of the kinetics and clinical correlates of serologic responses to Middle East respiratory syndrome coronavirus (MERS-CoV) infection is essential for diagnosing the disease, interpreting seroepidemiologic data to define prevalence and risk factors for infection, understanding pathogenesis, and assessing a potential role for passive immunotherapy. To address this knowledge gap, we investigated serologic responses to MERS-CoV in 17 patients.

The Study

During May–June 2015, an outbreak of MERS-CoV in South Korea resulted in 186 infections and 36 deaths (*1*–*3*); the outbreak strain was a clade B MERS-CoV closely related to viruses circulating in the Middle East (*1*). Seventeen patients with reverse transcription PCR–confirmed MERS-CoV infections were included in this study; the patients were hospitalized at Seoul National University (SNU) Hospital or SNU Boramae Medical Center in Seoul, South Korea, or at SNU Bundang Hospital, in Bundang, South Korea. We investigated early serologic responses; thus, patients who were transferred to these facilities ≥14 days after illness onset were excluded from study. Patients' demographic and clinical profiles are shown in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/12/15-1421-Techapp1.pdf). Of the 17 patients, 9 had severe disease (4 required mechanical ventilation, 4 required supplemental oxygen; 1 died) and 8 had mild disease. Serial serum samples were collected and analyzed. The study was approved by the SNU Institutional Review Board.

Antibody to MERS-CoV was detected by using the plaque reduction neutralization test (PRNT) and MERS-CoV S1 IgG ELISA (EUROIMMUN, Lübeck, Germany) (*4*,*5*) (online Technical Appendix). MERS-CoV EMC was used for the PRNT assay; a 50% PRNT endpoint ($PRNT_{50}$) was used because it was more sensitive than the 90% PRNT cutoff in detecting mild infections (*6*). The ELISA was based on the recombinant spike S1 region of strain EMC because that region is sufficiently divergent between different coronavirus species and expected to lead to less cross-reaction (*4*).

Overall, serologic responses were robust and were detected in most patients by week 3 of illness (Figure). Of the 12 patients who had serum samples tested beyond day 18 of illness, 9 had $PRNT_{50}$ titers >1:320 by day 21 and 2 more had titers >1:320 by day 28. Patient L, a 56-year-old woman with no underlying disease, had weakly positive $PRNT_{50}$ (1:20) and borderline ELISA responses (optical density ratio 1.0), even at day 32 of illness. A chest radiograph showed she had lung infiltrates, but she was not oxygen-dependent and was not administered antiviral drugs or corticosteroids; her recovery was uneventful.

Antibody responses in patient A, a 38-year-old man, were delayed up to 16–18 days after illness onset (Figure). He required mechanical ventilation, and on illness day 14, he was given convalescent-phase plasma (200 mL; antibody titer unknown) from the outbreak index patient's wife (*1*). The next day, antibody responses were undetectable in the patient's serum by PRNT or ELISA. By day 18, he had a $PRNT_{50}$ antibody titer of 1:10 and a negative ELISA response; strong antibody responses developed from day 21 onwards. We hypothesize that the data from the first 21 days of illness represent his own serologic response, unaffected by the passive transfusion with convalescent-phase plasma on day 14; thus, these data were included in the analysis. Patient A was given a second infusion of

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DOI: http://dx.doi.org/10.3201/eid2112.151421

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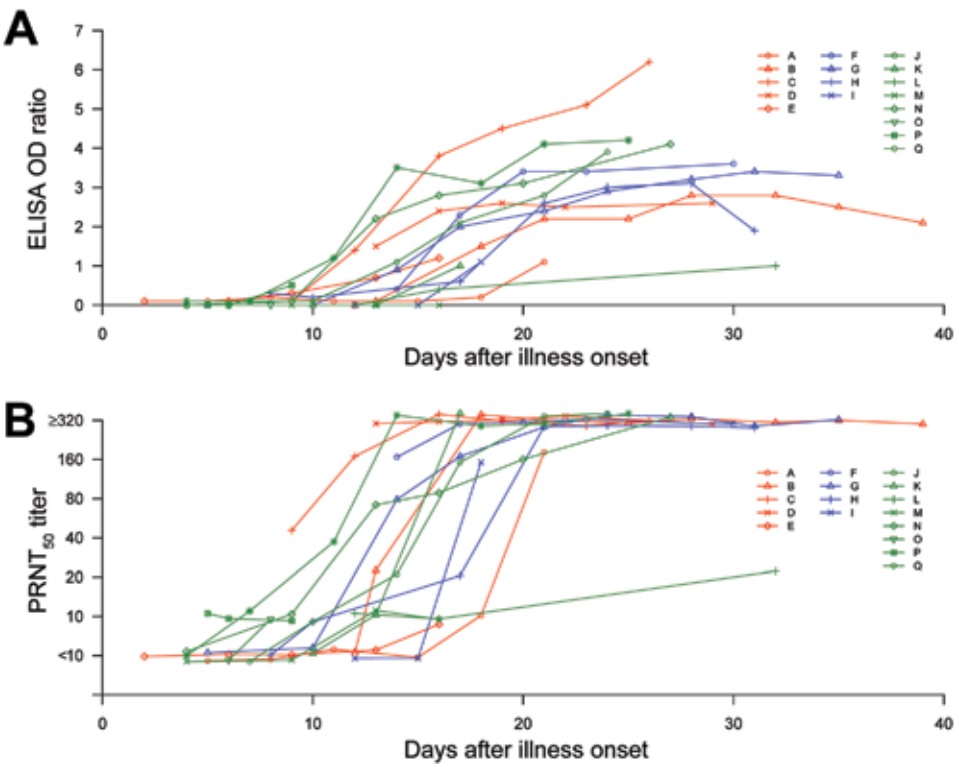

Figure. Antibody response kinetics in patients with Middle East respiratory syndrome coronavirus (MERS-CoV) infection, by days after illness onset, as determined by using a 50% endpoint plaque reduction neutralization test ($PRNT_{50}$) (A) and an S1 IgG ELISA (B). Key indicates individual patients; red inicates patients with severe illness requiring mechanical ventilation; blue indicates patients with severe illness requiring only supplemental oxygen therapy; and green indicates patients with mild illness. For better presentation, the $PRNT_{50}$ titers have been jittered vertically (random noise added to prevent overplotting) (*7*) by adding random numbers to the titers within the range of −0.2 to 0.2 at the log scale. OD, optical density.

convalescent-phase plasma on day 24, and serologic data after day 21 were excluded from analysis.

We constructed a statistical model in which age, sex, incubation period, concomitant conditions, and therapy with corticosteroids or antiviral drugs were adjusted for disease severity. We assessed how these factors were associated with the time from illness onset to commencement of the log-phase antibody response (Table 1) and the time for the antibody response to reach a titer of 1:40 ($PRNT_{50}$) or become positive in the ELISA (online Technical Appendix Table 2). An accelerated failure model was used for a more natural interpretation of the median time from illness onset to the aforementioned antibody responses (online Technical Appendix). Because the increase in antibody titers exhibited an S-shaped pattern, we assessed the rate of change in antibody response after the commencement of the exponential phase by manually removing data from the steady state, thus restricting antibody data to the log-phase response (Table 2). A linear mixed model was used to test the potential difference in the rate of increase by the above factors (online Technical Appendix). Patients with severe disease had significant delays in the commencement of $PRNT_{50}$ antibody responses (Table 1) but had a steeper slope to the antibody response once it began (Table 2). Thus, a delayed adaptive immune response may contribute to increased severity, and passive therapy with convalescent-phase immune plasma may be clinically beneficial. In avian influenza A(H7N9) virus infection of humans, earlier antibody responses and a faster rate of increasing antibody titers were associated with milder disease (*8*), but in SARS-CoV infection, earlier antibody responses were associated with an adverse outcome (*9*).

Table 1. Associations and p values for different clinical factors with time from illness onset to commencement of log phase of antibody response in $PRNT_{50}$ and S1-ELISA*

	Acceleration factor of time from illness onset to log phase of antibody response			
Clinical factors	$PRNT_{50}$ titer	p value	S1-ELISA OD ratio‡	p value
Severe disease	1.61	<0.001	1.19	0.21
Male sex†	0.90	0.52	0.90	0.48
Age ≥60 y†	0.95	0.73	1.08	0.60
Incubation period, d†	0.97	0.06	0.95	<0.001
Use of corticosteroid†	1.19	0.33	1.14	0.47
Use of antiviral drugs†	1.07	0.61	0.76	0.03
Concomitant conditions†	1.08	0.57	1.15	0.30

*Accelerated failure time models were used; acceleration factor >1 means a longer interval to commencement of antibody response. OD, optical density; $PRNT_{50}$, 50% endpoint plaque reduction neutralization test.
†Effects were adjusted for severity.
‡Increase over S1-ELISA OD ≥0.8.

Table 2. Testing potential difference in rates of change in antibody titers over day of illness during the exponential phase of the antibody response, accounting for sequential measurements taken at different days of illness and adjusted for severity*

	Difference in rates of change in log antibody titers			
Clinical factors	$PRNT_{50}$ titer	p value	S1-ELISA OD ratio	p value
Severe disease	0.09	0.01	0.08	0.07
Male sex†	0.07	0.05	0.14	0.01
Age ≥60 y†	0.05	0.22	–0.03	0.65
Incubation period, d†	0.01	0.16	0.02	0.004
Use of corticosteroid†	0.06	0.37	–0.04	0.58
Use of antiviral drugs†	0.06	0.10	0.05	0.35
Concomitant conditions†	0.06	0.06	0.07	0.16

*Differences in rates of change and p values were estimated by using linear mixed models; positive value indicates a faster increase in antibody titer. Given that the antibody titers exhibited an S-shaped pattern, the analysis was restricted to data for log-phase antibody responses by manually removing data from the inductive/steady-state phase. Increases in antibody titers during the log phase were compared by different factors, adjusted for disease severity, by using a linear mixed model to account for repeated measurements, assuming a linear increasing trend by days since illness onset. $PRNT_{50}$ titers were first log-transformed (with base 10). OD, optical density; $PRNT_{50}$, 50% endpoint plaque reduction neutralization test.
†Effects were adjusted for severity.

Extensive contact tracing during the outbreak enabled us to determine the date of MERS-CoV exposure and incubation periods for patients (online Technical Appendix Table 1). A longer incubation period was associated with earlier commencement of antibody responses detectable by ELISA (Table 1; online Technical Appendix Table 2) and with a steeper slope to the response once it began (Table 2). Even after adjusting for disease severity, the use of interferon and antiviral drugs was associated with earlier commencement of antibody responses detectable by ELISA (Table 1). The time to commencement of response was similar for men and women, but the slope of the response was steeper for male patients (Table 2).

Conclusions

An understanding of MERS-CoV antibody response kinetics helps in defining the window during which passive antibody therapy may be useful. In our study, this window was the first 21 days of illness for most patients. However, some patients may not develop strong antibody responses even after 4 weeks of illness, so therapy must be individualized.

Our study has some limitations. First, no MERS-CoV isolates from the study patients were available, so MERS-CoV EMC was the basis of the serologic assays we used. Strain EMC is a clade A virus, and the outbreak in South Korea was caused by a clade B virus (*1*). However, using serum from naturally infected camels, we previously showed that clade A and B viruses and genetically diverse MERS-CoVs from Egypt were serologically indistinguishable (*10*). Another study reported that isolates of MERS-CoVs circulating in Saudi Arabia in 2014 were antigenically indistinguishable from the EMC strain in neutralization tests with human convalescent-phase serum (*5*). Thus, it is unlikely that the use of MERS-CoV EMC in our study considerably affected the observed antibody titers. A second limitation was the small number of patients studied (n = 17) and that they were followed only through the acute stage of illness. Longer term follow-up is needed to define the duration of antibody responses. If MERS-CoV antibody responses wane, as has been reported with SARS (11), this is relevant for interpretation of seroepidemiologic studies and for finding convalescent-phase donors with high antibody titers for passive immunotherapy. It would be useful to investigate IgM antibody responses and antibody responses to other virus proteins, including the MERS-CoV nucleoprotein, especially in patient L, who had poor antibody responses.

In summary, our findings showed that an early MERS-CoV antibody response was associated with reduced disease severity. Robust neutralizing and S1 ELISA IgG antibody responses were mounted by the third week of illness in most patients. However, a robust response did not occur in a few patients, and infections in such patients may be undetectable by serologic and seroepidemiologic methods.

The study was supported by research grants from the Clinical Research Institute, Seoul National University Hospital (2015-1980), South Korea; the US National Institutes of Health (contract no. HHSN272201400006C); and the Health and Medical Research Fund, Food and Health Bureau, Government of Hong Kong Special Administrative Region (commissioned grant).

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No Evidence of Gouléako and Herbert Virus Infections in Pigs, Côte d'Ivoire and Ghana

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A recent report suggested that 2 novel bunyaviruses discovered in insects in Côte d'Ivoire caused lethal disease in swine in South Korea. We conducted cell culture studies and tested serum from pigs exposed to mosquitoes in Côte d'Ivoire and Ghana and found no evidence for infection in pigs.

Orthobunyaviruses and phleboviruses are transmitted to animals and humans by blood-feeding arthropods such as mosquitoes, sandflies, and ticks (*1,2*). Infection can cause systemic disease, including encephalitis or hemorrhagic fevers. Members of both genera of viruses encode a nonstructural (NS) protein that suppresses the antiviral interferon response of the vertebrate host (*3,4*). We recently discovered 2 novel prototypic bunyaviruses in mosquitoes in Côte d'Ivoire (*5,6*). Named Gouléako virus (GOLV) and Herbert virus (HEBV), the viruses tentatively define 2 novel bunyavirus-family genera that are in a sister relationship to the genera *Phlebovirus* and *Orthobunyavirus*, respectively. Neither virus encodes NS proteins, nor do the viruses infect vertebrate cells or cause disease in mice that have been intracerebrally inoculated with the viruses (*5*–*7*). Replication of both viruses is blocked at temperatures above 31°C, suggesting that the viruses are unlikely to infect mammals (*8*).

Chung et al. recently reported that, in 2013, GOLV and HEBV caused prevalent and lethal infections in swine in South Korea (*9*). In that study, >500 pigs from 40 farms were tested for both viruses, and viral RNA was detected in up to 79% of diseased and 55% of healthy pigs. Dead pigs carried virus in their lungs and intestines. GOLV was isolated from swine serum in porcine kidney 15 cells. These results suggest the discovery of disease caused by these 2 novel viruses in a major livestock species. Because of the implications of this finding, we attempted verification.

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DOI: http://dx.doi.org/10.3201/eid2112.141840

The Study

We first extended our recent cell culture studies to include porcine kidney 15 and human embryonic kidney 293 cells, which were the type of cells used by Chung et al. (*9*). Human hepatocellular 7 carcinoma cells were also included because they are highly susceptible to virus infection, as are Vero cells and several other cell lines we used in earlier studies (*5,6*). Infections with GOLV and HEBV were performed at multiplicities of infection of 1 in doublets in all cell lines. Vesicular stomatitis virus was used as a positive control at multiplicity of infection 1. Cell culture supernatants were analyzed for viral RNA after 0, 3, and 6 days by real-time reverse transcription PCR (RT-PCR) (*5,6*). No replication of GOLV and HEBV was detected, whereas vesicular stomatitis virus replicated to high concentrations (Figure 1). Three blind passages on fresh cells failed to yield virus.

Because cell culture experiments may not show the full host range of a specific virus, we tested serum samples collected in 2008 from *Sus scrofa domestica* pigs in Gouléako, the rural village where GOLV and HEBV were first isolated from mosquitoes in Côte d'Ivoire (*5,6*). The 28 tested samples represented nearly all the pigs kept in Gouléako at that time, all of which were constantly exposed to mosquitoes. We also tested 108 serum samples collected in 2011 from mosquito-exposed swine in Kumasi, Ghana, where mosquitoes were found to be infected with HEBV (*6*) and GOLV (S. Junglen, unpub. data).

All samples were tested for virus by real-time RT-PCR (*5,6*) and tested for antibodies against GOLV and HEBV nucleocapsid proteins by recombinant immunofluorescence assay (*10*). All samples were negative for the viruses (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/12/14-1840-Techapp.pdf). Online Technical Appendix Figure 1 shows antigen controls and results from 1 representative swine serum sample.

To compare the viruses found in pigs in South Korea with viruses found in mosquitoes in Africa, we replicated methods used by Chung et al. (*9*) and amplified a region of the GOLV glycoprotein precursor gene from 27 GOLV strains in mosquitoes (online Technical Appendix). Nucleotide sequence distance among mosquito strains was as high as 9.0%. The viruses found in the pigs fell within the genetic diversity of viral strains of GOLV and HEBV and

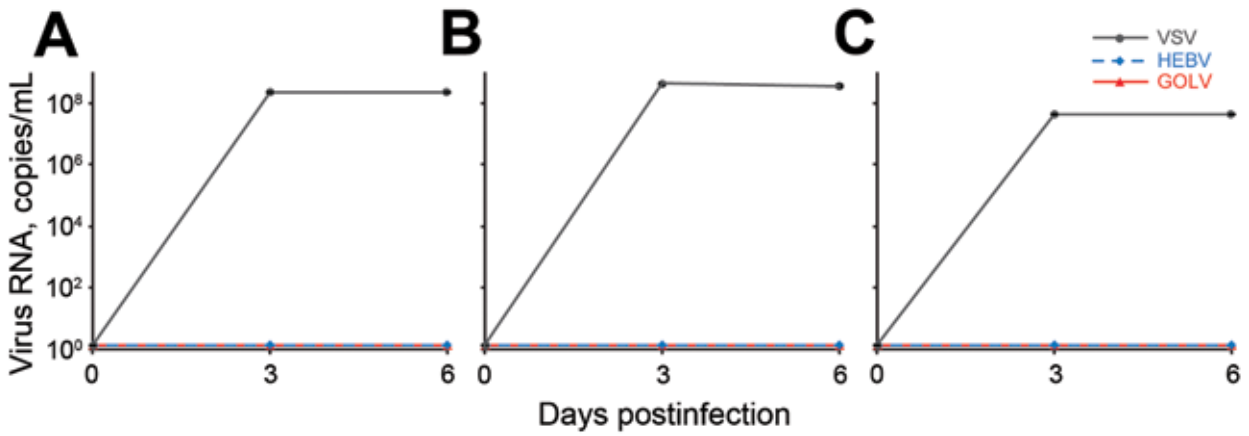

Figure 1. Infection of cells with vesicular stomatitis virus (VSV), Herbert virus (HEBV), and Gouléako virus (GOLV). A) Porcine kidney 15 cells; B) human embryonic kidney cells; C) human hepatocellular 7 cells. Cells were infected at a multiplicity of infection of 1. The number of viral genome copies in cell culture supernatants were measured at 0, 3, and 6 days postinfection by real-time reverse transcription PCR.

did not constitute phylogenetic outliers (Figure 2, panel A). The analyzed fragment had 6 aa exchanges, but they were insufficient for drawing conclusions about protein function because the fragment did not include domains putatively relevant for receptor binding (online Technical Appendix Figure 2).

Small RT-PCR fragments from the RNA-dependent RNA polymerase (RdRp) gene were presented by Chung et al. for HEBV. We performed phylogenetic analyses to compare these swine-derived sequences with sequences from all mosquito-derived viruses from which we could sequence the corresponding genome region (Figure 2, panel B). Comparison of swine-derived sequences with the phylogeny of mosquito-derived HEBV strains, constructed on the basis of the third conserved region of the RdRp (Figure 2, panel C), showed that the strains from South Korea fell within the phylogenetic diversity of HEBV strains identified in West Africa. Online Technical Appendix Figure 3 shows nucleotide- and amino acid–based alignments.

Conclusions

Our results contrast with those of Chung et al. (*9*) for several possible reasons. First, the viruses infecting swine in South Korea may constitute variants of GOLV and HEBV that can infect vertebrates. The presence of an NSs protein in phleboviruses and orthobunyaviruses provides interferon resistance required to infect vertebrates efficiently (*3,4*). Because full genome sequences from swine viruses detected by Chung et al. are not available, we have no information on the presence of NS proteins in these viruses. Furthermore, our detection assays might have failed to detect variant viruses. However, our RT-PCR assays have been shown to detect variant viruses, have been validated for sensitivity (≈100 viral genome copies per mL in liquid specimens), and provide high specificity by probe detection (*5,6*). A concern regarding the results of Chung et al. is the use of RT-PCR assays based on SYBR Green (Thermo Fisher Scientific, Lithuania) product detection, which, from our experience, is prone to yield nonspecific results because no probe is used in this assay. Nevertheless, RT-PCR products in Chung et al. have been confirmed by sequencing. Some sequences presented by these researchers contained stop codons in the HEBV RdRp and the GOLV glycoprotein precursor genes, making it unlikely that these sequences represent replicating viruses. Besides technical explanations, these sequences could represent viral genome fragments integrated in genomes of organisms, such as insects, that are eaten by pigs in the region. Integration of RNA virids derived from flaviviruses into the host genome has been described in insects (*11*). Testing food eaten by swine for insect DNA or viral RNA could yield insight. In addition, we may have collected serum when no active virus infections occurred in tested animals. However, past infections would have been shown by antibody tests. Because bunyaviruses from all vertebrate-infecting genera induce antibodies against the nucleoprotein (*12–14*), we are confident about our choice of antigen in our assays. Chung et al. presented no serologic results to support virus detections (*9*).

Several technical issues in the study by Chung et al. should be clarified further. First, RNA concentration in tissue, as determined by RT-PCR, did not correlate with the success of probe-based immunohistochemistry in several organ samples (*9*). Second, supernatants from the virus isolate from South Korea showed high cytopathogenic activity in cell culture (10^3–10^5 cytopathogenic units/mL) but low levels of concomitant viral RNA by RT-PCR. Because no antigen detection in cells was attempted, the cytopathogenic effect could have been caused by any other virus blindly isolated. One of the most infectious and deadly

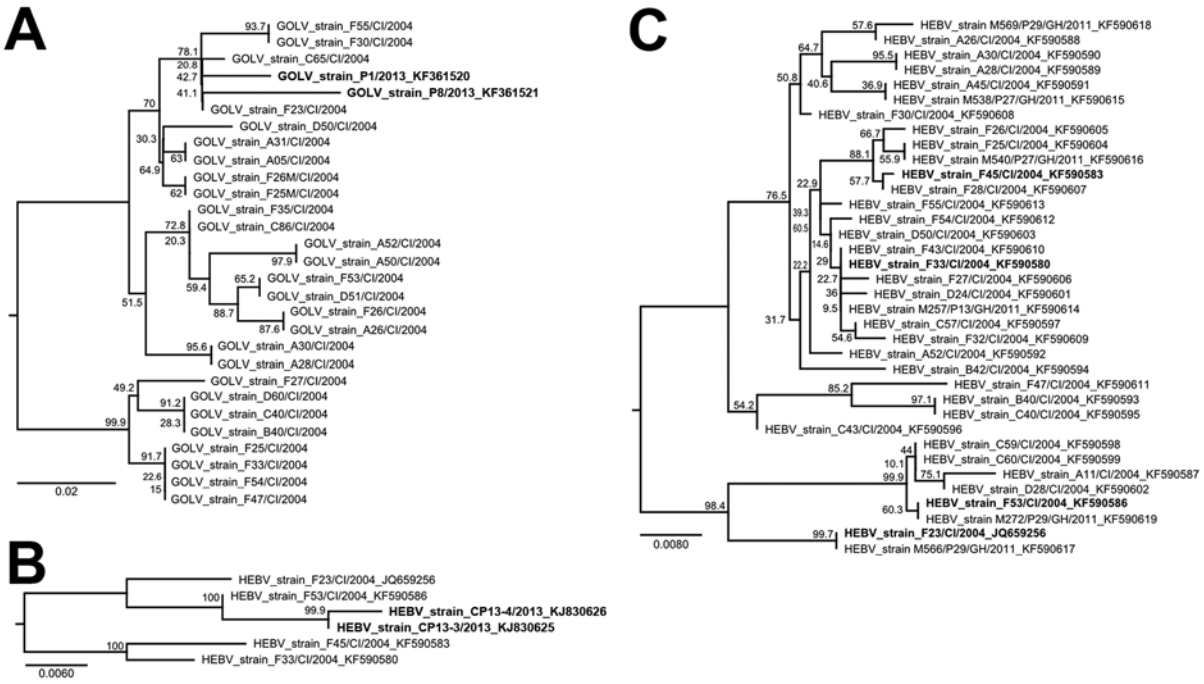

Figure 2. Maximum-likelihood phylogenetic analyses of Gouléako virus (GOLV) and Herbert virus (HEBV) strains from mosquitoes in Côte d'Ivoire, 2004, and Ghana, 2011, and virus strains detected by Chung el al. (*9*) in pigs in South Korea. A) Analysis of the glycoprotein precursor gene of GOLV strains identified in mosquitoes collected in Côte d'Ivoire and Ghana and of strains detected in swine in South Korea. Sequences originating from swine are shown in bold. B) Analysis of the RNA-dependent RNA polymerase gene of HEBV strains from mosquitoes and swine. Sequences originating from swine are shown in bold. C) Analysis of all identified HEBV strains found in mosquitoes. HEBV strains used for phylogenetic analyses in panel B are shown in bold. GOLV strains F25M/CI/2004 and F26M/CI/2004 were found in male mosquitoes. Scale bars indicate nucleotide substitutions per position in the alignment.

swine pathogens, the porcine reproductive and respiratory syndrome virus (*15*), was co-detected in lung samples of dead pigs in South Korea (*9*).

The finding of genome fragments of GOLV and HEBV in swine in South Korea needs to be more fully explored. However, with no further independent proof of infection of swine or other vertebrates, HEBV and GOLV should not be considered epizootic pathogens or arboviruses.

Acknowledgments

We thank Pascal Trippner for technical assistance and the Max Planck Society for providing field work assistance in Côte d'Ivoire.

The project was supported by the Deutsche Forschungsgemeinschaft (grant agreement no. DR 772/12-1) to C.D.

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Oropharyngeal Tularemia Outbreak Associated with Drinking Contaminated Tap Water, Turkey, July–September 2013

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In 2013, an oropharyngeal tularemia outbreak in Turkey affected 55 persons. Drinking tap water during the likely exposure period was significantly associated with illness (attack rate 27% vs. 11% among non–tap water drinkers). Findings showed the tap water source had been contaminated by surface water, and the chlorination device malfunctioned.

Tularemia, a severe epizootic disease caused by the gram-negative, intracellular coccobacillus *Francisella tularensis* (*1*,*2*), has 5 clinical forms: glandular/ulceroglandular, oculoglandular, pneumonic, typhoidal, and oropharyngeal (*2*). Oropharyngeal tularemia is caused by ingesting water or food contaminated with *F. tularensis*; the incubation period ranges from 1 to 14 days (*2*,*3*). Symptoms include sore throat, mouth ulcers, tonsillitis, and swollen lymph nodes in the neck.

Tularemia was first reported in Turkey in 1936. Subsequently, small outbreaks and sporadic cases have been reported, most of which were thought to be waterborne (*4*). In summer 2013, an outbreak of oropharyngeal tularemia occurred in a village in northeastern Turkey. We investigated the outbreak to identify the source of infection and mode of transmission.

The Study

On August 19, 2013, two persons from Sancaktepe Village, Turkey, sought care for influenza-like symptoms, tonsillitis, and swollen neck lymph nodes. Both patients tested positive for *F. tularensis* by blood microagglutination test. Clinicians treating the patients did not perform lymph node biopsies or conduct PCR testing of blood specimens to identify *F. tularensis*. Over the following weeks, dozens more patients were identified from the same village.

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DOI: http://dx.doi.org/10.3201/eid2112.142032

We conducted an investigation to identify potential exposures leading to *F. tularensis* infection (of any clinical form) among Sancaktepe Village residents. We defined a suspected case as onset of ≥1 specific symptoms (swollen lymph nodes in the neck or periauricular areas, sore throat, or swelling or redness of eyes) or ≥2 nonspecific symptoms (fever, chills, myalgia, or headache) during July 1–August 1, 2013. A probable case was onset of swollen lymph nodes plus sore throat or fever. A confirmed case was a suspected or probable case with a positive serologic test result.

We used the microagglutination test to detect *F. tularensis*–specific antibodies in patients' blood; a titer ≥1:160 was the cut-off (*2*). Because of inadequate laboratory capacity to handle heavily polluted water, we used culture, but not PCR, to identify *F. tularensis* in implicated environmental samples.

Of 350 Sancaktepe Village residents, we excluded 46 who were absent during Ramadan 2013, the likely exposure period (explained in the next paragraph). From the remaining 304 residents, we identified 122 suspected case-patients, of whom 94 underwent blood microagglutination testing; 39 were positive (titers 1:160–1:2,560) for *F. tularensis*. No patient had a 4-fold rise in antibody titers between acute and convalescent phases of illness. On the basis of symptoms, we identified 16 additional probable cases among suspected case-patients who were not tested (7/13) or who had a negative microagglutination test result (9/24). The 55 confirmed or probable cases/case-patients are henceforth referred to as cases/case-patients.

The outbreak began on July 9, peaked in late July, and ended in early September 2013. Of the 304 residents, 55 (18%) were infected. The epidemic curve indicated a continuous common-source exposure and a likely exposure period that roughly coincided with Ramadan 2013 (July 8–August 7) (Figure). Cases occurred in all age groups (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/14-2032-Techapp1.pdf) and village-wide. The attack rate did not differ significantly by sex: 18 (13%) of 137 male residents and 37 (22%) of 167 female residents were infected (relative risk [RR] 1.6, 95% CI 0.93–2.6). Clinical signs and symptoms included influenza-like symptoms and swollen lymph nodes in the neck or preauricular regions (online Technical Appendix Table 2).

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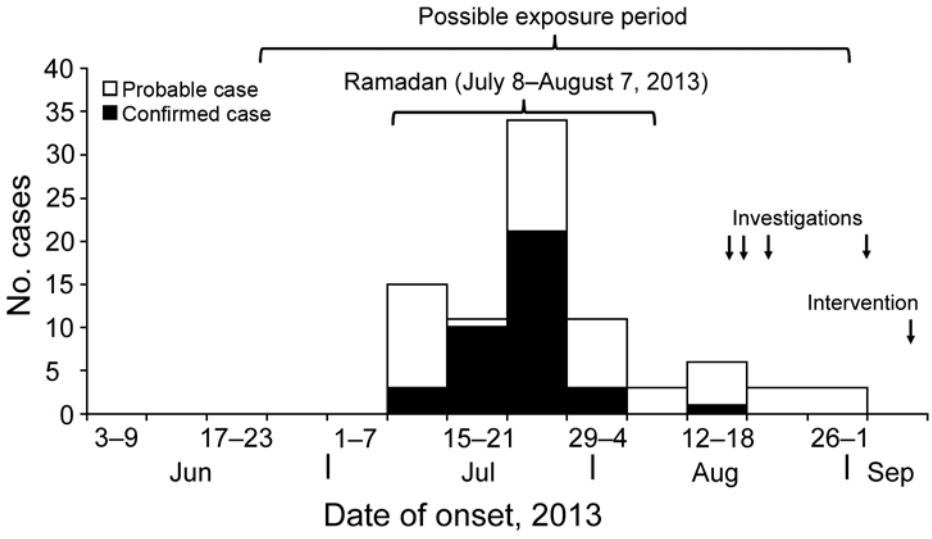

Figure. Epidemic curve indicating a continuous common-source exposure leading to an outbreak of oropharyngeal tularemia, Sancaktepe Village, Turkey, July–September 2013.

We hypothesized that the outbreak was caused by waterborne bacteria for 3 reasons: case-patients predominantly had oropharangeal symptoms; case-patients' age and geographic distributions suggested a ubiquitous exposure; and villagers reported that the tap water had a dead-animal smell during Ramadan. A retrospective cohort study (excluding 46 persons with sore throat or swollen lymph nodes but not meeting definitions for probable or confirmed case-patients) showed that illness developed in 27% (46/173) of persons who drank tap water versus 11% (9/85) of persons who did not (RR 2.5, 95% CI 1.3–4.9). Other types of water were not associated with illness (Table). Sensitivity analyses showed slightly stronger associations between drinking tap water and illness when only confirmed cases (RR 3.4, 95% CI 1.4–8.4) or cases with onset during the week of July 22 (RR 3.0, 95% CI 1.1–8.4) were included.

We asked villagers whether they had engaged in game hunting or eaten game meat around Ramadan; no villagers had such exposures. In addition, according to the village administrator, no large, village-wide gathering had occurred around Ramadan. Inspection of the village's main water storage tank revealed that the solar-powered chlorination device had malfunctioned. Water collected on August 22 had a chlorine level of 0 and elevated levels of total coliform (60 colony-forming units [CFUs]) and *Escherichia coli* (1 CFU). The main water storage tank was supplied by 2 water collection sites, A and B. Water from site A had unremarkable findings and low turbidity. Collection site B had 3 sources of water, 1 of which was surface water. A water sample from site B had high turbidity and contained a visible insect. Rodent activities, but not dead animals, were evident near the surface water ditch. Meteorologic data showed a lack of precipitation in this area for months. Water samples collected from site B on August 22 had high levels of total coliform (>100 CFU) and *E. coli* (50 CFU) (online Technical Appendix Figure). Culture of 2 water samples collected on August 28 and September 4, respectively, did not yield *F. tularensis*.

More than 300 wild and domestic animals worldwide have been found to be naturally infected with *F. tularensis* (*1*). *F. tularensis* subsp. *holarctica*, the only known disease-causing subspecies in Eurasia (*5*), is associated with water-associated rodents (e.g., beavers, muskrats). Humans can be infected with this subspecies by drinking contaminated water; having contact with contaminated streams, lakes, or rivers; having direct contact with contaminated objects (*1*,*2*); or eating uncooked contaminated food (*6*).

Tularemia surveillance in Turkey reported 4,827 tularemia cases nationwide during 2005–2011; contaminated water was presumed to have caused most cases, especially in rural areas (*4*). *F. tularensis* subsp. *holarctica*

Table. Risk for acquiring oropharyngeal tularemia among persons who drank water from different sources, Sancaktepe Village, Turkey, July–August 2013*

Source of water consumed	No. cases/total no. exposed (%)	No. cases/total no. not exposed (%)	Relative risk (95% CI)
Tap	46/173 (27)	9/85 (11)	2.5 (1.3–4.9)
Well	2/8 (25)	53/250 (21)	1.2 (0.35–4.00)
Underground spring	25/136 (18)	30/122 (25)	0.75 (0.47–1.2)
Bottled	5/31 (16)	50/227 (22)	0.73 (0.32–1.70)
Other	2/8 (25)	53/250 (21)	1.20 (0.35–4.00)

*The outbreak was associated with Ramadan, which occurred during July 8–August 7, 2013.

has been isolated from drinking water sources in places where tularemia outbreaks occurred (*7*). The bacteria presumably came from dead animals; a single infected water animal (e.g., vole, lemming, or mouse) can contaminate up to 500,000 L of water (*1*), and *F. tularensis* can survive in untreated water for months (*2*). Free available chlorine residual concentrations routinely maintained in tap water systems can reduce *F. tularensis* by 4 $\log_{10}$ in 2 hours (*8*). However, the malfunction of the chlorination device at Sancaktepe Village's main water storage tank enabled survival of the bacteria.

Our study had several limitations. *F. tularensis* was not isolated from water. *Francisella* species are fastidious and slow-growing and can be easily overwhelmed by competing organisms in environmental samples during culture (*9*). In addition, water samples were collected during late August–early September; by that time, the bacteria might have been cleared from the water. We spotted rodent activities, but no dead animals, near the implicated water source. The imperfect case definitions and potential subclinical infections in asymptomatic villagers might have led to misclassification, which tends to bias the association toward null; in other words, the observed association would have been stronger had there been no such bias, as evidenced by the sensitivity analysis that used laboratory-confirmed cases only.

Conclusions

This tularemia outbreak in northeastern Turkey was associated with drinking contaminated tap water. At our recommendation, the village administrator cut off the surface water source, repaired the chlorination device, and started checking chlorine levels regularly. No new outbreaks have subsequently occurred.

Acknowledgments

We are appreciative of staff at the Bayburt Provincial Public Health Directorate for their support and assistance with this study. We are also grateful to the 2013 fellows of the Turkey Field Epidemiology Training Program for their assistance with data collection and entry.

This study was supported by the EU project Surveillance and Control of Communicable Diseases (TR0802.16) and by the Ministry of Health, Public Health Institution of Turkey.

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Asymptomatic MERS-CoV Infection in Humans Possibly Linked to Infected Dromedaries Imported from Oman to United Arab Emirates, May 2015

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In May 2015 in United Arab Emirates, asymptomatic Middle East respiratory syndrome coronavirus infection was identified through active case finding in 2 men with exposure to infected dromedaries. Epidemiologic and virologic findings suggested zoonotic transmission. Genetic sequences for viruses from the men and camels were similar to those for viruses recently detected in other countries.

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) was first detected in humans in 2012 (*1*). Before 2015, most human infections occurred on the Arabian Peninsula. However, the recent occurrence of MERS in South Korea indicates that this pathogen can cause major outbreaks in other regions (*2*). Dromedaries are believed to be a source of MERS-CoV (*3*,*4*), but only a few case reports provide virologic and epidemiologic evidence that directly supports zoonotic transmission of the virus from dromedaries to humans (*5*–*7*). We report the detection of epidemiologically linked MERS-CoV infection in 2 men who had direct contact with infected dromedaries (*8*,*9*).

The Study

A 29-year-old man (contact 1) transported 8 dromedaries from Oman to United Arab Emirates on May 7, 2015 (Table 1). The same day, as part of a national policy for controlling MERS, samples were collected from the dromedaries at a screening center located at the United Arab Emirates border. The samples were tested by reverse transcription PCR (RT-PCR) on May 10 and found to be positive for the MERS-CoV open reading frame (ORF) 1A and upstream of E genes (*10*). This finding led local public health authorities to conduct active surveillance on humans who had contact with the infected dromedaries.

A sputum sample collected from contact 1 on May 10, 2015, was tested by RT-PCR on May 12 and found to be positive for MERS-CoV; the man was admitted to a hospital the same day. Follow-up respiratory samples obtained on May 13 and 14 were still RT-PCR–positive, but a sample obtained on May 18 was negative. The patient was asymptomatic at hospital admission and throughout his hospital stay (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/12/15-1132-Techapp1.pdf).

Contact 2 was a 33-year-old man who worked at the screening center mentioned above. He had direct contact with the same group of infected dromedaries during the sampling procedures. A nasal aspirate sample was obtained from the man on May 14, 2015, and found to be RT-PCR positive for MERS-CoV. Contact 2 was hospitalized on May 18. A follow-up sample obtained on May 18 was RT-PCR negative for MERS-CoV. Contact 2 was asymptomatic throughout his hospitalization (online Technical Appendix).

Samples from 32 other persons were also tested by RT-PCR (online Technical Appendix). None tested positive.

After the initial positive test results, the dromedaries were quarantined. Seven days later (May 14), follow-up nasal swab samples from 5 dromedaries were still positive by RT-PCR (Table 2); the animals also had mucopurulent nasal discharge. The animals were tested for the presence of MERS-CoV–specific neutralizing antibodies (*11*); all were seropositive. Two 4-month-old calves (ADFCA-HKU1 and ADFCA-HKU2) had the highest virus loads by real-time RT-PCR and the lowest neutralizing antibody titers (Table 2). Nasal swab samples from these 2 dromedaries were also MERS-CoV–positive by rapid antigen testing (*12*), which suggests the calves were still shedding virus 7 days after the first detection of v irus. Virus culture was not attempted. On May 25, 2015, the 2 calves were RT-PCR negative for MERS-CoV, and the whole group of camels was released from quarantine.

Respiratory specimens from the 2 infected humans and the 5 dromedaries that were still positive at the second

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DOI: http://dx.doi.org/10.3201/eid2112.151132

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Table 1. A chronology of major events in a study of asymptomatic MERS-CoV infection in 2 humans after direct contact with infected dromedary camels imported from Oman to United Arab Emirates, May 2015*

Date, May 2015	Event
7	Contact 1 transported 8 dromedaries from Oman to the United Arab Emirates border. Contact 2 had direct contact with the dromedaries during sampling procedures at the camel screening center at the border. All 8 dromedaries were quarantined until test results were available on May 10.
10	All 8 dromedaries were found to be RT-PCR positive for MERS-CoV and were quarantined in a separate structure located at the same border location. Active surveillance of persons with direct or indirect contact with the infected dromedaries was initiated. A sputum sample was obtained from contact 1; it tested positive for MER-CoV by RT-PCR on 12 May, 2015.
12	A sample obtained from contact 1 on May 10 tested positive for MERS-CoV by RT-PCR; contact 1 was hospitalized in a negative-pressure room.
13	A follow-up sample was obtained from contact 1, and it tested positive for MERS by RT-PCR.†
14	A follow-up sample was obtained from contact 1, and it tested positive for MERS by RT-PCR. A nasal aspirate sample was obtained from contact 2; it tested positive for MERS by RT-PCR on May 17.† Samples were obtained from the infected dromedaries, and 5 were still MERS-CoV–positive by RT-PCR (Table 2).†
17	A sample obtained from contact 2 on May 14 tested positive for MERS-CoV by RT-PCR.
18	Contact 2 was admitted to a negative-pressure room in the same hospital as contact 1. Follow-up samples were obtained from contacts 1 and 2, and they tested negative for MERS-CoV by RT-PCR.
20	A follow-up sample was obtained from contact 2, and it tested negative for MERS-CoV by RT-PCR.
21	A follow-up sample was obtained from contact 2, and it tested negative for MERS-CoV by RT-PCR.
25	Follow-up samples from the 5 dromedaries tested negative for MERS-CoV by RT-PCR. All dromedaries were released from quarantine.
End of month‡	Contacts 1 and 2 were released uneventfully from the hospital.

*Contacts 1 and 2, humans who had direct physical contact with infected dromedaries; MERS-CoV, Middle East respiratory syndrome; RT-PCR, reverse transcription PCR.
†Samples subjected to sequencing analyses.
‡Exact date unknown.

sampling were analyzed by dideoxy sequencing as previously described (*13*); the nucleocapsid gene sequences of all dromedary samples were found to be identical. Samples from dromedaries ADFCA-HKU1–3 were selected for further analysis, and a sequence contig encompassing the 3′ end of the ORF1AB gene through the 3′ untranslated region of the MERS-CoV genome (≈8,900 nt; sequence coverage 4) was obtained from each sample. Contigs from the 3 samples were identical, with the exception of a V221I (GTT→ATT) mutation in the ORF4b protein of the sample from dromedary ADFCA-HKU2. The viral RNA content of the 2 human samples available for analysis was too low to provide long PCR amplicons (cycle threshold 35.5 and 36.9 by upstream of E gene assay). However, partial sequences of MERS-CoV spike (466 nt, contacts 1 and 2), ORF3–4a (273 nt, contact 1), and nucleocapsid (451 nt, contacts 1 and 2) gene regions could be detected from the samples. All of these sequences were identical to those deduced from the dromedary specimens. Genomic sequences determined from this study were submitted to GenBank (accession nos. KT275306–KT275315).

The 3 sequence contigs obtained from the dromedary samples were phylogenetically closely related to those of viruses detected in humans in the Saudi Arabia, China, and South Korea in 2015 (Figure). All sequences from this cluster, together with the partial ORF3–4a sequence detected in the sample from contact 1, shared 2 cluster-specific mutations, 79S (TCA→TCT) and P86L (CCT→CTT), in the ORF3 protein, suggesting that these viruses may share a common lineage. Apart from the unique V221I mutation, the sequences for viruses from the 3 dromedaries shared a unique ORF4a-Q102E (GAG→CAG) mutation that was not found in any published MERS-CoV genomes. Other than those mutations,

Table 2. Demographic data and clinical test results for MERS-CoV–infected dromedary camels imported from Oman to United Arab Emirates, May 2015*

				Test results		
Camel ID	Age/sex	Purpose of importation	Mucopurulent nasal discharge†	RT-PCR (C_t)†‡	Rapid antigen test†§	Serum neutralizing antibody titer†¶
ADFCA-HKU1	4 m/F	Breeding	Moderate	Pos (24.54)	Pos	1:40
ADFCA-HKU2	4 m/F	Breeding	Moderate	Pos (27.59)	Pos	1:40
ADFCA-HKU3	4 m/F	Breeding	Moderate	Pos (28.82)	Neg	1:80
ADFCA-HKU4	7 m/F	Breeding	Moderate	Pos (29.81)	Neg	1:80
ADFCA-HKU5	10 y/F	Breeding	Mild	Pos (30.05)	Neg	1:160

*C_t, cycle threshold; ID, identification; MERS-CoV, Middle East respiratory syndrome; Neg, negative; Pos, positive; RT-PCR, reverse transcription PCR.
†Observations or samples from the second sampling on May 14, 2015.
‡Results were determined by open reading frame 1A and upstream of E gene RT-PCR assays; C_t values were from the open reading frame 1A assay. C_t values from both assays were comparable (data not shown).
§Results were determined by immunochromatographic tests for MERS-CoV nucleocapsid protein (*12*).
¶Results were determined by pseudoparticle neutralization assays (*11*).

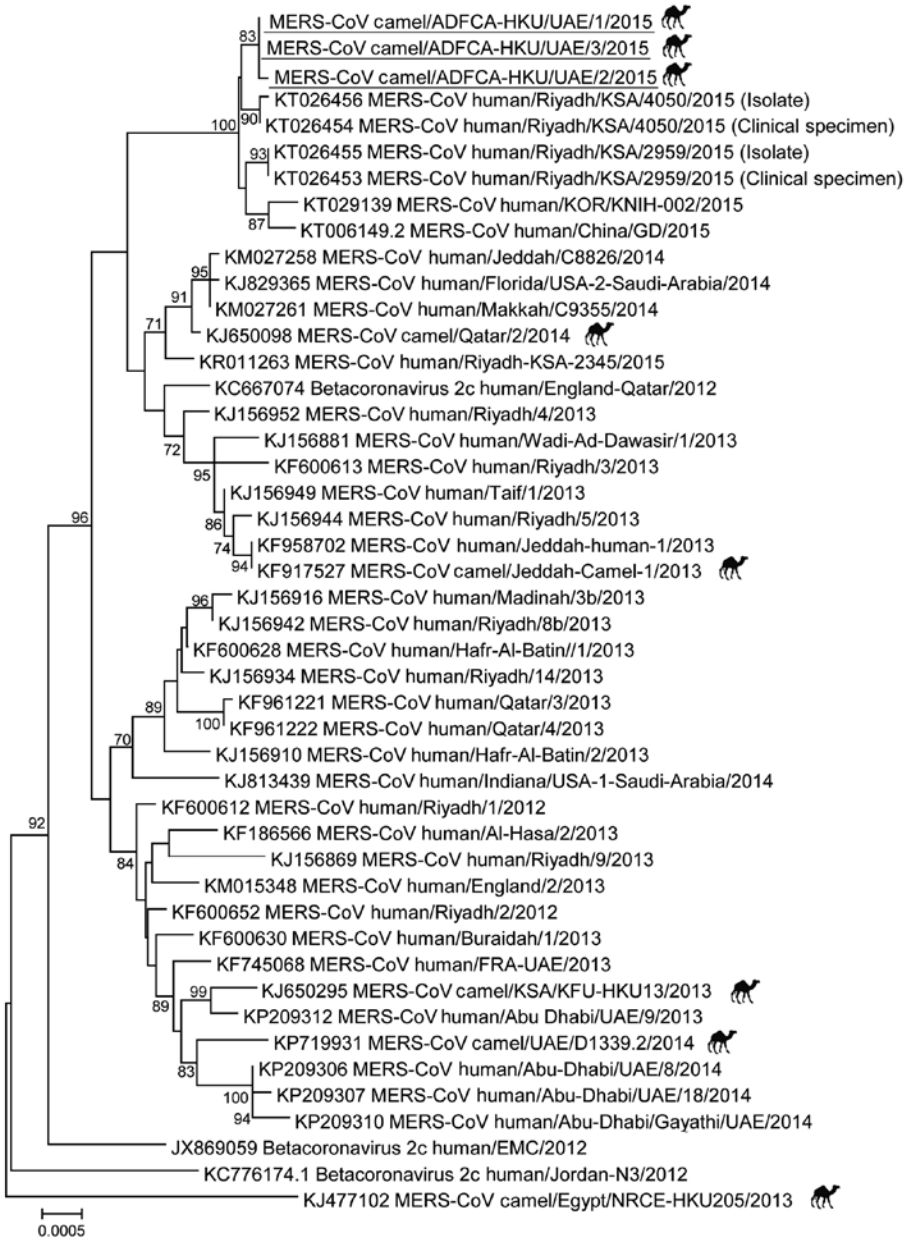

Figure. Phylogenetic analyses of partial Middle East respiratory syndrome coronavirus (MERS-CoV) genomic sequences for viruses detected in dromedaries imported from Oman to United Arab Emirates, May 2015. A partial viral RNA sequence spanning the 3′ end of the open reading frame 1AB gene through the 3′ untranslated region of the MERS-CoV genome (≈8,900 nt) was used in the analysis. The phylogenetic tree was constructed with MEGA6 software (http://www.megasoftware.net/) by using the neighbor-joining method. Numbers at nodes indicate bootstrap values determined by 1,000 replicates. Only bootstrap values >70 are denoted. Underlining indicates sequences for viruses detected in this study. GenBank accession numbers are shown for published sequences. Symbols indicate MERS-CoVs detected from dromedaries. Scale bar indicates the estimated genetic distance of these viruses.

all of the ORFs (nonstructural protein 13, spike, ORF3, ORF5, envelope, membrane, nucleocapsid, and ORF8b) of these virus sequences were unremarkable.

Conclusions

We report 2 cases of MERS-CoV infection in men who had direct contact with the same group of infected dromedaries. Neither man had a concurrent medical condition or a history of exposure to human MERS cases in the 14 days before their first MERS-CoV–positive test results. Genomic sequences for the viruses derived from the men and dromedaries and findings from the epidemiologic investigation suggest possible zoonotic transmission of MERS-CoV from dromedaries to humans. Although it is unlikely, we cannot exclude the possibility that the men and dromedaries were independently infected by other sources.

Both infected humans were kept in the hospital for ≈2 incubation periods and were asymptomatic during this period. Clinical observations and positive RT-PCR results suggest that the men were asymptomatically infected with MERS-CoV. Asymptomatic infections have been detected previously (*14*). Our findings provide further evidence that asymptomatic human infections can be caused by zoonotic transmission. It is not clear whether asymptomatic infection can lead to transmission between humans. Nonetheless, our findings highlight the importance of systematic surveillance

of persons who have frequent contact with dromedaries. A recent study demonstrated that persons who have frequent exposure to camels are more likely than the general population to be seropositive for MERS-CoV (*4*). The unique border screening program and multisectoral collaborations highlighted in this investigation serve as a model for effective MERS-CoV surveillance at the animal–human interface.

Our study had some limitations. We did not test serum samples from the human contacts; such testing would be of interest for follow-up investigation of the patients' serologic responses. We also obtained limited RNA samples from these persons, which prevented us from conducting more extensive viral sequence analyses.

MERS-CoV genomic sequences determined in this study are similar to those of viruses detected in 2015 in patients in Saudi Arabia and South Korea with hospital-acquired infections. The infected dromedaries in this study were imported from Oman, which suggests that viruses from this clade are widely circulating on the Arabian Peninsula. Sequence analyses of MERS-CoVs found in South Korea and China do not suggest that viruses from this clade are necessarily more transmissible variants (*15*). However, given that a single introduction of MERS-CoV from this clade caused >180 human infections in hospital settings (*2*) and that viruses of this clade are causing other human infections in Saudi Arabia, further phenotypic risk assessment of this particular MERS-CoV clade should be a priority.

Acknowledgment

We acknowledge the team from Health Authority Abu Dhabi, Abu Dhabi Health Services Company, that supported the epidemiologic investigation, including the laboratory team in Sheikh Khalifa Medical City.

The sequence analyses and serologic work were partly funded by a research contract from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (contract no. N272201400006C), and by a grant from Area of Excellence Scheme, University Grants Committee of the Hong Kong Special Administrative Region (no. AoE/M-12/06).

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Aquatic Bird Bornavirus 1 in Wild Geese, Denmark

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To investigate aquatic bird bornavirus 1 in Europe, we examined 333 brains from hunter-killed geese in Denmark in 2014. Seven samples were positive by reverse transcription PCR and were 98.2%–99.8% identical; they were also 97.4%–98.1% identical to reference strains of aquatic bird bornavirus 1 from geese in North America.

Avian bornaviruses were first identified as the probable causative agent of proventricular dilatation disease in parrots in 2008 (*1*,*2*). Eight psittacine viruses and 5 passerine viruses have since been described (*3*,*4*). In 2009, aquatic bird bornavirus 1 was detected in free-ranging Canada geese (*Branta canadensis*) and trumpeter swans (*Cygnus buccinator*) in Ontario, Canada (*5*). Subsequently, this bornavirus has been detected across North America in at least 15 species of free-ranging wild birds (*6*,*7*). Initially designated as ABV-CG due to the high prevalence in Canada geese, the virus has been renamed aquatic bird bornavirus 1 (ABBV-1) in a reorganization of the taxonomy of the *Bornaviridae* (*4*). A second waterfowl-associated virus (ABBV-2) was isolated from ducks in North America in 2014 (*8*). Despite the fact that North American and European waterfowl are known to share breeding grounds in the Arctic, avian bornaviruses had not been detected in wild birds outside North America. The purpose of this study was to investigate the presence of aquatic bird bornavirus 1 in wild waterfowl in Denmark.

The Study

By using real-time reverse transcription PCR (RT-PCR), we screened brain tissue from 333 hunter-killed geese from 9 locations in Denmark (Table; online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/12/15-0650-Techapp1.pdf), collected during November and December 2014. Each screened sample consisted of pooled tissue from 5 individual birds, of the same species, collected at the same location. Primers and probe targeting the matrix (M) gene specific for ABBV-1 (*9*) were used. RNA was purified from 35 mg of pooled or individual brain samples with the QIAGEN RNeasy Mini Kit (QIAGEN, Copenhagen, Denmark), according to instructions from the supplier. Each PCR reaction contained 5 μL RNA; 1× RT-PCR buffer (AgPath-ID One-Step RT-PCR Kit; Life Technologies, Naerum, Denmark); 0.5 μmol/L of each primer, 0.25 μmol/L FAM-BHQ-1 labeled probe; and 1× RT-PCR enzyme mix in a total volume of 25 μL. The reactions were run on Rotor-Gene Q (QIAGEN) at 45°C, for 600 s, 95°C for 600 s, followed by 45 cycles of 94°C for 5 s and 60°C for 60 s. Data were analyzed with Rotor-Gene Q Series Software version 2.3.1 (QIAGEN). Parameters were adjusted as follows: dynamic tube, on; slope correct, on; ignore first cycle, 1; outlier removal, 10%; threshold fixed, 0.01. All other settings were default.

Samples from birds in positive pools were purified and tested individually by quantitative -PCR and confirmed by endpoint conventional RT-PCR with a 2.200-bp amplicon covering the nucleocapsid (N), X protein, phosphoprotein (P), and partial M genes. Primers were previously published (*7*). Each reaction contained 1× buffer, 1.2 μmol/L of each primer, 0.4 μmol/L dNTP mix, 0.4 μmol/L enzyme mix (QIAGEN OneStep RT-PCR Kit; QIAGEN), and 5 μL purified RNA, in a total volume of 25 μL. Amplification was performed on a T3 PCR machine (Biometra, Fredensborg, Denmark) with cycling conditions 30 min at 50°C, 15 min at 95°C, and 120 s at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 150 s at 68°C, and a final elongation at 68°C. Products were analyzed on 0.8% agarose E-Gels (Invitrogen, Naerum, Denmark) and verified by Sanger sequencing (LGC Genomics, GmbH, Berlin, Germany) with primers previously published (*1*) and the following primers: 5′-CAGCTCCAGTAAGGTGAGTTG-3′, 5′-CGCCGACTAGTGGACAGCCC-3′, and 5′-CTGCGGCATTCTACTGGAG-3′. Sequence data were edited with CLC Main Workbench 7.0 (CLC bio, QIAGEN, Aarhus, Denmark).

Seven ABBV-1–positive brain samples from individual birds were identified (Table). These samples were from 3 species of geese originating in 6 of 9 locations sampled. The N, X, P, and partial M fragment sequences were aligned, and a neighbor-joining tree with 1,000 bootstrap replicates was constructed with the CLC software and edited by using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/) (Figure). The European sequences from this study

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DOI: http://dx.doi.org/10.3201/eid2112.150650

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Table. Number and species of wild geese tested for ABBV-1 in 9 locations, Denmark, 2014*

Species	No. positive/no. tested (%)									
	Randbøl	Wadden Sea	Lolland	Skelby	Værnengene	Mandø	Møn	Skjern Enge	Western coast of Jutland	All locations
Greylag goose	0/8	1†/37 (2.7)	1‡/41 (2.4)	0/2	0/3	–	1§/10 (1)	1¶/28 (3.6)	1#/6 (16.7)	5/135 (3.7)
Pink-footed goose	–	–	–	0/1	0/8	–	–	1**/41 (2.4)	0/53	1/103 (1.0)
Barnacle goose	–	–	–	0/3	–	1††/47 (2.1)	–	–	–	1/50 (0.2)
Taiga bean goose	–	–	0/7	–	–	–	–	–	–	0/7
Tundra bean goose	–	–	0/7	0/1	–	–	–	–	–	0/8
White-fronted goose	–	–	0/6	0/1	–	–	–	0/1	0/8	0/16
Canada goose	–	–	–	–	–	–	0/13	–	–	0/13
Hybrid	–	–	–	–	–	–	0/1	–	–	0/1
All species	0/8	1/37 (2.7)	1/61 (1.6)	0/8	0/11	1/47 (2.1)	1/24 (4.1)	2/70 (2.9)	1/67 (1.5)	7/333 (2.1)

*ABBV-1, aquatic bird bornavirus 1. Dashes indicate none tested.
†GenBank accession no. KR075030.
‡GenBank accession no. KR075031.
§GenBank accession no. KR075033.
¶GenBank accession no. KR075035.
#GenBank accession no. KR075036.
**GenBank accession no. KR075034.
†† GenBank accession no. KR075032.

clustered together with North American ABBV-1, and a pairwise comparison in CLC software showed 98.2% to 99.8% identity among the 7 sequences and 97.4% to 98.1% identity to the reference strain of ABBV-1 (GenBank accession no. KF578398).

The 2.1% (95% CI 0.6%–3.6%) prevalence of ABBV-1 in wild geese in Denmark is considerably lower than the prevalences reported in North America, which average 10%–30% but in some studies have exceeded 50% (*6*,*7*,*9*,*10*). In 1 study, the prevalence of ABBV-1 was higher in stable nonmigrating populations of Canada geese than in migratory birds, suggesting that prevalence may vary with population density and intensity or duration of use of geographic locations. In the investigation described here,

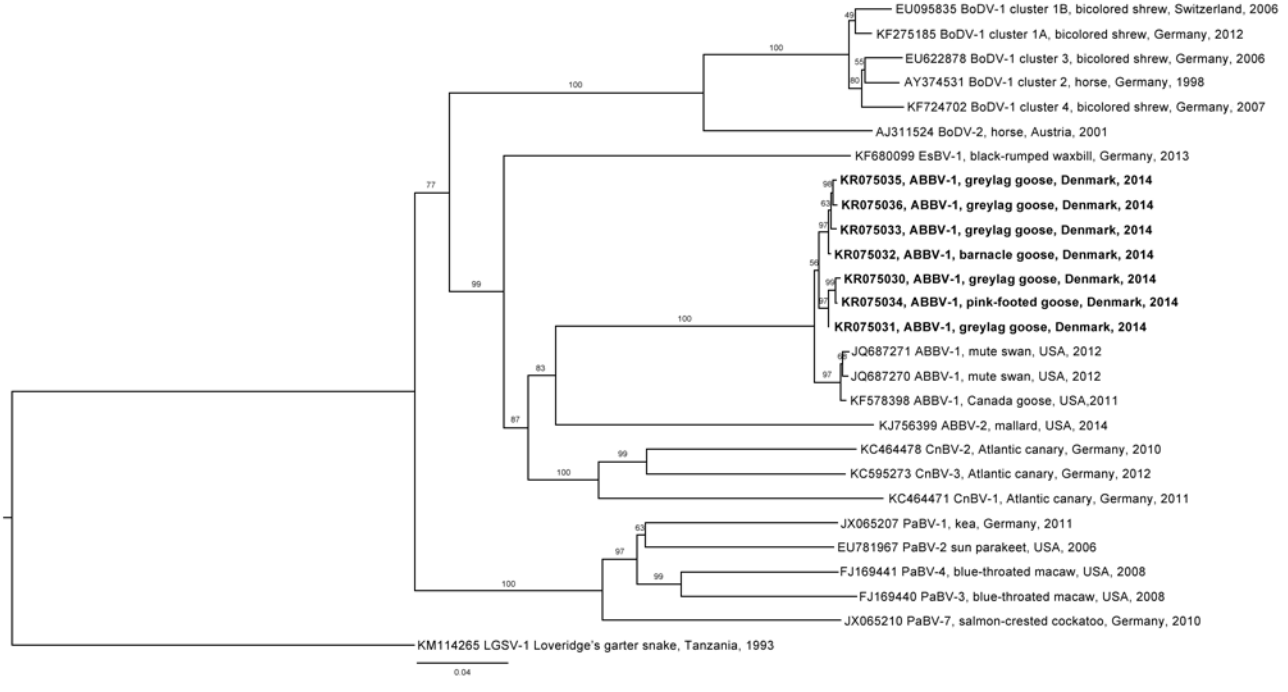

Figure. Phylogenetic tree comparing aquatic bird bornavirus 1 sequences obtained from waterfowl in Europe with selected bornavirus sequences from GenBank. Bold indicates viruses isolated in this study. Numbers along branches indicate bootstrap values. Scale bar indicates nucleotide substitutions per site.

we surveyed only a limited number of species previously identified as positive for ABBV-1 but identified 3 novel host species. Thus, the low prevalence found in geese in Denmark might be due to the sampling of a high proportion of transient migratory and apparently healthy birds as well as a possible variation in species susceptibility.

The finding of ABBV-1 in migratory waterfowl in Denmark suggests that the virus is widespread in waterfowl populations in Europe, but further investigation is needed to verify this claim. Pink-footed geese (*Anser brachyrhynchus*) and greylag geese (*A. anser*) migrate through the western part of Europe from Svalbard, Norway, to Spain. Barnacle geese (*A. leucopsis*) migrate from wintering grounds in the Wadden Sea area to northern Scandinavia and Russia. Because migrating waterfowl often gather in flocks of mixed species, transmission of pathogens between species is possible, and even likely, on the basis of our findings of nearly identical (99.7%) sequences in 1 pink-footed goose and in 1 greylag goose. The origin of ABBV-1 cannot be determined from this study, but the presence of highly homologous viruses in North America and Europe promotes speculation on possible transmission routes between these continents. Avian populations in Greenland could be the link between American and European flocks; the country is host to large breeding populations of geese that winter in both North America and in Europe.

The results here do not allow the clinical implications of ABBV-1 infections in waterfowl in Denmark to be determined, because none of the sampled geese were reported to be ill, and only goose heads were examined in the study. In North America, birds infected with ABBV-1 have exhibited nonsuppurative inflammation of the central, peripheral and autonomous nervous systems and associated neurologic and gastrointestinal clinical signs, including proventricular stasis.

Conclusions

This study identifies ABBV-1 in wild geese in Europe; phylogenetic analyses demonstrated that the sequences from our investigation cluster with those from North America in the waterbird-1 cluster. The barnacle goose, greylag goose, and pink-footed goose were added to the list of waterfowl known to be hosts of ABBV-1. On the basis of the migration patterns of the affected species, we propose that the virus is distributed widely in Europe, but further investigation is needed to determine the validity of this hypothesis.

Acknowledgments

We thank Davor Ojkic for assistance with PCR development and validation and Sari Mia Dose and Tanja Normann Gade for technical assistance in testing the samples.

Drs. Thomsen and Nielsen recently received their doctor of veterinary medicine degrees from University of Copenhagen, Denmark. Their main interests are avian medicine and pathology, along with molecular detection of disease.

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Life-Threatening Sochi Virus Infections, Russia

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Release date: November 12, 2015; Expiration date: November 12, 2016

Learning Objectives

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

- Analyze the demographics of patients infected with the Sochi virus in the current study
- Assess laboratory data available from patients infected with Sochi virus in the current study
- Distinguish the anatomic site of the highest concentration of Sochi virus among infected individuals
- Evaluate the prognosis of infection with Sochi virus.

CME Editor

Karen L. Foster, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Karen L. Foster has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Clinical Professor of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed the following financial relationships: served as an advisor or consultant for Lundbeck, Inc.; McNeil Pharmaceuticals; Takeda Pharmaceuticals North America, Inc.*

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DOI: http://dx.doi.org/10.3201/eid2112.150891

Sochi virus was recently identified as a new hantavirus genotype carried by the Black Sea field mouse, *Apodemus ponticus*. We evaluated 62 patients in Russia with Sochi virus infection. Most clinical cases were severe, and the case-fatality rate was as high as 14.5%.

Hantaviruses are zoonotic pathogens transmitted from small animals to humans. Hantavirus disease in the Americas is called hantavirus pulmonary syndrome and in Asia and Europe is called hemorrhagic fever with renal syndrome (HFRS). Both syndromes can lead to cardiopulmonary and renal failure (*1*). Recently we described a new hantavirus, Sochi virus, from the administrative region Krasnodar (including the city of Sochi), southern European Russia, which was isolated in cell culture from a Black Sea field mouse (*Apodemus ponticus*) and a

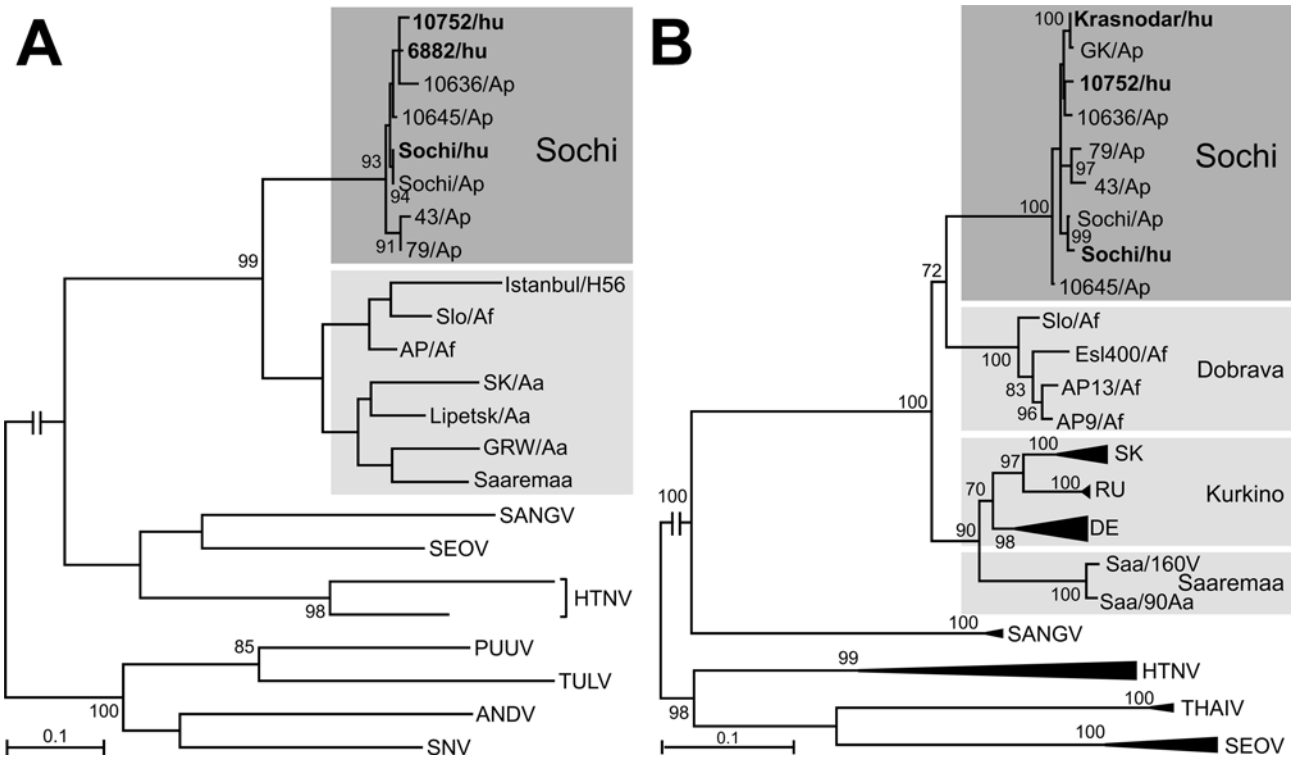

Figure 1. Phylogenetic analysis segment sequences of Sochi virus, Russia: A) 347-bp large (L) segment sequence; B) 1,197-bp small (S) segment sequence. Virus sequences derived from patients (shown in bold type) and *Apodemus ponticus* mice cluster within the Sochi genotype of DOBV. Evolutionary analysis was conducted in MEGA6 (*6*). The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura 3-parameter model with a discrete gamma distribution and 5 rate categories (analysis in panel A) and on the general time reversible model with gamma rates and heterogeneous patterns (analysis in panel B), respectively, which were estimated to be the best-fit substitution model according to the Bayesian information criterion. Scale bars indicate an evolutionary distance of 0.1 substitutions per position in the sequence. Bootstrap values ≥70%, calculated from 500 replicates, are shown at the tree branches. GenBank accession numbers of all sequences used in the analysis are listed in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/12/15-0891-Techapp1.pdf). Dark gray shading iindicates cluster of DOBV-Sochi strains; light gray shading indicates different clusters of strains from other DOBV genotypes. ANDV, Andes virus; DOBV, Dobrava-Belgrade virus; HTNV, Hantaan virus; PUUV, Puumala virus; SANGV, Sangassou virus; SEOV, Seoul virus; SNV, Sin Nombre virus; THAIV, Thailand virus; TULV, Tula virus.

patient with fulminant hantavirus disease who died of shock and combined kidney and lung failure (*2–4*). Molecular taxonomical analyses identified Sochi virus as a new genotype within the Dobrava-Belgrade virus (DOBV) species (*5*). Here we show that HFRS caused by Sochi virus infection occurs in the geographic region where *A. ponticus* mice are prevalent. For 62 patients infected by this virus during 2000–2013, we evaluated clinical and epidemiologic data.

The Study

Serum of patients with suspected acute hantavirus disease from the Krasnodar region were screened for hantavirus antibodies by indirect immunofluorescence assays and ELISA. Sixty-two patients showed clear DOBV IgG seropositivity. During the acute phase of illness, all patients tested positive for DOBV IgM (data not shown). For 26 patients, sufficient volumes of follow-up serum were available for additional focus reduction neutralization assays to specify neutralizing antibodies. All serum samples exhibited substantially higher neutralizing titers toward DOBV than toward Puumala virus, Hantaan virus, and Seoul virus. When the neutralizing effect of DOBV-positive patients' serum were compared against the different human pathogenic genotypes of DOBV (Dobrava, Kurkino, and Sochi), all serum predominantly reacted with the Sochi genotype (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0891-Techapp1.pdf).

We successfully obtained virus genomic large (L) segment sequences from 2 patients (no. 51, specimen no. 6882; no. 59, specimen no. 10752). In the neighborhood

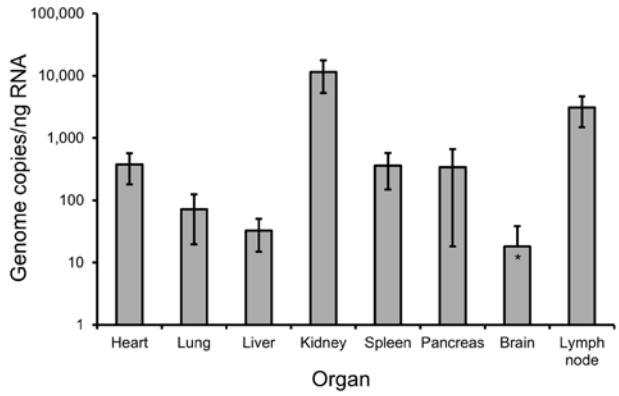

Figure 2. Quantification of hantavirus RNA in tissue biopsies from a 50-year-old Sochi virus–infected man (patient no. 59), Russia. Two independent approaches were performed to extract RNA from each organ. Quantitative reverse transcription PCR previously developed for DOBV (*7*) was used to measure virus load in the analyzed biopsy samples. Three quantitative reverse transcription PCR estimations were conducted for every RNA extraction, followed by calculation of mean values and SDs. Viral RNA levels are shown as genome copies per nanogram of total RNA isolated from the samples. Error bars indicate SD.

of the residence of patient no. 59, mice were trapped, and hantaviral L and small (S) segment regions from 2 *A. ponticus* animals (specimen nos. 10636, 10645) were amplified. The sequences obtained were deposited in GenBank under accession nos. KM192207–09 and KP878308–10 (L segment) and KP878311–13 (S segment) (online Technical Appendix Table 2). Samples from virus-positive mice were phylogenetically characterized by analysis of a 242-bp region of their *cytB* gene; all of them clustered with the previously identified *A. ponticus* animals (*3*) (data not shown). In addition, the *A. ponticus*–derived isolate Sochi/Ap (*4*), the patient-derived isolate Sochi/hu (*5*), an S segment sequence from a mouse (GK/Ap) trapped near the home of the previously described Krasnodar patient (*4*), and sequences originating from 2 *A. ponticus* mice sampled near the Black Sea coast, 43/Ap and 79/Ap, were included in the molecular analyses of the virus.

The patient-derived sequences 6882/hu, 10752/hu, and Sochi/hu clearly cluster with *A. ponticus*–derived sequences 43/Ap, 79/Ap, 10636/Ap, 10645/Ap, and Sochi/Ap (Figure 1, panel A). In the analysis of the S segment, we obtained a very similar result; the patient-derived sequences 10752/hu, Krasnodar/hu, and Sochi/hu cluster with *A. ponticus*–associated sequences 43/Ap, 79/Ap, 10636/Ap, 10645/Ap, GK/Ap, and Sochi/Ap (Figure 1, panel B). In analysis of both L and S segments, the Sochi virus strains form a unique group, clearly distinguishable from all other DOBV genotypes.

Specimens from different organs of deceased patient no. 59 were analyzed for virus load. The highest concentration was detected in kidney (11,446 copies/ng RNA) and lymph node (3,086 copies/ng RNA), whereas the least virus RNA (10–100 copies/ng RNA) was detected in lung, brain, and liver (Figure 2).

The clinical disease severity of the 62 Sochi virus–infected patients investigated (Table 1) was subdivided into mild, moderate, or severe following the standard Russian criteria (i.e., length of febrile phase, minimal blood pressure in the hypotonic phase, extent of hemorrhagic symptoms, minimal urine production, serum creatinine level, and extent of proteinuria) (online Technical Appendix Table 3). The case-fatality rate (CFR) was as high as 14.5% (9/62 patients). Including fatalities, severe disease developed in nearly 60% of patients, whereas the remaining 40% of cases were moderate. The average age of all patients was 33 years. A significantly higher proportion of patients were males ($p = 1.05 \times 10^{-9}$). Moreover, severe disease developed in most affected male patients (66.7%) but in only 35.7% of affected female patients ($p = 0.037$). The fact that only 2 of 9 fatal cases occurred in female patients (Table 1) underscores this finding.

All 9 patients with fatal infections died of multiorgan failure and shock (Table 2). Postmortem examination showed multiple hemorrhages and edema in internal organs, including kidneys and lungs. The patients died within 8.2 days (range 3–16 days) after disease onset. An extraordinary fulminant course was observed for patient no. 47, who died 3 days after onset and before he could be hospitalized. This 19-year-old man was the son-in-law of patient no. 48, who also died after Sochi virus infection. Both men lived at the same rural address, and rodent contact during work in haystacks was reported.

Table 1. Comparisons in clinical outcome, age, and sex of 62 patients with Sochi virus infection, Russia*

	Total		Sex, no. (%)		Age, y, n/N (%)	
Characteristic	No. (%)	Median age, y (range)	M, n = 48	F, n = 14	7–15	>15
No. patients	62 (100)	33.3 (7–57)	**48 (77.4)**	**14 (22.6)**	**6/62 (9.7%)**	**56/62 (90.3)**
Outcome						
Died	9 (14.5)	38.6 (19–53)	7 (14.6)	2 (14.3)	0/6	9/56 (16.1)
Survived	53 (85.5)	32.4 (7–57)	41 (85.4)	12 (85.7)	6/6 (100)	47/56 (83.9)
Illness course						
Severe, including fatal	37 (59.7)	33.1 (10–57)	**32 (66.7)**	**5 (35.7)**	3/6 (50)	34/56 (60.7)
Moderate, mild	25 (40.3)	33.6 (7–57)	**16 (33.3)**	**9 (64.3)**	3/6 (50)	22/56 (39.3)

*Bold type indicates statistically significant differences between sex or age groups. Comparison of binomial population proportions analysis as implemented in Statlets (NWP Associates, Inc., http://www.mrs.umn.edu/~sungurea/statlets/statlets.htm) indicates rejection of the null hypothesis (claiming that the 2 proportions are equal) at significance level of $p<0.05$.

Table 2. Characteristics of 9 deceased patients with Sochi virus infection, Russia*

Patient no.	Age, y/sex	Hospitalized, no. d after onset	GI symptoms	Max serum creatinine, μmol/L†	Min platelet count, $\times 10^9$/L‡	Died, no. d after onset	Clinical and postmortem findings
23	33/M	5	No	148	70	8	Pneumonia; renal, cardiovascular, multiorgan failure; multiple internal hemorrhages, edema
29	29/M	Same day	Yes	282	115	6	Renal, cardiovascular, multiorgan failure; multiple internal hemorrhages, edema
30	47/F	5	Yes	391	38	12	Renal, lung failure; shock; coagulation disturbance; hemorrhagic gastroenteritis; multiple internal hemorrhages, edema
34	53/M	3	Yes	250	110	10	Multiorgan failure; coagulation disturbances; multiple internal hemorrhages
42	30/M	14	Yes	186	67	16	Uremic coma; multiorgan failure; multiple internal hemorrhages
47§	41/M	Died before hospitalization	Yes	NR	NR	3	Renal failure; multiple internal hemorrhages, edema
48§	19/M	4	Yes	192	54	6	Renal, cardiovascular failure; RDS, DIC syndrome; bleedings in pituitary, adrenal gland, intestinum, etc.
56	35/F	4	Yes	410	49	6	Cardiovascular, renal, lung, liver failure; renal tubular necrosis; lung, brain edema
59	50/M	5	Yes	310	3	7	Renal,cardiovascular failure; RDS; multiple internal hemorrhages; pleurorrhea; lung, brain edema

*DIC, disseminated intravascular coagulation; GI, gastrointestinal; max, maximum; min, minimum; RDS, respiratory distress syndrome; NR, not reported.
†Reference range <96 μmol/L for female patients, <110 μmol/L for male patients.
‡Reference range 150–400 $\times 10^9$/L
§Patient no. 47 was the father in-law of patient no. 48; both lived in the same rural residence.

Conclusions

We have demonstrated the occurrence of human infections by Sochi virus and studied the clinical outcome for 62 patients. This virus is carried by the Black Sea field mouse (*A. ponticus*), which occurs naturally in the Transcaucasian region between the Black and Caspian Seas, including a part of southern European Russia. In anecdotal field studies in the coast region near Sochi, *A. ponticus* was the most abundant mouse species (71% of all trapped mice were identified as *A. ponticus*); moreover, 14% of trapped *A. ponticus* mice were serologically proven to be DOBV infected (*8*). This finding indicates that DOBV is the hantavirus indigenous in this geographic area and that *A. ponticus* mice are highly relevant as a hantavirus reservoir. All evidence from the natural virus reservoir, as well as serologic and molecular diagnostics of patients' serum, shows that the virus responsible for the infections is the DOBV genotype Sochi.

Most investigated patients found to be infected by Sochi virus exhibited a severe clinical course. With a calculated CFR of 14.5%, Sochi virus might be the most deadly hantavirus outside the Americas, where 35%–50% of hantavirus infections are fatal (*1*,*9*). Even Asian Hantaan virus is estimated to be less deadly; recent studies show CFRs of 1%–3% in China and South Korea, where Hantaan virus infections play an important role in HFRS morbidity (*10*,*11*). On the other hand, increased awareness in diagnostics, treatment, and prevention by local physicians and public health authorities is expected to improve survival rates for Sochi virus infections.

Among the related viruses of the DOBV species, Sochi virus seems to have the highest level of virulence, similar to Dobrava virus (carried by *A. flavicollis* mice), which has a CFR of up to 10%–12% (*12*,*13*). As shown in larger studies, disease caused by infection with the related Kurkino genotype (carried by the western lineage of *A. agrarius* mice) is associated with a CFR of only 0.3%–0.9% (*3*,*14*). These phylogenetically related viruses exert a quite different pathogenicity in humans.

Acknowledgments

We thank Brita Auste for careful execution of the molecular diagnostics.

This work was supported by Russian Science Foundation (grant no. 14-15-00619), Robert Koch Institute on behalf of the German Ministry of Public Health (grant no. 1369-382), and Deutsche Forschungsgemeinschaft (Priority Program 1596 "Ecology and species barriers in emerging viral diseases," grant no. KR1293/13-1).

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Vectorborne Transmission of *Leishmania infantum* from Hounds, United States

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Leishmaniasis is a zoonotic disease caused by predominantly vectorborne *Leishmania* spp. In the United States, canine visceral leishmaniasis is common among hounds, and *L. infantum* vertical transmission among hounds has been confirmed. We found that *L. infantum* from hounds remains infective in sandflies, underscoring the risk for human exposure by vectorborne transmission.

Leishmaniasis is endemic to 98 countries (*1*). Canids are the reservoir for zoonotic human visceral leishmaniasis (VL) (*2*), and canine VL was detected in the United States in 1980 (*3*). Subsequent investigation demonstrated that many US hounds were infected with *Leishmania infantum* (*4*). Evidence has demonstrated that *L. infantum* was spread by vertical transmission over many canine generations; no evidence of vector transmission has been reported (*5*,*6*). Vertical transmission may lead *L. infantum* to adapt to vectorless transmission and shed largely unrecognized factors needed for vector infection. Continuous axenic cell culture conditions without vector involvement have been shown to attenuate pathogen infectivity (*7*). Similarly, *L. infantum* circulating primarily via vertical transmission within US hunting hounds may lose its ability to infect and may be transmitted by traditional vectors.

In North America, 3 species of sandfly (*Lutzomyia anthophora, Lu. diabolica,* and *Lu. shannoni*) are known vectors of *Leishmania* spp. Reported cases of autochthonous cutaneous leishmaniasis in the United States include 9 cases in northeastern Texas (*8*), 2 in Oklahoma (*9*), and 1 in North Dakota (*10*). In the Americas, the principal sandfly vector is *Lu. longipalpis*, which can transmit *Leishmania* of multiple species. (*11*); its northernmost distribution is limited to Mexico. *Lu. shannoni* sandflies have been found in Kansas and Missouri (total range 21 states) (*12*). During 2010–2013, we assessed whether *L. infantum* circulating among hunting dogs in the United States can fully develop within sandflies and be transmitted to a susceptible vertebrate host.

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DOI: http://dx.doi.org/10.3201/eid2112.141167

The Study

A total of 300 laboratory-reared female *Lu. longipalpis* sandflies were allowed to feed on 2 hounds naturally infected with *L. infantum,* strain MCAN/US/2001/FOXY-MO1 or a closely related strain. During 2007–2011, the hounds had been tested for infection with *Leishmania* spp. by ELISA, PCR, and Dual Path Platform Test (Chembio Diagnostic Systems, Inc. Medford, NY, USA (Table 1). *L. infantum* development in these sandflies was assessed by dissecting flies starting at 72 hours after feeding and every other day thereafter. Migration and attachment of parasites to the stomodeal valve of the sandfly and formation of a gel-like plug were evident at 10 days after feeding (Figure 1), indicating successful parasite development.

Next, to determine sandfly capacity to transmit the US strain of *L. infantum* to a susceptible vertebrate host, we allowed *L. infantum*–naive and *L. infantum*–infected sandflies to feed on 7 *L. infantum*–naive hamsters for 13 days. For confirmation of *L. infantum* infection, we dissected the alimentary tract of sandflies that fed on the hamsters (Table 2). A total of 30 sandflies were used for feeding on hamsters; 11 flies fed and were subjected to *Leishmania* detection by PCR, which confirmed *Leishmania* positivity for 5 sandflies. Hamster blood samples were collected 2 weeks after infection and monthly for 5 months. *L. infantum* DNA was detected in hamster blood by quantitative PCR (qPCR) and was present in hamster nos. 1 (at 2 mo), 2 (at 3 mo), 5 (at 4 mo), and 6 (at 4 mo) with cycle thresholds of 43.88, 28.27, 34.38, and 45 respectively. Cycle thresholds $\leq$45 were considered positive for *L. infantum* (*5*).

On hamster no. 5, a cutaneous lesion consistent with *Leishmania* infection persisted for 1 month. Tissue from this lesion was harvested to assay for *Leishmania* infection. Increased numbers of macrophages and granulocytes were present in the dermal layer. Bacteria found in the tissue probably represented secondary infection, a common sequela of canine VL. Cellular infiltrate was observed, indicative of inflammation and infection (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/12/14-1167-Techapp1.pdf). No *L. infantum* parasites were observed on slides stained

Table 1. *Leishmania infantum* status of US foxhounds on which infected sandflies fed*

Sex	Age, y	Type of test, date of testing					
		PCR, 2007	PCR, 2008	Serology/PCR, 2009	Serology/PCR, 2010	Serology/PCR, 2011	DPP, 2011
M	7	Borderline	–	–/–	32–	≥512/+	+
F	6	–	+	64/–	≥512/+	≥512/+	+

*Serologic results determined by immunofluorescence antibody testing: <64 indicates negative (–). PCR results: – indicates no amplification; borderline indicates amplification on 1 of 3 tests; positive (+) indicates amplification on 2 of 3 or 3 of 3 tests. DPP indicates K39/22 Dual Path Platform Test (Chembio Diagnostic Systems, Inc. Medford, NY, USA), to detect antibodies against *Leishmania* spp.

with hematoxylin and eosin, periodic acid–Schiff, or Giemsa, and lesion tissue was negative for *L. infantum* by qPCR (data not shown).

qPCR was performed to quantify parasite load within common *Leishmania*-infected organs from all hamsters. The mean quantities of *Leishmania* DNA amplified from spleen, bone marrow, and lymph node from hamsters on which *Leishmania*-infected sandflies had fed were 12-, 22-, and 11-fold greater than that from hamsters on which *Leishmania*-naive sandflies had fed (Figure 2). According to extrapolation from a PCR standard curve similar to one previously used (*5*), the highest parasite load was in bone marrow, which contained an average of 1,238 (±282) parasites/mg tissue.

Conclusions

In the United States, parasites from VL-symptomatic, naturally infected hunting hounds remain highly infectious to *Leishmania*-competent *Lu. longipalpis* sandflies. Parasites that had fed on *L. infantum*–infected hounds were able to develop fully within sandflies and to be subsequently transmitted to and disseminated within hamsters.

The capacity of *Lu. shannoni* and *Lu. longipalpis* sandflies to acquire *L. infantum* from naturally infected dogs has been compared in leishmaniasis-endemic Brazil (*13*). Although lower infection rates were observed in *Lu. shannoni* (9%) than in *Lu. longipalpis* (36%) sandflies, the intensity of infection (200–500 promastigotes/fly) was higher in *Lu. shannoni* sandflies. This finding demonstrates a potentially lower threshold for acquiring infection from *Lu. shannoni* sandflies because the infectious dose per sandfly was greater. *Lu. shannoni* sandflies are commonly found within the United States and also in areas where *L. infantum*–infected hounds were reported (*4*). These data demonstrate the risk for vectorborne transmission of zoonotic VL from these dogs in the United States.

Despite our use of an *L. infantum* strain that is primarily, if not solely, transmitted via vertical transmission between dogs in the United States, we were able to measure substantial parasite loads in sandflies that fed on these dogs and in the bone marrow, spleen, and peripheral lymph nodes of hamsters on which infected sandflies had fed (Figures 1, 2). Parasite DNA was not amplified in the liver, possibly because of lower parasite loads in the liver during

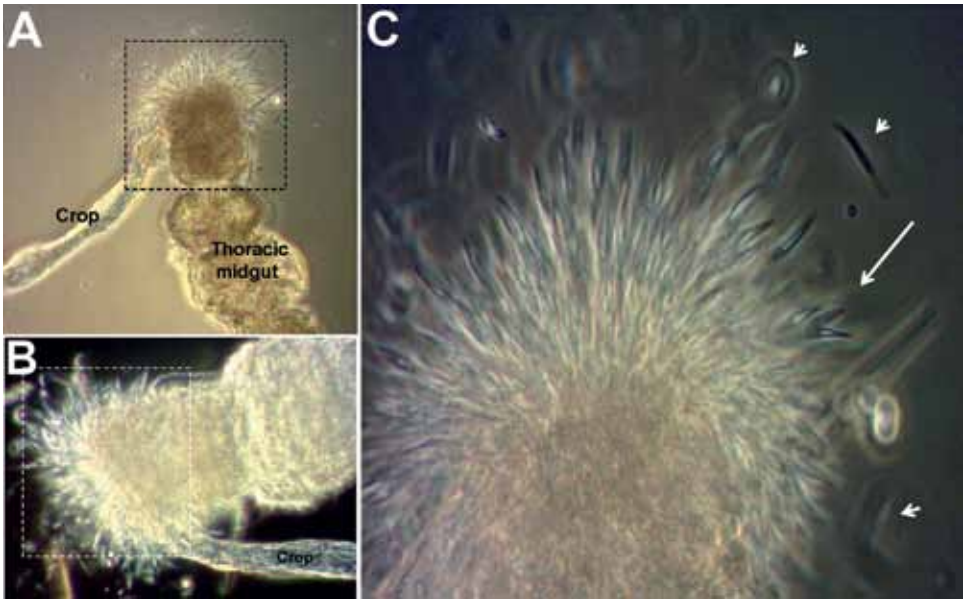

Figure 1. Sandflies infected with *Leishmania infantum* from US foxhounds, showing blocked stomodeal valve. Development of *L. infantum* (MCAN/US/2001/FOXYMO1) in laboratory-reared *Lutzomyia longipalpis* sandflies led to stomodeal valve blockage 10–13 days after infection. A) Dissected gut of infected sandfly, showing stomodeal valve (cardia) obstructed by *Leishmania* parasites (dashed box). Foregut removed during dissection and parasites entangled by flagella are visible. Original magnification ×10. B) Parasites obstructing stomodeal valve and parasite-secreted plug (dashed box). Original magnification ×40. C) Parasite plug dissected from the stomodeal valve, showing metacyclic promastigote parasites attached to plug (arrow), as well as free-swimming parasites (arrowheads). Original magnification ×100 with oil.

Table 2. Blood meal feeding and *Leishmania infantum* infection status of sandflies that fed on *L. infantum*–infected hamsters

Hamster no.	No. sandflies in which blood was visible/no. examined	PCR result for *L. infantun* from sandfly DNA extraction
1	1/5	–
2	2/5	+
3	1/5	–
4	0/5	Not applicable
5	3/5	2 + /1 –
6	2/5	1 +/ 1 –
7	2/5	–

later infection, as demonstrated in experimental VL infections of mice (*14*). Therefore, the US strain of *L. infantum* that is circulating in North American hunting hounds has not lost virulence factors that facilitate adherence to sandfly gut and facilitate transmission, and subsequent dissemination, in a secondary host.

This study focused on the possibility that domestic hounds serve as reservoir hosts for *L. infantum* within the United States; however, other potential *L. infantum* reservoirs include coyotes, foxes, and opossums. When leishmaniasis was found to be reemerging among hounds in the United States in 2000, a total of 291 wild canids were trapped and tested (*15*). No serologic evidence of infection was found, but these studies were limited to the southeastern United States; further study is needed to rule out the possibility that enzootic cycles of transmission do not exist within wild canids. The range covered by *Lu. shannoni* sandflies overlaps that of reservoir species including coyotes, foxes, and hunting hounds. Occurrence of *Leishmania* vectors in areas of naturally infected hounds indicates a coalescence of components for establishment of a sylvatic and/or domestic cycle of *L. infantum*. Diagnostic testing and preventive measures should be considered for dog breeds known to harbor *L. infantum*.

In the United States, *L. infantum* is circulating among dogs. Despite the fact that vertical transmission maintains VL within the hound population (*5*), *L. infantum* was able to fully develop in sandflies and be further transmitted to a susceptible vertebrate host. Symptomatic hounds were highly infectious to sandflies. *L. infantum* strain MCAN/US/2001/FOXYMO1, similar to the common European zymodeme MON-1, circulating dog-to-dog in North America maintained all necessary requirements for complete development within sandflies. Overlap of sandfly infections (e.g., *Lu. shannoni*, and *L. infantumi*) in hounds may put companion dogs and humans at risk and could pose an emerging risk for *L. infantum*–triggered clinical disease in at-risk populations in North America.

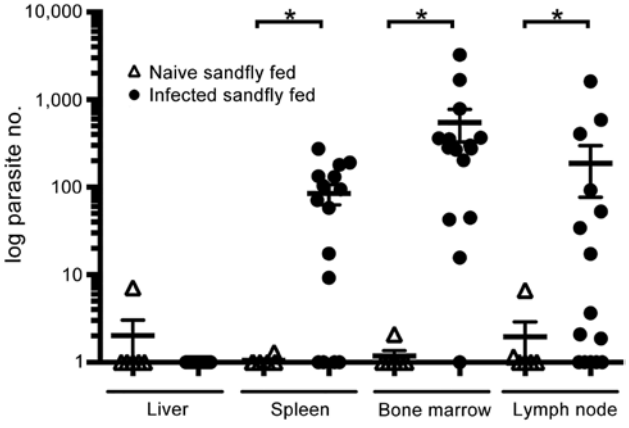

Figure 2. Visceralization of *Leishmania infantum* from US foxhounds, transmitted by sandflies into hamsters. *Leishmania* spp.–specific quantitative PCR was performed, and parasite load was calculated from a standard curve. Horizontal bars indicate mean values for 3 experiments run in duplicate. Statistical significance was determined by 1-way analysis of variance with Bonferroni posttest between 6 naive and 15 infected groups, by tissue type. Error bars indicate ± SEM. *p<0.05.

Acknowledgments

We thank members of the laboratory of C.A.P. for helpful suggestions; Douglas Jones for troubleshooting assistance; Chembio Diagnostic Systems, Inc. (Medford, NY, USA) for donation of *Leishmania* dual-path platform tests; collaborating foxhunting clubs for use of their priceless hounds; and Iowa State University Laboratory Animal Resources for valuable assistance.

This work was funded by grant AI088051 from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, to M.R.O. and C.A.P. K.J.E. was funded by a fellowship from the Morris Animal Foundation and Pfizer.

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Water as Source of *Francisella tularensis* Infection in Humans, Turkey

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Francisella tularensis DNA extractions and isolates from the environment and humans were genetically characterized to elucidate environmental sources that cause human tularemia in Turkey. Extensive genetic diversity consistent with genotypes from human outbreaks was identified in environmental samples and confirmed water as a source of human tularemia in Turkey.

Tularemia is a disease caused primarily by 2 subspecies of *Francisella tularensis*: *F. tularensis* subsp. *tularensis*, which is restricted to North America; and *F. tularensis* subsp. *holarctica*, which is found widely throughout the northern hemisphere but is the only subspecies in most of Eurasia (*1*). Through whole-genome sequencing and canonical single-nucleotide polymorphism (canSNP) genotyping, *F. tularensis* subsp. *holarctica* has been divided into 4 major genetic groups (B.4, B.6, B.12, and B.16) consisting of multiple subgroups (Figure 1) (*1*–*3*). Geographic distribution of these subgroups in Europe, Japan, and the USA are well described (*1*–*3*).

The phylogeography of *F. tularensis* in Asia is poorly understood because of undersampling in many regions, but recent studies have revealed new insights. A report has described rich phylogenetic diversity of the bacterium in China (*4*), including the rare B.16 group (biovar *japonica*). Previously, B.16 was known only in Japan (*1*) and Turkey (*6*). Sweden reportedly has the highest overall phylogenetic diversity among regions worldwide (*2*).

In Turkey, tularemia cases in humans have increased since 2009 (*7*), but little is known about environmental sources. Tularemia was first reported in Turkey in 1936 and then was sporadically reported for several decades (*7*). After improved surveillance, the number of tularemia cases increased in the 1980s and led to registration of tularemia as a reportable disease in 2004 (*7,8*). Incidence has continued to increase since then (*7*), and tularemia is now considered a reemerging zoonotic disease in Turkey.

Patients with oropharyngeal signs and symptoms account for ≈90% of tularemia cases in Turkey (*8*), and cases emerge seasonally from August–March (*7*). Seasonality of incidence of cases is presumably associated with consumption of contaminated water (*9*), but confirming sources is difficult. Reports of confirmation of *F. tularensis* from water samples by PCR (*10*) or culture (*6*) are rare, and definitive studies that link water to tularemia in humans are lacking. How water sources become seasonally contaminated is also unknown, but contamination could be caused by rodents. Recently, *F. tularensis* was confirmed by PCR from 2 mice captured in Thrace (*11*), but in Turkey, confirmation has not been obtained from ticks or mosquitoes, which are known vectors of *F. tularensis* (*1*,*4*).

Genetic characterization of clinical samples from tularemia outbreaks in Turkey in 2011 showed that multiple phylogenetic groups cause disease in multiple regions across Turkey (*5*); however, no environmental samples were assessed in that study. We report our findings from genetically characterized samples positive for *F. tularensis* from environmental and human sources located in multiple active tularemia areas in Turkey. Our results provide new insights into *F. tularensis* transmission from environmental sources to humans.

The Study

To examine environmental reservoirs that could be possible sources for human infections, during 2010–2013, we sampled water sources and rodent populations from suspected sites where transmission of *F. tularensis* infection could occur in Turkey. To survey and compare phylogenetic diversity of environmental samples and clinical samples, we examined 33 clinical samples of mostly oropharyngeal tularemia cases from approximately the same sites where environmental samples were collected. DNA was extracted (DNeasy Blood & Tissue Kit, QIAGEN GmbH, Hilden, Germany) from 6 water, 1 rodent spleen, and 33 human samples (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0634-Techapp.pdf).

The extractions were confirmed *F. tularensis*–positive by using PCR and targeting the *tul*4 gene (*12*). Analysis

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DOI: http://dx.doi.org/10.3201/eid2112.150634

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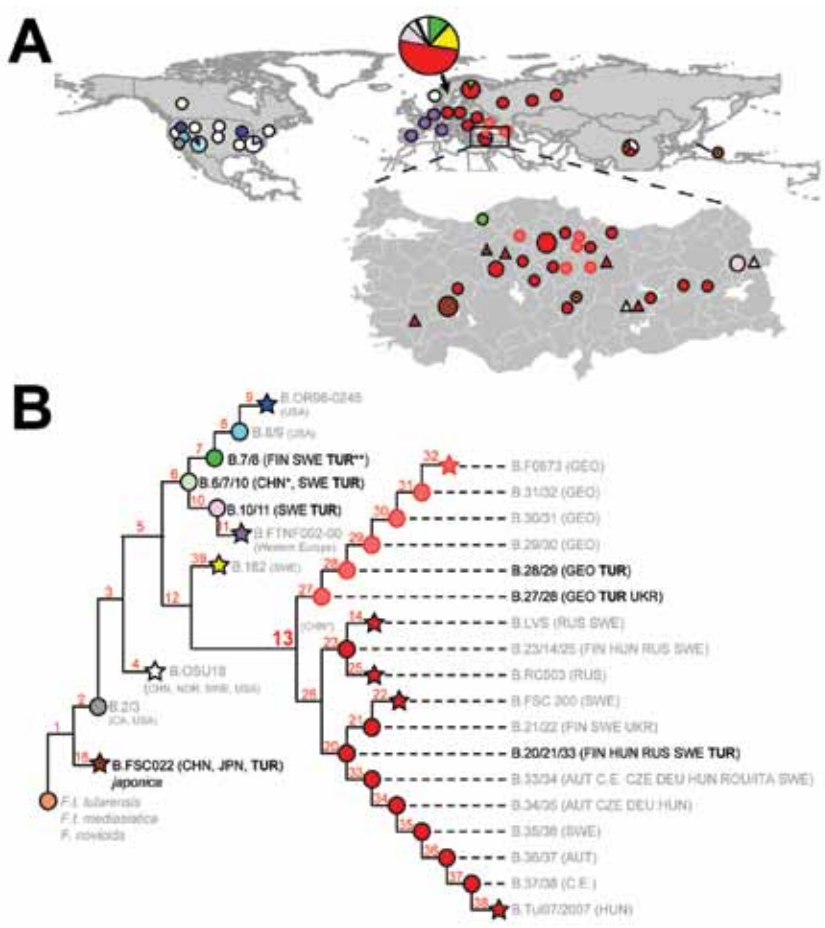

Figure 1. Phylogeography of *Francisella tularensis* subsp. *holarctica*. A) Global distribution of known phylogenetic groups determined on the basis of previous studies (*2*–*4*); enlarged map of Turkey shows locations of phylogenetic groups identified among the 40 samples positive for *F. tularensis* examined in this and previous studies (*5*). Circle size indicates number of samples (small circles, 1–3; medium circles, 4–6; large circles, 7–9). Colors of circles (human samples) and triangles (environmental samples) represent the phylogenetic subgroups to which these samples were assigned (see panel B). Subgroup B.16 (biovar *japonica*) is represented by the dot inside the brown circles and triangles. B) Phylogenetic tree for *F. tularensis* subsp. *holarctica* constructed on the basis of current canonical single-nucleotide polymorphism genotyping. Red numbers indicate nomenclature of canonical single-nucleotide polymorphism groups. Terminal subgroups representing sequenced strains are shown as stars, and intervening nodes representing collapsed branches are indicated by circles. Countries of origin for samples assigned to relevant phylogenetic groups are as follows: AUT, Austria; CE, central Europe, unknown country; CHN, China; CZE, Czech Republic; DEU, Germany; FIN, Finland; GEO, Georgia; HUN, Hungary; ITA, Italy; NOR Norway; ROU, Romania; RUS, Russia; SWE, Sweden; TUR, Turkey; UKR, Ukraine; USA, United States. CHN* indicates approximate phylogenetic placement because of a lack of resolved information on single-nucleotide polymorphisms (*4*). TUR** indicates identification from a previous study (*5*).

by using 21 published canSNP assays, as previously described (*5*), assigned these samples to 3 major phylogenetic groups and distinct subgroups: B.16 (n = 11); B.6 (2 subgroups: B.6/7/10, n = 1; and B.10/11, n = 6); and B.13 (2 subgroups: B.27, n = 5; and B.20/21/33, n = 17) (Figure 1; online Technical Appendix Table 1). Of the subgroups, 3 were previously unknown in Turkey: B.6/7/10, B.10/11, and B.16. The 7 environmental samples collected included most of the known phylogenetic diversity in Turkey and represented the 3 major groups: B16, B6 (B.6/7/10 and B.10/11), and B.13 (the group previously known to be in Turkey). Of the subgroups identified, all but B.6/7/10 were also found in the human samples.

To determine detailed associations between environmental and human clinical samples, we examined the genetic diversity among these samples by using multilocus variable number of tandem repeats analysis (MLVA) (*13*). All samples contained a single MLVA genotype (online Technical Appendix Figure, panels A–C); no mixed allele calls were observed at any of the examined variable number of tandem-repeats loci. Three different environmental samples (F0922, F0910, and F0916) had canSNP and MLVA genotypes that were identical to those of clinical samples (online Technical Appendix Table 1). In 2 instances (F0910 and F0916), the environmental sample and its respective genetically identical clinical sample(s) were recovered from different geographic regions, resulting in identical genotypes being found in different localities and suggesting that close genotypes are dispersed widely in Turkey. One environmental sample (F0922) had genetic, geographic, and temporal data (online Technical Appendix Figure, panel A) concordant with data from human samples. This water sample shared identical canSNP and MLVA genotypes with 5 clinical samples recovered 2 weeks previously at the same locality, strongly suggesting that the human cases are linked with this infected water source.

The genetic characterization of *F. tularensis* from environmental sources provides insights into transmission of tularemia from the environment to humans, but little is known about how water is contaminated. The seasonal

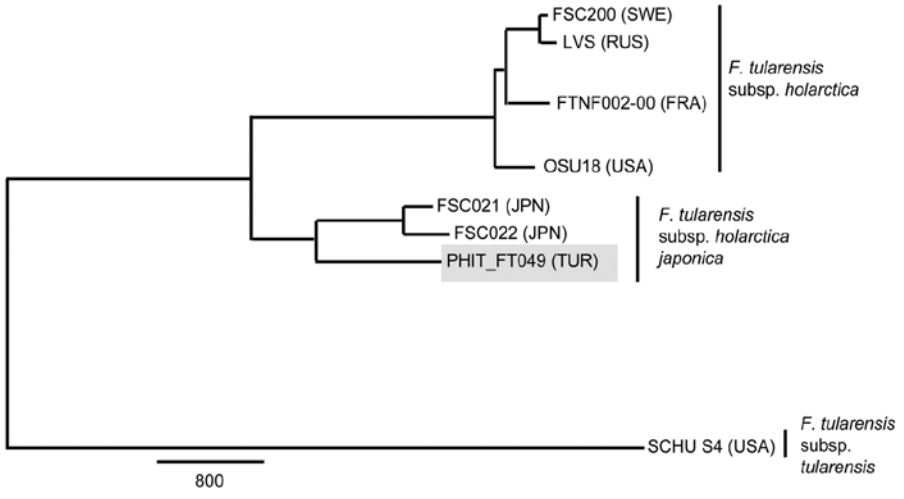

Figure 2. Maximum-parsimony phylogeny constructed by using 10,443 putative single-nucleotide polymorphisms discovered from whole-genome sequences of 8 *Francisella tularensis* strains. Gray shading indicates the B.16 (biovar *japonica*) strain from Turkey (PHIT_FT049). Detailed methods are described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/articles/21/12/15-0634-Techapp.pdf). Reference strains were retrieved from GenBank (online Technical Appendix Table 2). Countries of origin are indicated as follows: FRA, France; JPN, Japan; RUS, Russia; SWE, Sweden; TUR, Turkey; USA, United States. Scale bar indicates single-nucleotide polymorphisms.

nature of human outbreaks suggests that water sources are not constant reservoirs but rather are contaminated by another source. Rodents were identified as reservoirs (21% tularemia positive) in Bulgaria, where mainly oropharyngeal tularemia is endemic (*14*). We found a rodent sample (F0910) with canSNP and MLVA genotypes identical to an oropharyngeal clinical sample (F0898) (online Technical Appendix Table 1), a finding consistent with water contamination that originates from animal sources. However, the converse is also possible: animals could become infected by contaminated water.

Analysis of the 7 environmental *F. tularensis* subsp. *holarctica* samples from Turkey revealed extensive phylogenetic diversity that represents most known major groups in the world. Three of the 4 major *F. tularensis* subsp. *holarctica* phylogenetic groups (B.4, B.6, B.12, and B.16) are found in Turkey, including the highly basal B.16 group (biovar *japonica*) (Figure 1). This finding indicates that no single phylogenetic type is dominant in Turkey, unlike in Western Europe (*3*). Diversity was also represented in the clinical samples, suggesting that all major groups have similar capacities to cause disease, as other studies have suggested (*15*).

To gain insights into the evolutionary origin of the B.16 group, we examined the phylogenetic relationships among 3 published B.16 strains: 1 from Turkey (PHIT-FT049) (*6*) and 2 from Japan (FSC021 and FSC022) (GenBank accession nos. CP007148.1, SRX147922, and DS264138.1, respectively; Figure 2). We generated a global core-genome SNP phylogeny (online Technical Appendix) for these 3 B.16 strains and 5 strains from other groups (online Technical Appendix Table 2). As expected, PHIT-FT049 clusters with the Japanese B.16 strains from Japan and shares 448 putative SNPs; however, it is also distinct from the 2 strains from Japan, which together share 640 putative SNPs (Figure 2). The distinctiveness of the B.16 strain from Turkey strongly suggests that it has an evolutionary history different from that of the Japanese strains. The MLVA phylogeny of B.16 strains (online Technical Appendix Table 1) reveals greater diversity among the 8 strains from Japan than among the 8 strains from Turkey. These data show that the B.16 strains from Turkey and Japan are highly distinct, and the greater diversity in strains from Japan supports the possibility that the place of ancestral origin of the B.16 group is Asia.

Conclusions

Phylogenetically diverse strains of *F. tularensis* subsp. *holarctica* are environmentally established in Turkey and cause human disease. The strains in Turkey now include many phylogenetic groups previously found only in Scandinavia or Asia.

Acknowledgments

We thank Charles Williamson, Katy Califf, Bridget Barker, and Heidie Hornstra-O'Neill for assistance with the manuscript.

This study was funded by the US Department of Homeland Security, Science and Technology Directorate, Award NBCH2070001, and by the Cowden Endowment in Microbiology at Northern Arizona University.

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Association of Human Q Fever with Animal Husbandry, Taiwan, 2004–2012

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In Taiwan, Q fever cases in humans began increasing in 2004 and peaked in 2007 but dramatically declined in 2008 and 2011. Cases were significantly correlated with the number of goats. The decline might be associated with the collateral effects of measures to control goat pox in 2008 and 2010.

Q fever is a zoonosis caused by infection with *Coxiella burnetii*, a gram-negative bacterium and obligate intracellular pathogen. The major animal reservoirs are goats, sheep, and cattle. Humans are infected mainly through inhalation of aerosolized particles contaminated with *C. burnetii* excreted by an infected animal, particularly in fetal products, milk, urine, and feces (*1*). Nearly 60% of primary infections (acute Q fever) are asymptomatic. Symptomatic infection is characterized by influenza-like symptoms, occasionally accompanied by hepatitis or pneumonia.

Historically, the largest outbreak of Q fever caused nearly 4,000 human infections in the Netherlands during 2007–2010 (*2*). Epidemiologic studies implicated infected farm animals (sheep and goats) as the source for human infection (*2*,*3*). In the United States, an outbreak was associated with fetal products from aborted goats (*4*). Because of improved recognition and reporting of Q fever, it became reportable in the United States in 1999, and the number of cases increased by 250% during 2000–2004 (*5*).

In Taiwan, Q fever is endemic, particularly in the south (*6*). We previously identified the epidemiology and clinical characteristics of Q fever in Taiwan, but the association between human Q fever and animal husbandry has not been investigated (*7*–*9*). Our objective was to investigate the epidemiology of human Q fever and its association with animals by analyzing nationwide databases of human Q fever and animal husbandry during 2004–2012.

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DOI: http://dx.doi.org/10.3201/eid2112.141997

The Study

The Ethics Committee of E-Da Hospital (EMRP-103–042) approved this study. We collected data on confirmed human Q fever cases in Taiwan that occurred during 2004–2012 from the notifiable infectious diseases statistics system established by the Centers for Disease Control and Prevention of Taiwan (Taiwan CDC), which is an open and public website (http://nidss.cdc.gov.tw/ch/SingleDisease.aspx?dc=1&dt=4&disease=0830). The data included the number of confirmed cases, patient sex, age groups (5-year groups), and the geographic locations of cases (county and district) in Taiwan every month from 2004 through 2012. Reported cases included suspected cases of Q fever reported to the Taiwan CDC by clinicians. Generally, paired blood specimens (acute or convalescent phase) from reported case-patients are collected and sent to the contracted laboratories of the Taiwan CDC for laboratory testing of Q fever. Confirmed cases are reported cases that are confirmed positive for Q fever by laboratory tests. Q fever was confirmed either by serologic detection of a ≥4-fold increase in specific antibodies against *C. burnetii* phase II antigen by using an indirect immunofluorescence antibody assay or by a molecular method consisting of positive detection of *C. burnetii* DNA in blood using PCR.

We collected husbandry data on goats and cattle from 2004 through 2012 from the open and publicly available data released by the Council of Agriculture, Executive Yuan, Taiwan (http://agrstat.coa.gov.tw/sdweb/public/official/OfficialInformation.aspx). The maps of the geographic distributions of Q fever cases and animals were created by using SuperGIS Desktop software (Supergeo Technologies Inc., Taipei, Taiwan).

We identified 879 (6.3%) confirmed cases of Q fever among the 13,962 cases reported during 2004–2012. The number of confirmed cases increased dramatically starting in 2004 and peaked in 2007 but declined in 2008 and 2011 (Figure 1, panel A). Additionally, the annual incidence increased from 0.44 cases per 100,000 population in 2004 to 0.68 in 2007 and decreased from 0.40 in 2008 to 0.15 in 2011. Overall average annual incidence was 0.43 cases per 100,000 population. Cases occurred mainly in southern Taiwan (674 [76.7%] of 879) and particularly in the southern (17.4%) and Kaohsiung-Pingtung (59.3%) regions (Figure 1, panels A, B), and were most prevalent from March through September (669 [76.1%] cases) (Figure 1, panel C). Most case-patients were 30–69

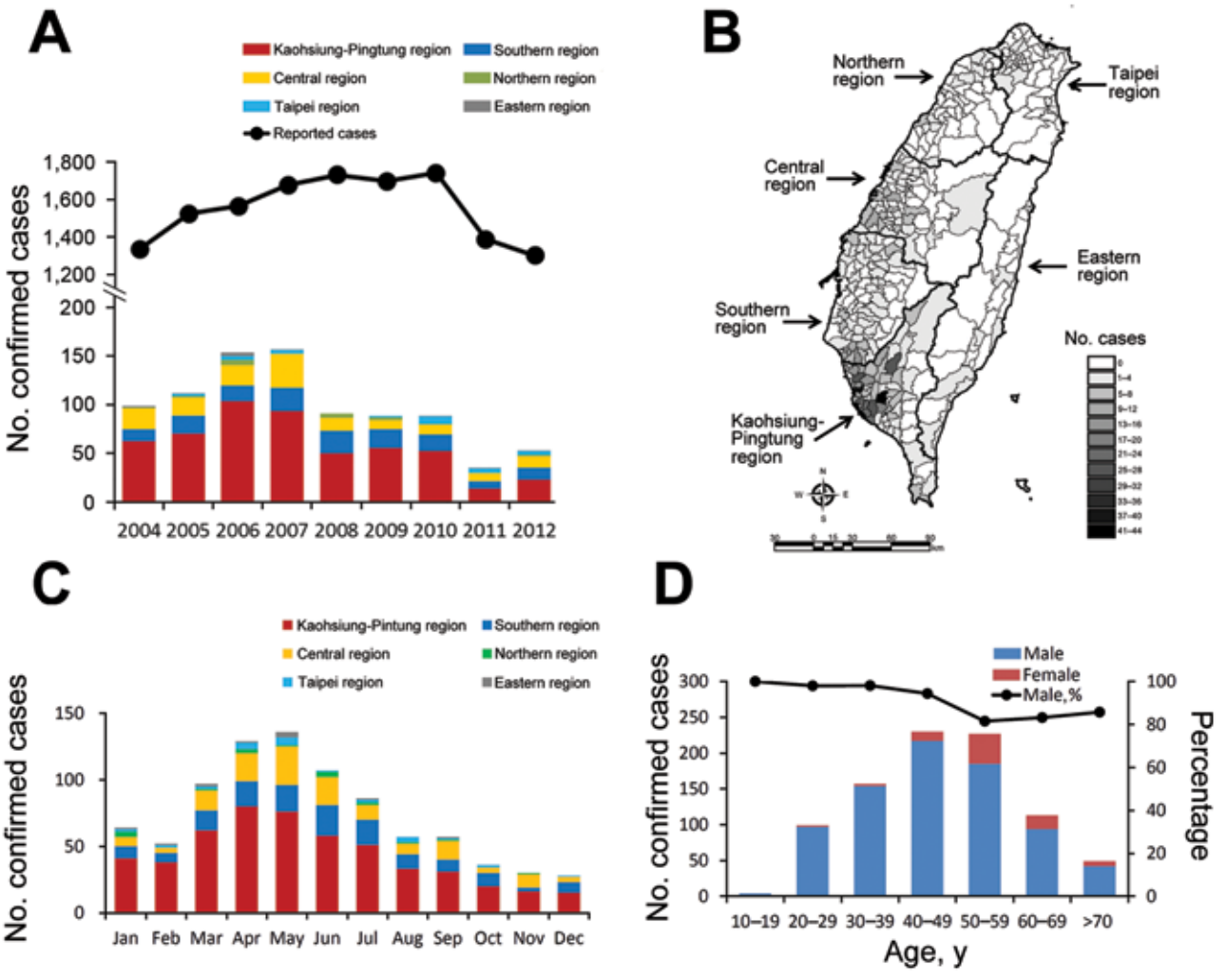

Figure 1. Q fever in humans, Taiwan, 2004–2012. A) Trends in reported and confirmed cases of Q fever. B) Geographic distribution of confirmed cases of Q fever. C) Monthly distribution of the confirmed cases. D) Age and sex distributions of patients with confirmed Q fever.

years old (727 [82.7%]) and male (793 [90.2%]) (Figure 1, panel D).

During 2004–2014, cattle and goats were distributed primarily in the southern and Kaohsiung-Pingtung regions (Figure 2, panels A, C). The trend of human Q fever cases was significantly correlated with the number of goats, rather than with the number of cattle (Figure 2, panels B, D).

Conclusions

During 2004–2012, the average incidence of Q fever in Taiwan was 0.43 cases per 100,000 population, which was higher than the incidence in the United States (0.04) (10) but lower than that in France (2.5) (11). Regardless of these differences in incidence, Q fever cases increased after Q fever became notifiable in the United States (5,10) and France (11). This increase might be attributed to improved recognition and increased reporting of notifiable infectious diseases to the authorities. In Taiwan, we have previously illustrated that reported and confirmed Q fever cases dramatically increased beginning in 2004, 3 years before it became notifiable in October 2007 (6). However, confirmed cases of Q fever decreased in 2008, even though the number of reported cases remained steady during 2007–2010 (Figure 1, panel A). Accordingly, the changing number of confirmed cases could not be explained by a change in the number of reported cases or by the advent of Q fever as a notifiable disease in Taiwan.

Most cases occurred in southern Taiwan, particularly in the Kaohsiung-Pingtung region, despite fluctuations in the yearly number of cases (Figure 1, panels A, B). Geographic distribution was correlated with the distribution of cattle and goat husbandry, which was predominant in southern Taiwan (Figure 2, panels A, C). A serologic study in the Kaohsiung-Pingtung region found a high seroprevalence of Q fever in animals (*12*). Seroprevalence rates in the overall herd and in individual animals were, respectively,

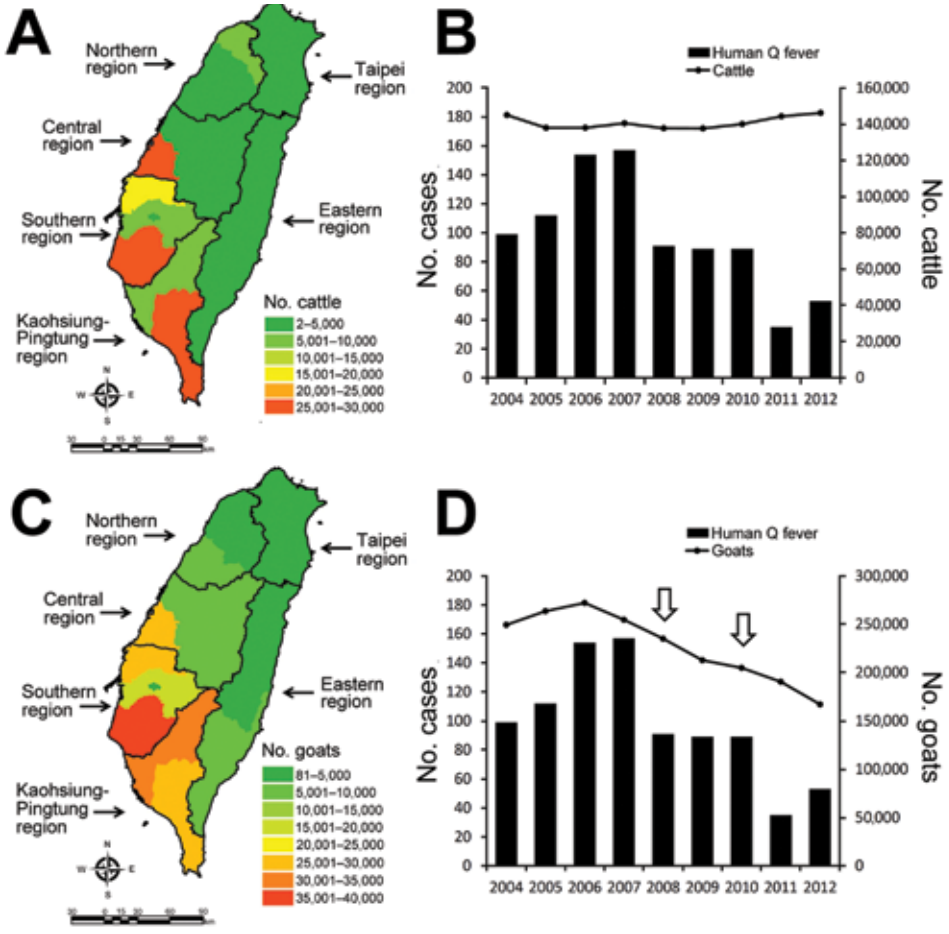

Figure 2. Q fever in cattle and goats and comparison with number of human Q fever cases, Taiwan, 2004–2012. A) Average number and distribution of cattle during 2004–2012; B) comparison of human Q fever cases and number of cattle showing no correlation ($p = 0.123$). C) Average number and distribution of goats during 2004–2012; D) comparison of human Q fever cases and the number of goats showing a significant correlation ($p = 0.003$). Arrows indicate goat pox epidemics of 2008 and 2010. The correlation between human Q fever, cattle, and goat was analyzed by Pearson's correlation.

73.6% and 48.3% in goats and 66.7% and 19.5% in cattle. In addition, the 26.3% seroprevalence in persons engaging in veterinary and animal-related work was higher than in the reference population (2.7%). Thus, we suspected that the decrease in the number of human cases might have been associated with animal reservoirs, particularly goats.

Goats and cattle are the major animal reservoirs of *C. burnetii*. We illustrated that the increase and decrease in human Q fever cases was correlated with variation in the number of goats, rather than cattle (Figure 2, panel B). After the number of goats began to decrease in 2007, human Q fever cases dramatically decreased in 2008 and 2011. The decrease in the number of goats was possibly associated with 2 episodes of goat pox epidemics in July 2008 and April 2010 (Figure 2, panel D) and the culling of 210 and >20,000 goats in the 2008 and 2010 epidemics, respectively (*13*,*14*). To control the goat pox epidemics, several measures were enacted, including animal and vehicle movement control within infected areas, cleaning and disinfection of infected farms and equipment, culling of infected animals, and vaccination. Although these measures were applied to control goat pox, collateral effects that diminished the spread of *C. burnetii* from infected goats or a contaminated environment to humans also might have existed.

This study has certain limitations. The association between livestock numbers and human Q fever might be ecologic because data on individual exposures and on Q fever in goats and cattle over time were not available for analysis.

In conclusion, Q fever is an endemic disease in Taiwan. Human cases increased beginning in 2004 and decreased in 2008 and 2011, which was correlated with the number of goats and possibly was associated with the collateral effects of measures taken to control goat pox in 2008 and 2010.

This work was supported by a research grant from E-Da Hospital (EDAHP103001) and the Ministry of Science and Technology (MOST 103-2314-B-650-008). The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

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Factors Associated with Severe Leptospirosis, Martinique, 2010–2013

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To identify factors associated with disease severity, we examined 102 patients with quantitative PCR–confirmed leptospirosis in Martinique during 2010–2013. Associated factors were hypotension, chest auscultation abnormalities, icterus, oligo/anuria, thrombocytopenia, prothrombin time <68%, high levels of leptospiremia, and infection with *L. interrogans* serovar Icterohaemorrhagiae/Copenhageni.

Leptospirosis is a bacterial zoonosis of worldwide distribution; incidence is highest in impoverished populations in developing countries and tropical regions (*1*). Humans are usually infected through contact with water or soil contaminated with the urine of carrier animals (*2*). The disease is caused by pathogenic strains of bacteria of the genus *Leptospira*, which is composed of 21 genomic species; 9 of them are pathogenic and comprise >200 serovars (*3*). To reduce the effects of severe leptospirosis, early diagnosis and prompt triage of high-risk patients is critical. Quantitative PCR (qPCR) might provide rapid diagnosis during the acute stage of the illness, offers the ability to measure the level of leptospiremia, and provides genomic identification (*4*–*6*). Our objectives were to determine if qPCR-determined leptospiremia was associated with severe evolution of the disease and to identify clinical and biological variables associated with severity.

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DOI: http://dx.doi.org/10.3201/eid2112.141099

The Study

From December 2010 through February 2013, blood samples were obtained from a cohort of 102 adult patients with qPCR-confirmed leptospirosis at the University Hospital of Martinique. The study was approved by the French ethics committee. At the time of admission, clinical characteristics, biological findings, and potential exposures were recorded. Severe leptospirosis was defined by the presence of ≥1 of the following: shock treated with vasoactive drugs, acute renal failure requiring dialysis, internal bleeding requiring blood transfusion, respiratory insufficiency requiring mechanical ventilation, or death.

After EDTA-treated plasma was concentrated by centrifugation, DNA was extracted and used to perform a SYBR green assay (Bio-Rad, Hercules, CA, USA) selective for *lfb1* as previously described (*7*–*9*). The sensitivity of the assay was evaluated by using DNA extracted from 10-fold dilutions of reference strains (at 10^7–10^2 leptospires/mL) belonging to *L. borgpetersenii*, *L. interrogans*, and *L. kirschneri*. Serum samples were subjected to microscopic agglutination testing, and 45 available samples of *Leptospira* were cultured as previously described (*8*). Genomic DNA was extracted from cultures or from human plasma, and then *Leptospira* species and subspecies were identified as previously described (*10*,*11*).

Statistical analyses were performed by using Stata software version 12 (StataCorp LP, College Station, TX, USA). Leptospiremia was log-transformed. Receiver operating characteristics curve analysis was used to determine the critical threshold for leptospiremia as the marker for severity. Logistic regression was used to identify factors associated with severity. Continuous variables were summarized by using median, first quartile, and third quartile and compared by using nonparametric tests (Mann-Whitney or Kruskal-Wallis, as appropriate). A p value of <0.05 was considered statistically significant.

Most (86.3%) of the 102 patients were men; median age was 49 (37–57) years. Of these patients, 89 were hospitalized, 23 required treatment in intensive care units, and 12 (11.7%) had severe leptospirosis according to our clinical definition. The median delay between symptom onset and qPCR diagnosis was 3 days first quartile and third quartile = 2, 5 days, respectively); blood tests were sampled from day 1 through day 11 after symptom onset, before administration of antimicrobial drugs. The median delay between symptom onset and antimicrobial drug receipt was 4 (3, 5) days. This delay did not differ significantly among patients with severe disease.

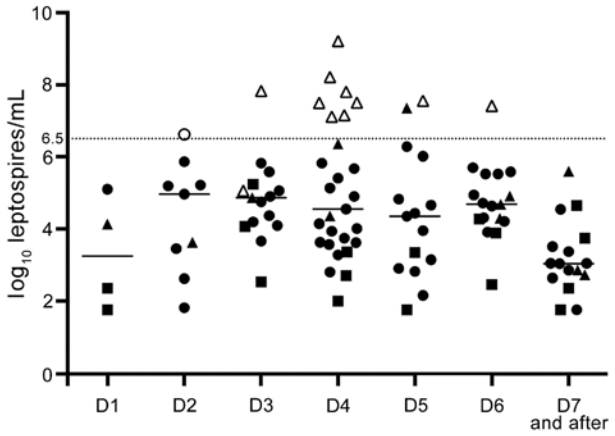

Figure 1. Leptospiremia in 102 patients with quantitative PCR–confirmed leptospirosis and day of sample collection since symptom onset, Martinique, 2010–2013. Each symbol (triangle, circle, or square) represents the leptospiremia of 1 leptospirosis patient on the day when the sample was collected. D indicates day since symptom onset. Open symbols indicate severe cases; closed symbols indicate nonsevere cases. Triangles correspond to *Leptospira interrogans* species, circles to other identified species, and squares to cases without genomic identification. Dotted line indicates the threshold for severe diseases determined by receiver operating characteristic curve analysis.

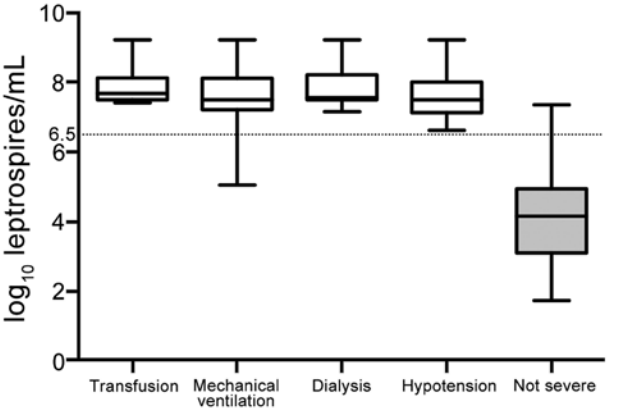

Figure 2. Distribution of leptospiremia among 102 patients with quantitative PCR–confirmed leptospirosis, grouped by severity criteria, Martinique, 2010–2013. Criteria that met our clinical definition for severe leptospirosis were shock treated with vasoactive drugs, acute renal failure requiring dialysis, internal bleeding requiring blood transfusion (e.g., alveolar hemorrhage), and respiratory insufficiency requiring mechanical ventilation or death during hospitalization. Horizontal lines in box-and-whisker plots indicate (top to bottom) maximum value, third quartile, median (second quartile), first quartile, minimum value.Dotted line indicates the threshold for severe diseases determined by receiver operating characteristic curve analysis.

Leptospiremia, determined by qPCR (Figure 1), was significantly higher among patients with severe disease (7.49 $\log_{10}$ [7.13, 7.81] vs. 4.16 $\log_{10}$ [3.14, 4.93]; p = 0.00001). Among those with severe disease, 9 had shock requiring vasoactive drugs, 8 had pulmonary involvement requiring mechanical ventilation, 8 had internal bleeding requiring blood transfusion, and 7 had acute renal failure requiring dialysis. No patient died. The median length of evolution before occurrence of severe leptospirosis was 3 (3, 4) days. Using a receiver operating characteristic curve analysis, we found a critical threshold of 6.5 $\log_{10}$ leptospires/mL that could be considered severe leptospirosis (Figures 1, 2). Except for acute renal failure, all complications were associated with a higher level of leptospiremia (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/14-1099-Techapp1.pdf).

The only epidemiologic characteristic associated with severity was presence of rats in the house or the surrounding vicinity (p = 0.02). Clinical and biological findings recorded at admission were associated with severity (Tables 1, 2) as follows: hypotension, chest auscultation abnormalities, icterus, oligo/anuria, bilirubin >49 μmol/L, creatinine >154 μmol/L, creatine phosphokinase >443 U/L, C-reactive

Table 1. Clinical characteristics of 102 patients with quantitative PCR–confirmed leptospirosis, by disease severity, Martinique, 2010–2013

Characteristic	All patients, n = 102, no. (%)	Patients with severe disease, n = 12, no. (%)	Patients with nonsevere disease, n = 90, no. (%)	p value
Fever >38°C	88 (86.3)	9 (75)	79 (87.8)	0.364
Hypotension*	10 (9.8)	5 (41.7)	5 (5.6)	0.002
Cough	12 (11.8)	3 (25)	9 (10)	0.148
Abnormalities at chest auscultation	7 (6.9)	4 (33.3)	3 (3.3)	0.003
Abdominal pain	30 (29.4)	5 (41.7)	25 (27.8)	0.329
Vomiting	42 (41.2)	5 (41.7)	37 (41.1)	1
Diarrhea	30 (29.4)	3 (25)	27 (30)	1
Icterus	39 (38.2)	9 (75)	30 (33.3)	0.009
Conjunctival suffusion	20 (19.6)	1 (8.3)	19 (21.1)	0.45
Consciousness disorders	2 (1.6)	1 (8.3)	1 (1.1)	0.2
Hemorrhage†	6 (5.9)	1 (8.3)	5 (5.6)	0.54
Oliguria** or anuria‡	8 (7.8)	5 (41.7)	3 (3.3)	0.0001

*Systolic blood pressure <90 mm Hg.
†Hemoptysis, hematuria, bleeding of the gums, or hematemesis.
‡<500 mL urine/day.

Table 2. Initial laboratory findings among 102 patients with quantitative PCR–confirmed leptospirosis, by disease severity, Martinique, 2010–2013

Initial laboratory findings*	All patients, n = 102, no. (%)	Patients with severe disease, n = 12, no. (%)	Patients with nonsevere disease, n = 90, no. (%)	p value
Bilirubin				
μmol/L (Q1, Q3)	20 (12, 49)	56.5 (35.5, 103)	18 (12, 38)	0.0035
>49 μmol/L, no./total (%)	25/99 (25.2)	7/12 (58.3)	18/87 (20.7)	0.01
Creatinine				
μmol/L (Q1, Q3)	104 (88, 154)	169.5 (132.5, 217.5)	100 (87, 137)	0.0084
>154 μmol/L, no./total (%)	26/101 (25.7)	7/12 (58.3)	19/89 (21.3)	0.011
Urea nitrogen (mmo/Ll)				
mmol/L (Q1, Q3)	5.7 (4.2, 9.3)	10.1 (8, 18.5)	5.5 (4, 8.6)	0.0068
>9.3, mmol/L, no./total (%)	21/84 (25)	4/8 (50)	17/76 (22.4)	0.103
Creatine phosphokinase				
U/L (Q1, Q3)	170 (70, 443)	953 (204, 1332)	145 (64, 390)	0.0202
>443 U/L, no./total (%)	19/75 (25.3)	5/9 (55.6)	14/66 (21.2)	0.041
C-reactive protein				
mg/L (Q1, Q3)	188.5 (108, 282)	338.5 (197.5, 464.5)	177.9 (89, 265)	0.0017
>282 mg/L, no./total (%)	26/102 (25.5)	7/12 (58.3)	19/90 (21.1)	0.011
Potassium, mmol/ L (Q1, Q3)	3.7 (3.4, 4.1)	3.75 (3.35, 4.15)	3.7 (3.3, 4.1)	0.8
Sodium, mmo/L (Q1, Q3)	134 (132, 136)	134 (131.5, 135)	134 (132, 136)	0.44
Aspartate aminotransferase, U/L (Q1, Q3)	61.5 (32, 102)	73.5 (59, 126.5)	57.5 (31, 102)	0.19
Alanine aminotransferase, U/L(Q1, Q3)	55 (30, 96)	49 (33.5, 74.5)	55 (30, 99)	0.69
Hemoglobin				
g/dL (Q1, Q3)	13.2 (12.2, 14.5)	12.2 (11.6, 13)	13.3 (12.4, 14.7)	0.027
<12.2 g/dL, no./total (%)	26/102 (25.5)	6/12 (50)	20/90 (22.2)	0.071
Leukocytes, $\times 10^9$ cells/L (Q1, Q3)	8.51 (6.2, 10.9)	10.3 (9.1, 11.4)	7.8 (6.1, 10.5)	0.07
Lymphocytes				
$\times 10^9$ cells/L (Q1, Q3)	0.7 (0.49, 1)	0.5 (0.2, 0.7)	0.7 (0.5, 1)	0.043
$<0.49 \times 10^9$ cells/L, no./total (%)	24/92 (26)	4/8 (50)	20/84 (23.8)	0.19
Platelets				
Concentration, $\times 10^9$/L (Q1, Q3)	138 (92, 183)	70.5 (32.5, 115)	141 (99, 191)	0.0011
$<92 \times 10^9$/L, no./total (%)	26/101 (25.7)	7/12 (58.3)	19/89 (21.3)	0.011
Prothrombin time				
% (Q1, Q3)	74 (68, 90.5)	66.5 (56, 74.5)	75.5 (69, 91)	0.0166
<68%, no./total (%)	20/76 (26.3)	7/12 (58.3)	13/64 (20.3)	0.011

*Continuous variables are summarized by using median, first quartile (Q1), and third quartile (Q3). Hematologic and biochemical variables are categorized into 2 groups, using Q1 or Q3 as appropriate.

protein >282 mg/L, hemoglobin <12.2 g/dL, lymphocytes $<0.49 \times 10^9$ cells/L, platelets $<92 \times 10^9$/L, and prothrombin time <68%.

Molecular typing of genomic DNA was performed from the 102 acute-phase blood samples (online Technical Appendix Table 2). Leptospire species determination was successful for 85 (83%) patients and corresponded to 1 of the following 6 pathogenic species: *L. interrogans* (n = 23), *L. santarosai* (n = 22), *L. borgpetersenii* (n = 18), *L. kirschneri* (n = 15), *L. kmetyi* (n = 4), and *L. noguchii* (n = 3). Among the genomic species identified, *L. interrogans* was associated with severity (p = 0.001), highest level of leptospiremia (p = 0.0001), and previous exposure to rats (p = 0.02). The level of leptospiremia in specimens for which species was not identified was significantly lower (p = 0.0001). The median melting peak for *L. interrogans* strains was 83.1°C (82.8°C, 83.4°C), which differed significantly from that of other species, for which the median melting peak was 85°C (84°C, 85.9°C) (p = 0.0001).

Microscopic agglutination testing enabled identification of the putative serogroups (highest titer >400) for 70 (68.6%) patients; the 3 most frequently identified serogroups were Icterohaemorrhagiae (n = 39), Ballum (n = 11), and Celledoni (n = 10). Serogroup Icterohaemorrhagiae can be subdivided into serovars Icterohaemorrhagiae/Copenhageni (n = 20) and Bogvere (n = 10); the remaining 9 serogroups cannot be unambiguously typed at the serovar level. Serovar Icterohaemorrhagiae/Copenhageni was identified for 11 of the 12 patients with severe disease (p = 0.03). The identification of the putative serogroup was not possible for 32 patients (online Technical Appendix Table 3).

Conclusions

This prospective study enabled us to report the potential contribution of qPCR to timely diagnosis and leptospirosis severity evaluation at the point of care in a disease-endemic area. We based our classification of severity on treatment-related criteria to reflect everyday patient management, as previously reported (*12*,*13*). The fact that no patient died could be associated with factors such as reduced diagnosis time and early treatment. Currently, only qPCR enables unequivocal diagnosis during

the acute phase of illness, when antimicrobial drugs are most likely to have the greatest benefit (*6*,*14*). Our results show a strong association between leptospiremia levels and disease severity. A lower critical threshold was reported in New Caledonia, and differences between critical thresholds may be associated with the variability of virulence among serovars, host factors, or qPCR technique (*13*).

The samples used for qPCR diagnosis were also used for direct *Leptospira* genomic identification, although molecular typing performance was impaired for samples with the lowest leptospiremia, as previously reported (*15*). The factors significantly associated with severity were infection with the species *L. interrogans,* the serogroup Icterohaemorrhagiae, and the presence of rats (usual carriers of that serogroup). In that context, melting curve analysis of the assay may provide rapid and useful additional information because it can differentiate between *L. interrogans* and other pathogenic species (*7*,*9*). The potential correlation between disease severity and serogroup Icterohaemorrhagiae has been reported in other tropical islands, and our results also emphasize the need for public health action to control rodents (*12*,*13*).

qPCR can be used for rapid diagnosis of acute leptospirosis and may provide timely information useful for evaluation of disease severity. Use of qPCR to determine leptospiremia seems increasingly accessible and should be evaluated in other disease-endemic areas. Whether high levels of leptospiremia are associated with factors such as pathogen virulence characteristics or host factors should also be explored.

Acknowledgments

We thank Olivier Verlaeten and Ludovic Jeanneau, who introduced the qPCR technique in the University Hospital of Fort de France; Dorothée Haug and the technical staff at the Service de Bactériologie of the University Hospital of Fort-de-France; Janick Jean-Marie and the staff at the Centre d'Investigation Clinique et d'Epidémiologie Clinique Antilles-Guyane (Institut National de la Santé et de la Recherche Médicale CIE 802); Christopher Pappas for editorial assistance; and Eric Caumes for critical review of the manuscript. We also thank the French Ministry of Health with the participation of the Groupement Interrégional de Recherche Clinique et d'Innovation Sud-Ouest Outre-Mer for funding and the Direction de la Recherche Clinique et de Innovation of the University Hospital of Fort-de-France, Martinique, France, for methodologic support.

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Sindbis and Middelburg Old World Alphaviruses Associated with Neurologic Disease in Horses, South Africa

Stephanie van Niekerk, Stacey Human, June Williams, Erna van Wilpe, Marthi Pretorius, Robert Swanepoel, Marietjie Venter

Old World alphaviruses were identified in 52 of 623 horses with febrile or neurologic disease in South Africa. Five of 8 Sindbis virus infections were mild; 2 of 3 fatal cases involved co-infections. Of 44 Middelburg virus infections, 28 caused neurologic disease; 12 were fatal. Middelburg virus likely has zoonotic potential.

Alphaviruses (*Togaviridae*) include zoonotic, vectorborne viruses with epidemic potential (*1*). Phylogenetic analysis defined 2 monophyletic groups: 1) the New World group, consisting of Sindbis virus (SINV), Venezuelan equine encephalitis virus, and Eastern equine encephalitis virus; and 2) the Old World group, consisting of Semliki Forest virus (SFV), Middelburg virus (MIDV), Ndumu virus, Chikungunya virus (CHIKV), and Barmah Forest virus (*2*). Old World alphaviruses are associated mainly with febrile disease and arthralgia, are often accompanied by a maculopapular rash, and are rarely fatal, although neurologic cases have been reported (*3*). In contrast, New World alphaviruses are associated with neurologic disease in horses and, potentially, humans (*4*).

We previously investigated horses as sentinels for detection of neurologic arboviruses and described West Nile virus (WNV) lineage 2 (*5*) and Shunivirus (SHUV) as previously missed causes of fatal encephalitis in Africa (*6*), with zoonotic potential (*7*). Five alphaviruses have been detected in vectors in southern Africa: SINV, CHIKV, MIDV, Ndumu virus, and SFV (*8*); however, little is known about prevalence, pathogenicity, and host range (*9*).

The Study

To determine if alphaviruses may contribute to undefined neurologic infections, we investigated specimens (blood, cerebrospinal fluid, or tissue from brain, spinal cord, or visceral organs) from 623 horses with unexplained febrile and acute neurologic infections reported to our surveillance program by veterinarians across South Africa during January 2008–December 2013. Of reported cases, 346 horses had neurologic signs; 277 had mainly febrile illness and other miscellaneous signs, including colic and sudden death (online Technical Appendix Figure 1, panel A, http://wwwnc.cdc.gov/EID/article/21/12/15-0132-Techapp.pdf). Formalin-fixed tissue samples from horses that died were submitted for histopathology. Horses ranged from <1 to 20 years of age and included thoroughbred, Arabian, warmblood, and part-bred horses; most were bred locally.

A generic nested alphavirus nonstructural polyprotein (nsP) region 4 gene reverse transcription PCR (*10*) was used to screen total nucleic acids. TaqMan probes (Roche, Indianapolis, IN, USA) were developed for rapid differentiation of MIDV and SINV by real-time PCR (online Technical Appendix).

PCR-positive cases were confirmed by sequencing the nsP4 amplicon (200 bp), followed by maximum-likelihood analysis (Figure 1, panel A). Additional amplification, sequencing, and phylogenetic analysis of a 349-bp E1 gene fragment (online Technical Appendix) was attempted (Figure 1, panel B) to investigate recombination events.

Baby hamster kidney cell–culture isolates were obtained for 2 MIDV strains under Biosafety Level 3 conditions. Isolate SAE25/2011 from blood of a horse with neurologic signs was visualized by electron microscopy (Figure 2); the full genome was sequenced as described (*11*). By using maximum-likelihood and P-distance analysis (online Technical Appendix Figure 2), we compared isolate SAE25/2011 with MIDV-857, which was isolated in 1993 from spleen of a horse with African horse sickness virus (AHSV)–like signs in Zimbabwe (GenBank accession no. EF536323) (*12*). Differential diagnosis for flaviviruses, WNV, Wesselsbron virus, SHUV, and equine encephalosis viruses was performed on all specimens, which were sent to other laboratories for testing for AHSV, equine herpes viruses, and rabies (*5*,*6*).

Of the 623 horses, 8 (1.3%) tested positive for SINV and 44 (7.1%) for MIDV (Table 1). Of the 8 horses with SINV, 3 survived febrile illness, 2 survived neurologic disease, and 3 died from neurologic disease. Two of the 3 horses that died with SINV had WNV co-infection and were positive by PCR for both viruses in brain tissue. In those 2 horses, lesions of meningoencephalitis were visible

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DOI: http://dx.doi.org/10.3201/eid2112.150132

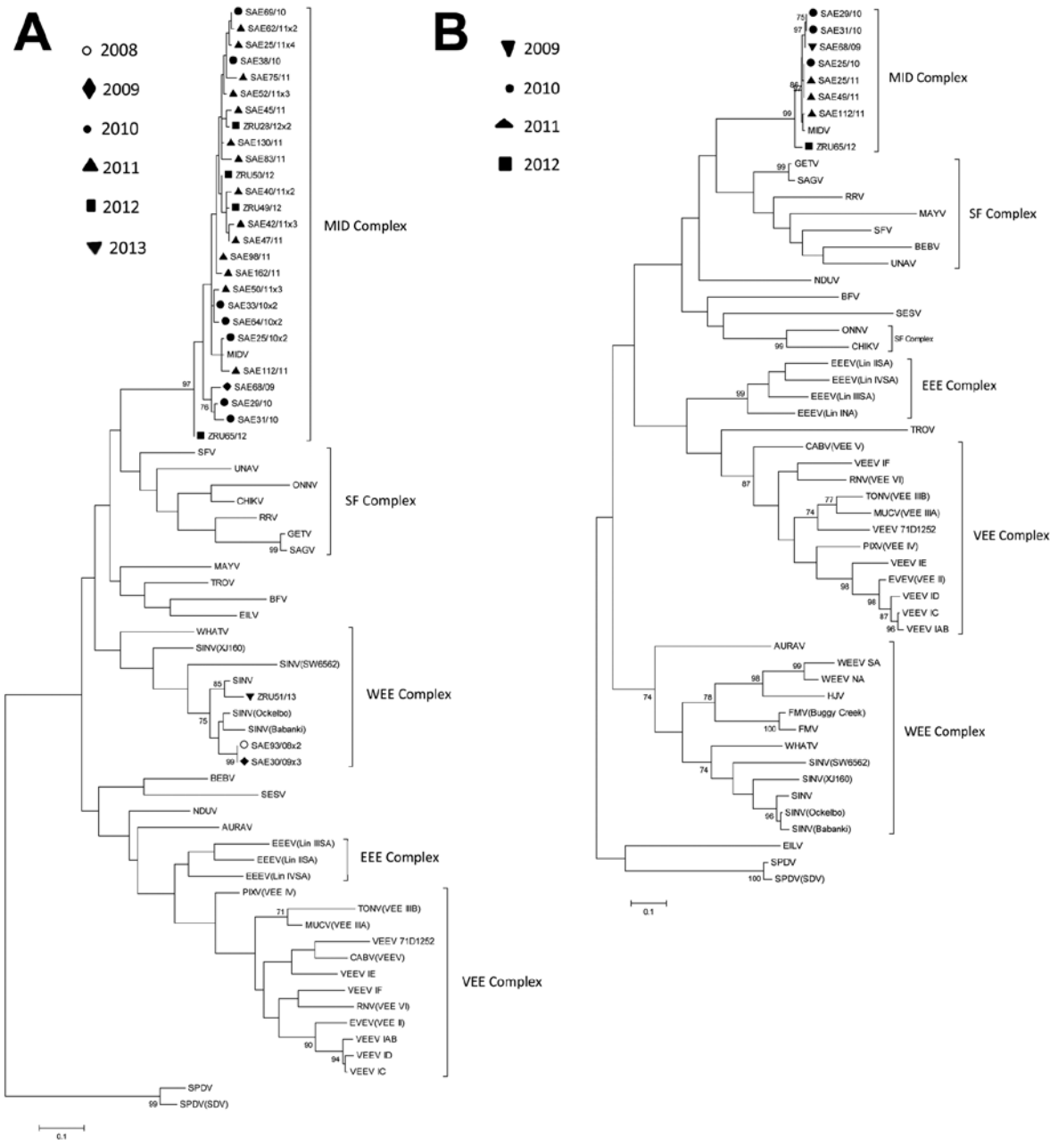

Figure 1. Maximum-likelihood trees of strains of Middelburg virus and Sindbis virus identified in horses in South Africa relative to other members of the alphavirus genus. Trees were constructed by using the Tamura-Nei substitution model and midpoint rooted with MEGA5 (http://www.megasoftware.net/). Scale bar indicates 0.1 nt substitutions. Estimates were constructed on the basis of bootstrap resampling performed with 1,000 replicates. Confidence estimates >70 are shown. A) A 200-bp fragment of the nonstructural polyprotein region 4 gene of MDV- and SINV-positive cases. B) A 348-bp fragment of the E1 gene of 7 MIDV cases identified in horses in southern Africa (genome position 10543–10911 corresponding with the MIDV-857 strain in GenBank accession no. EF536323). Reference sequences used in these trees are as previously described (*2*). Complexes are identified as follows: EEE, Eastern equine encephalitis; MID, Middelburg; SF, Semliki Forest; VEE, Venezuelan equine encephalitis; WEE, Western equine encephalitis. Viruses are identified as follows: AURAV, Aura virus; BEBV, Bebaru virus; BFV, Barmah Forest virus; CABV, Cabassou virus; CHIKV, Chikungunya virus; EEEV, Eastern equine encephalitis virus; EILV, Eilat virus; EVEV, Everglades virus; FMV, Fort Morgan virus; GETV, Getah virus; HJV, Highlands J virus; MAYV, Mayaro virus; MIDV, Middelburg virus; MUCV, Mucambo virus; NDUV, Ndumu virus; ONNV, O'nyong nyong virus; PIXV, Pixuna virus; RNV, Rio Negro virus; RRV, Ross River virus; SAE, South Africa equine virus; SAGV, Sagiyama virus; SESV, Southern elephant seal virus; SFV, Semliki Forest virus; SINV, Sindbis virus; SPDV, Salmon pancreatic disease virus; TONV, Tonate virus; TROV, Trocara virus; UNAV, Una virus; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WHATV, Whataroanvirus; ZRU, Zoonoses Research Unit virus.

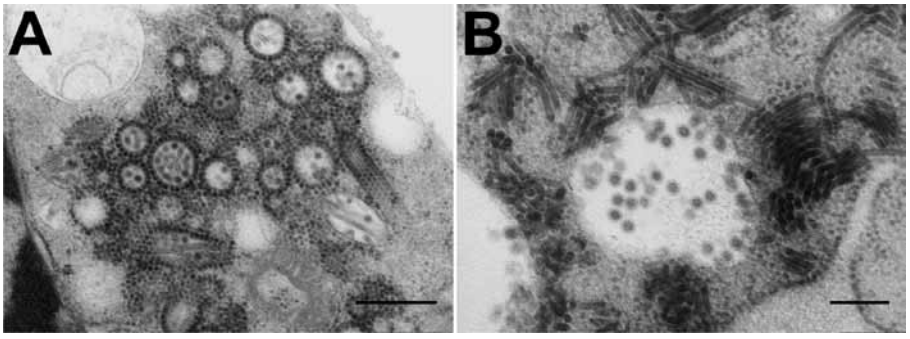

Figure 2. Electron micrographs of Middelburg virus isolate SAE25/2011 in baby hamster kidney cell culture. A) Several enveloped virions consisting of a dense core and surrounded by a translucent layer are shown in the vesiculated endoplasmic reticulum. The virus has elongated forms and numerous precursor nucleocapsids in the cytoplasm. Many of the nucleocapsids are associated with the outer surfaces of the vesiculated endoplasmic reticulum. Scale bar indicates 500 nm. B) Virions in a cytopathic vacuole are surrounded by elongated forms of the virus. Scale bar indicates 200 nm. Micrographs courtesy of Stephanie van Niekerk et al.

by histopathology but were nonspecific and could not be ascribed to either virus. Immunohistochemistry (IHC) for WNV in equine cases is reportedly a poor diagnostic tool (*13*), although IHC for SINV and MIDV needs to be established. In the third fatal case, SINV was detected in blood; neurologic samples were unavailable for testing. One horse with SINV had mild colic, dysphagia with tongue paralysis, and pale mucus membranes (Table 2).

Of 44 horses with MIDV, 16 had febrile disease, 28 had neurologic disease, and 12 died. Five dual infections were detected: 1 horse with MIDV and SHUV infections and 2 with MIDV and EEV infections survived; 1 horse with MIDV and AHSV infections and 1 with MIDV and WNV infections died (Table 2). For 6 of 12 horses that died with MIDV, central nervous system samples were available, and MIDV was detected in brain of all 6 horses. AHSV was detected in lung and spleen and MIDV in brain of 1 horse. MIDV and WNV was found in brain of another; only MIDV was detected in brain of the other 4.

Altogether, 26 (66.7%) of 39 horses with MIDV single infection showed signs of neurologic disease (Table 2). Clinical signs with less severe illness included fever, stiffness, swollen limbs, hyperreactiveness, and depression; signs of severe neurologic disease included ataxia, paresis, paralysis, recumbency, and seizures. One horse exhibited icterus, 1 had a pregnancy abort, and 1 had laminitic stance. Brain and cord tissues from 4 horses with neurologic disease caused by MIDV single infections were examined microscopically. MIDV was detected in blood of 1 horse but in brain of the others. Lesions of mild to moderate meningoencephalitis were observed in all 4, including perivascular cuffing involving mainly mononuclear cells, glial nodules, and diffuse gliosis.

No clear associations with age, gender, or breed were apparent for either virus. Number of cases peaked during the rainy season (February–May), consistent with vectorborne diseases, although sporadic cases were detected year-round (online Technical Appendix Figure 1, panel B). Both viruses were widely distributed across South Africa (online Technical Appendix Figure 3).

The nsP4 gene fragment for 6 SINV-positive specimens had ≤7% nt differences but 100% aa identity, clustering closely to the Ockelbo strain from Sweden and Babanki strain from Cameroon. One SINV isolate was of unrecorded origin (Figure 1, panel A) (*2*).

MIDV nsP4 partial sequences of 26 unique strains clustered with MIDV-857 (*12*), (Figure 1, panel A), with 3.8% nt and 2.8% aa differences from South Africa strains and ≤5% nt and 3.6% aa differences from MIDV-857. No specific relationships to time, geographic origin, or outcome were evident, although the length and conserved nature of the diagnostic amplicon limited the phylogenetic analysis. The E1 gene fragments from 7 MIDV cases (GenBank accession nos. JN226792–JN226795, KF680222–KF680224) clustered with MIDV-857 (Figure 1, panel B), differing by ≤1.2% nt and 0.5% aa levels from each other and by ≤1.4% nt and 0.6% aa levels from MIDV-857.

The genome of MIDV isolate SAE25/2011 (GenBank accession no. KF680222) was 11,674 nt in length, excluding the poly (A) tail, with 98.5% nt and 99.4% aa identity to MIDV-857 (*12*), clustering similarly as previous alphavirus phylogenetic investigations (*2*). Seven aa differences exist in the structural polyprotein (98.7% identity) and 17 in the nsP region (99.3% identity). Three aa changes in the nsP and 3 in the structural polyprotein altered hydrophilicity. The nsP1 amino acid sequences differed by 0.8%; the nsP2 and nsP3 were identical; and nsP4 differed by 0.5%. The capsid protein differed by 1.1%; E1 and E2 differed by 0.5%; and E3 and 6K proteins were identical. SAE25/2011, like MIDV-857, also contained the recombinant SFV domains identified previously (*12*).

Table 1. Prevalence of neurologic and febrile cases of MIDV and SINV infections in horses, South Africa, 2008–2013*

Alphavirus results	2008	2009	2010	2011	2012	2013	Total
MIDV							
Specimens, no.	76	50	137	166	107	100	636 (100)
PCR+, no. (% of total specimens)	0	1 (2)	10 (7)	26 (16)	4 (4)	3 (3)	44 (7)
Deaths, no. (% of no. PCR+)	NA	0	2 (50)	7 (27)	3 (75)	0	12 (27)
Detected in CNS, no. (% of no. PCR+)	NA	0	0	6 (23)	0	0	6 (14)
Neurologic disease, no. (% of no. PCR+)	NA	1	7 (70)	16 (62)	4 (100)	2 (67)	30 (68)
Fever only, no. (% of no. PCR+)	NA	0	6 (60)	5 (19)	2 (50)	0	13 (30)
Co-infections, no. and type	NA	0	1 SHUV, 1 EEV	1 WNV,† 1 EEV, 1 AHSV†	0	0	2 EEV, 1 ASHV,† 1 SHUV, 1 WNV†
SINV							
Specimens, no.	76	50	137	166	107	100	636
SINV+, no. (% of total specimens)	2 (3)	4 (8)	0	0	1 (1)	1 (1)	8 (1)
Deaths, no. (% of no.+)	0 (0)	2† (50)	NA	NA	1 (100)	0	3 (38)
Detected in CNS, no. (% of no.+)	–	2 (50)	NA	NA	0	NA	
Neurologic disease, no. (% of no.+)	1 (50)	2 (50)	NA	NA	1 (100)	0	4 (50)
Fever only, no. (% of no.+)	1 (50)	2 (50)	NA	NA	0	1 (100)	4 (50)
Co-infections, no. and type	1 EEV, 1 AHSV	2 WNV†	NA	NA	0	0	1 EEV, 1 AHSV, 2 WNV†

*AHSV, African horse sickness virus; CNS, central nervous system sample; EEV, equine encephalosis virus; MIDV, Middelburg virus; NA, not applicable; SHUV, Shunivirus; SINV, Sindbis virus; WNV, West Nile virus; –, undetectable in central nervous system sample; +, positive.
†Fatal cases with co-infections.

Conclusions

Besides CHIKV, SINV is the most widely distributed Old World alphavirus. It is associated with fever, rash, and arthralgia in humans in Europe, Asia, Africa, and Australia (*8*) and has been isolated from mosquitoes, birds, and humans in South Africa. Although antibodies described in livestock and horses, it has been unrecognized as a potential pathogen of horses. Less is known about MIDV. Isolated in 1957 from mosquitoes in South Africa, subsequent surveys identified antibodies in humans, horses, and livestock, and the virus has been isolated in mosquitoes and 2 humans elsewhere in Africa (*14*). In 1974, MIDV was isolated from blood of a horse during an outbreak of fever and icterus and was implicated in a horse with anorexia and muscular stiffness in South Africa (*14*) and from spleen of a horse in Zimbabwe in 1993 (*11*). Additional investigations have been limited, and the virus has not previously been associated with neurologic disease.

Table 2. Clinical signs associated with MIDV and SINV infections in horses, South Africa, 2008–2013*

Clinical sign	SINV, no. (%), n = 8	MIDV, no. (%), n = 44
Fever	5 (62.5)	20 (45.5)
Ataxia	2 (25.0)	16 (36.4)
Unspecified neurologic signs	2 (25.0)	14 (31.8)
Muscle fasciculation	0	4 (9.0)
Muscle weakness	1 (12.5)	2 (5.0)
Depression, listlessness	1 (12.5)	10 (22.7)
Hepatitis/Icterus	0	1 (2.0)
Limb paresis, paralysis	0	7 (15.9)
Recumbency	2 (25.0)	6 (13.6)
Tachycardia	0	6 (13.6)
Tachypnea, dyspnea	0	5 (11.4)
Fasciculations	0	4 (9.1)
Seizures	0	2 (4.5)
Abortion	0	1 (2.0)

*MIDV, Middelburg virus; SINV, Sindbis virus.

This study was not structured to provide definitive information on the prevalence and incidence of disease and likely underestimates the situation. Development of serologic assays for diagnosis of cases past the viremic stage are needed to establish the true prevalence and effects of disease, and IHC is needed for investigating the pathogenesis in horses and other species. Our findings strongly suggest that Old World alphaviruses, particularly MIDV, may constitute an overlooked cause of febrile and neurologic disease in horses and, like New World alphaviruses, may pose threats to horses, livestock, and humans.

Acknowledgments

The authors thank all veterinarians and horse owners who contributed cases to this study.

The study received funding through a Cooperate agreement grant with the Global Disease Detection Center, US Centers for Disease Control and Prevention, South Africa.

The study had Section 20 clearance from the Department of Agriculture Forestry and Fisheries of South Africa and ethical clearance from the University of Pretoria Faculty of Health ethics committee and Veterinary Science animal ethics committee.

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Spillover of Peste des Petits Ruminants Virus from Domestic to Wild Ruminants in the Serengeti Ecosystem, Tanzania

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We tested wildlife inhabiting areas near domestic livestock, pastures, and water sources in the Ngorongoro district in the Serengeti ecosystem of northern Tanzania and found 63% seropositivity for peste des petits ruminants virus. Sequencing of the viral genome from sick sheep in the area confirmed lineage II virus circulation.

Peste des petits ruminants (PPR) is caused by peste des petits ruminants virus (PPRV), a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, and primarily affects sheep and goats. Although PPRV can infect a wide range of domestic and nondomestic species, the disease has not been confirmed in free-ranging wildlife species in sub-Saharan Africa. Increased understanding of the epidemiology of PPRV infection in mixed species environments is urgently needed, especially because the virus range has apparently expanded in recent years, with associated social and economic effects of epidemics in areas where the disease had not been circulating, including programs for ongoing control (*1*).

PPR was first reported in northern Tanzania in 2008 and resulted from a southward spread from Kenya and Uganda by migrating livestock (*2*,*3*). No serologic and clinical reports of PPRV infection in wildlife occurred in sub-Saharan Africa during 2005–2013, although seropositivity was recorded in Uganda, Ethiopia, and other countries in West and Central Africa before this period (*4*). A study of serum samples collected from 331 wildlife 1–12 years of age in Tanzania, including in the Serengeti, Arusha, Katavi, and Tarangire National Parks and in the Ngorongoro Crater in the Ngorongoro Conservation Area (NCA), showed seronegative results for PPRV (*5*); however, that study did not include animals from our study area. Another study of >500 serum samples from wildlife in northeastern Kenya, tested during 2008–2010 for the Somali Ecosystem Rinderpest Eradication Coordination Unit program, also showed seronegative results for PPRV (F. Gakuya, pers. comm., 2012). In both studies, samples taken in Kenya and Tanzania were banked samples collected opportunistically during other research activities in the study areas. These results indicated that wildlife were not being infected by PPRV in this region; however, because of the opportunistic sampling, more targeted surveillance was considered necessary to confirm this PPRV-seronegative status (*5*). Given the proposed global eradication of PPRV by 2030 (*6*), we sought to determine the role of wildlife as possible hosts and sentinels of PPRV infection in northern Tanzania.

The Study

In June 2014, we investigated whether evidence could be found for PPRV infection in resident wildlife as a result of possible spillover from domestic animals (i.e., nomadic pastoral and agropastoral livestock), with whom they may share pasture and water resources in the Ngorongoro district within NCA (Figure 1). After obtaining ethical approval through the Animal Health and Welfare European Research Area Network; the Biotechnology and Biological Sciences Research Council; and Tanzania government departments responsible for research on wildlife and livestock, we collected serum samples from wildlife near resident livestock or from pastures and water sources shared with resident and nomadic livestock. Sampling was performed during the dry season, so only resident wildlife were sampled; migratory populations had already moved northwest from NCA. Both netting and chemical immobilization were used in 11 sampling sites, and samples were collected from 46 wild animals (Table 1). After the animals were restrained, they were clinically examined, and age was determined on the basis of incisor tooth eruption. Whole blood samples were collected with and without anticoagulant, and eye and nasal swabs were taken.

Similar sets of blood, eye, and nasal samples were collected from 5 domestic sheep and 5 goats that were reportedly ill with suspected signs of PPR (2 herds) or opportunistically sampled (1 herd) in locations across the wildlife range, which included 1 resident livestock herd

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DOI: http://dx.doi.org/10.3201/eid2112.150223

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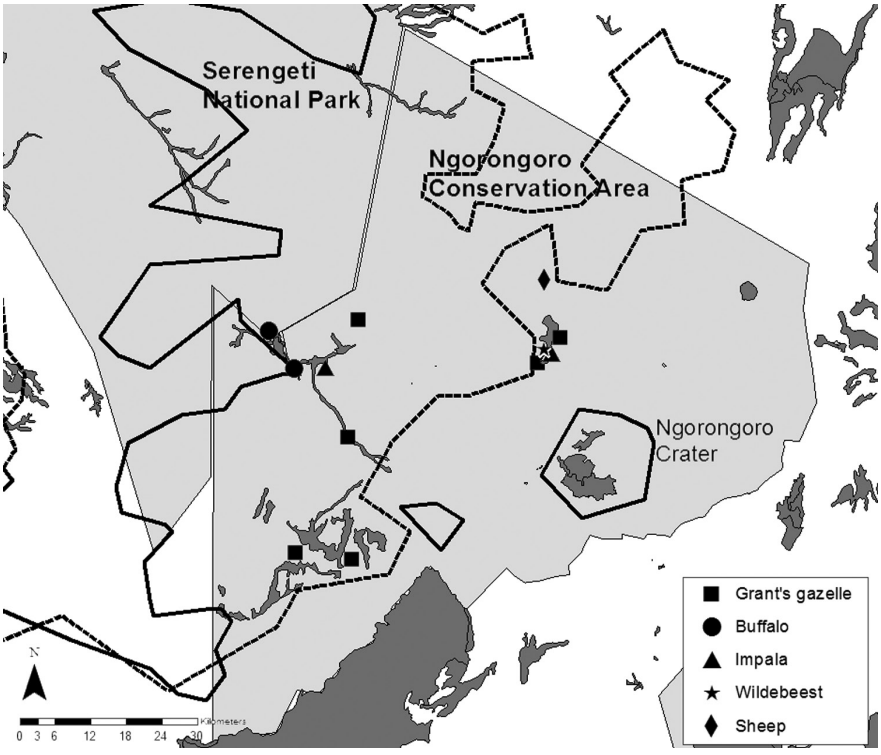

Figure 1. Geospatial map of sampling area showing distribution of buffalo (2014) and nonbuffalo (2006) species in the Greater Serengeti Ecosystem, northern Tanzania. Map is constructed on the basis of aerial census data and sites of livestock and wildlife herds sampled in June 2014 and found to be positive for peste des petits ruminants virus (PPRV) infection in the Ngorongoro Conservation Area.

in a high-altitude location and 2 nomadic livestock herds in low-altitude locations. No positive results were obtained from small livestock sampled in the highlands of Ngorongoro Crater. Three sheep (1 young, 1 semi-adult, and 1 adult) from the Esieki plains in the northern part of NCA were fresh cases with PPRV-specific clinical signs; their eye and nasal swab samples tested positive for PPRV antigen by a lateral flow device (*7*) (Table 2; online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0223-Techapp1.pdf). Although the adult animals had been vaccinated in April 2013, lambs <4 months of age and those born after that month were unvaccinated, creating a window for PPRV infection in the vaccinated herd and indicating a vital need to vaccinate all kids and lambs immediately after weaning, when they lose protection by maternal antibodies.

Real-time reverse transcription PCR (*9*) confirmed PPRV infection in all 3 PPRV-positive sheep from the Esieki plains. In addition, a sample from 1 Grant's gazelle in the Esieki plains was also positive (cycle threshold 34). Samples from 2 domestic young goats from Ngoile, NCA, were also positive (cycle threshold 32 and 37). Amplification of the PPR genome in gel-based PCR was possible only from swabs from the 3 clinically positive animals by using N-gene primers (*10*). The partial N-gene sequences available in GenBank for Africa through December 2014 were aligned and used for constructing a neighborhood-joining phylogenetic tree (Figure 2) that confirmed co-circulation of lineage II PPRV along with lineage III and IV in Tanzania (*11*–*14*). Recently, lineage II has been circulating in Central Africa (*12*,*13*). Possible incursion of lineage II from Central to East Africa, particularly to Tanzania, may have been overlooked because not all outbreaks are reported or investigated by viral genome sequencing.

In addition to infection among domestic livestock, detection of antibodies in blood samples by H c-ELISA (Biological Diagnostic Supplies Ltd., Ayrshire, UK) (*8*) showed PPRV seropositivity in all wildlife species and herds sampled across NCA except for Thomson's gazelle, but only 1 animal of this species was sampled (Table 1). Age-specific

Table 1. Seroprevalence of peste des petits ruminants virus in wildlife in the Ngorongoro Conservation Area, Tanzania, 2014

Species	No. sampled	No. positive/no. negative	Individual prevalence, %	No. herds sampled	No. positive/no. negative	Herd prevalence, %
Buffalo	10	5/5	50	2	2/0	100
Grant's gazelle	30	20/10	66	8	8/0	100
Thomson's gazelle	1	0/1	0	1	0/1	0
Wildebeest	2	1/1	50	1	1/0	100
Impala	3	3/0	100	1	1/0	100
Total	46	29/17	63	13	12/13	92

Table 2. Seroprevalence of peste des petits ruminants virus in domestic small ruminants in the Ngorongoro Conservation Area, Tanzania, 2014

Species	No. sampled	No. positive/no. negative	
		H c-ELISA*	LFD antigen test†
Goat	5	2/0	0/5
Sheep	5	0/2	3/2
Total	10	2/2	3/7

*Serum samples from 2 animals from each species were used to detect antibodies by H c-ELISA (*8*).
†Eye and nasal swab samples from 5 animals from each species were used for antigen testing with a lateral flow device (LFD) (*7*).

data for the buffalo and Grant's gazelle showed seropositivity increasing with age (online Technical Appendix Figure 2, panels A and B) and included animals <6 months of age.

Conclusions

Our findings provide evidence for PPRV infection in wildlife in East Africa. Recurring outbreaks in NCA in Tanzania confirm that PPRV, having recently emerged in this region, is likely now endemic in this area and is circulating

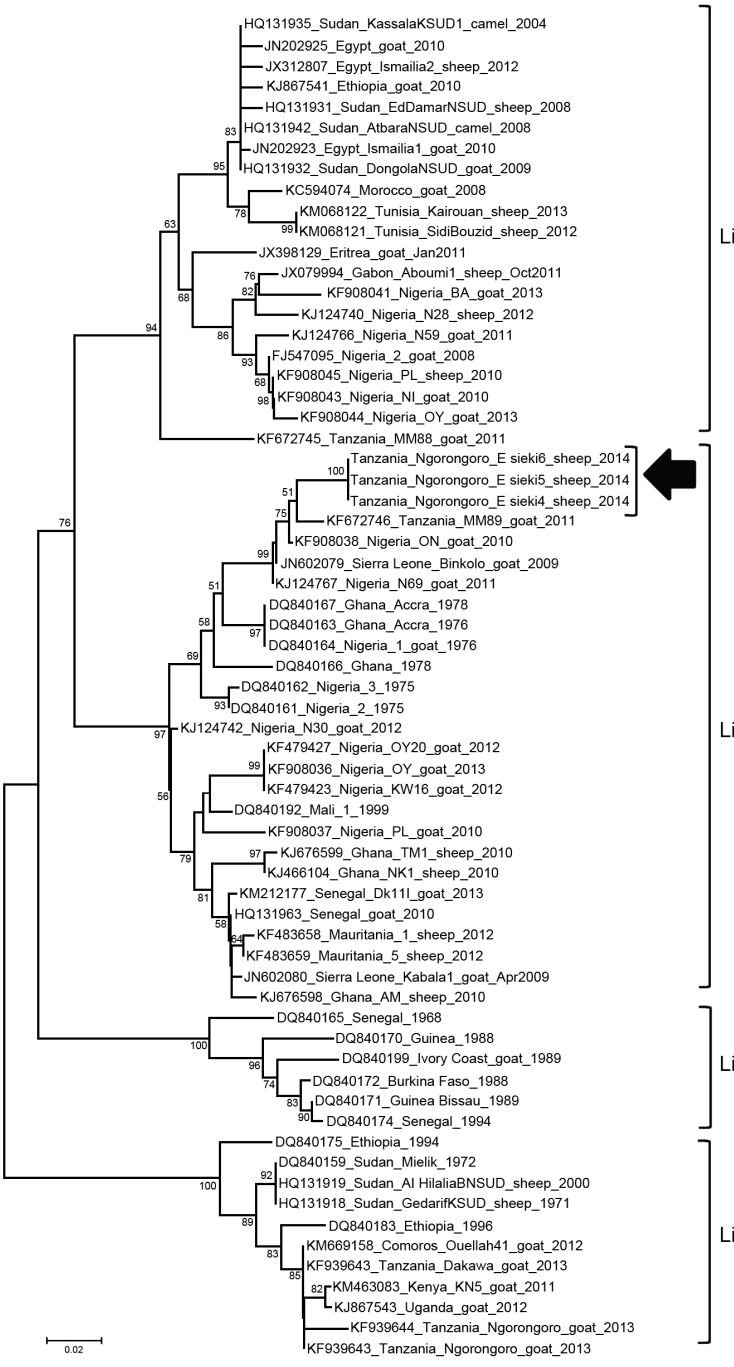

Figure 2. Neighbor-joining tree constructed on the basis of partial N-gene sequences of peste des petits ruminants virus (PPRV), showing relationships among the PPRV isolates from Africa. The Kimura 2-parameter model was used to calculate percentages (indicated by numbers beside branches) of replicate trees in which the associated taxa clustered together in 1,000 bootstrap replicates. Arrow indicates isolates sequenced in this study; sequences have been submitted to GenBank and are awaiting accession numbers. Scale bar indicates nucleotide substitutions per site.

among sheep, goats, and wildlife despite several rounds of mass vaccination. Most wild ruminant species and sampled subpopulations or herds sharing range with small livestock in NCA have been infected with PPRV, with the youngest wild animal confirmed antibody positive at ≈6 months of age, suggesting recent exposure.

Our sample represents resident wildlife in NCA and not migrating populations in the ecosystem. The positive result from a small resident herd of wildebeest near Olbalbal warrants closer examination of the PPRV status of migrating populations of wildebeest, Thomson's gazelle, and topi (a type of antelope, *Damaliscus lunatus*), which moved out of the area during April–May 2014. The single Thomson's gazelle sample is inconclusive. Age-specific data show that antibody prevalence rises with age, suggesting intermittent but regular exposure in the wildlife populations; however, circulation of the virus within and between the populations of each wildlife species is also possible.

The transmission and spread of PPRV appears to be considerable; high seroprevalence is observed at individual and herd levels, without all animals being infected, suggesting lower infective loads in the wildlife and a possibility that most infections could result from direct spillover of virus from infected livestock. The possibility of spillover infections is supported by the apparent absence of antibodies in the wildlife populations that have no contact with livestock (*5*).

Absence of clinical evidence in wildlife does not constitute evidence of absence of the disease. Antibodies were present in many wildlife we sampled, and the genome was present in 1 Grant's gazelle in the Esieki plains, where ongoing outbreaks were confirmed in domestic sheep. Clinical infections caused by PPRV have been recorded often in captive gazelle (*Gazella* species) (*15*) in United Arab Emirates. Currently, no evidence of wildlife disease exists, but cases or carcasses might go unnoticed because of deaths from other causes and rapid removal of dead animals by scavengers. These findings confirm endemic PPRV in the Greater Serengeti Ecosystem and suggest that free-ranging wildlife are susceptible to infection and can act as sentinels of livestock disease but do not appear to be maintaining infection across their populations.

Acknowledgments

We thank the Ministry of Livestock and Fisheries Development; Tanzania Wildlife Research Institute; Ngorongoro Conservation Area Authority; and the Tanzania Commission for Science and Technology for permissions and assistance with field activities.

We also thank the Biotechnology and Biological Sciences Research Council and the European Commission Animal Health and Welfare European Research Area Network fund for providing support for epidemiologic research under the Improved Understanding of the Epidemiology of Peste-des-Petits Ruminants project led by The Pirbright Institute (BB/L013657/1) and the Royal Veterinary College (BB/L013592/1).

Dr. Mahapatra is trained as a veterinarian, has a doctoral degree in molecular virology, and currently works as a senior scientist at the Pirbright Institute, UK. Her research interests include the epidemiology of peste des petits ruminants disease and the development of a DIVA vaccine that can be used in eradication of this disease.

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Porcine Epidemic Diarrhea Virus among Farmed Pigs, Ukraine

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An outbreak of porcine epidemic diarrhea occurred in the summer of 2014 in Ukraine, severely affecting piglets <10 days of age; the mortality rate approached 100%. Full genome sequencing showed the virus to be closely related to strains reported from North America, showing a sequence identity of up to 99.8%.

Porcine epidemic diarrhea (PED), caused by PED virus (PEDV) was first recognized in the United Kingdom in 1971 (*1*). Since then, outbreaks of PED have been documented in several European and Asian countries (*2*). PED has been reported in China since the 1980s; however, in October 2010, more virulent strains of PEDV emerged there, causing high fatality rates among suckling piglets (*3*). Since May 2013, similar virulent PEDV strains have been reported in the Unites States (*4*), and a report from Ohio indicated a second incursion into the USA by a slightly different PEDV strain (USA/OH851/2014, GenBank accession no. KJ399978), characterized by focal deletions in the S gene (*5*). PEDV was subsequently reported from Mexico (*6*), Canada (*7*), and other countries in the Americas (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/12/15-0272-Techapp1.pdf).

In May 2014, an outbreak of PED among pigs in the finishing stage of growth was reported in Germany and associated with a PEDV strain very similar to the OH851 variant of PEDV (*8*). Reports also indicate the presence of this PEDV variant in other European countries, including the Netherlands (*9*), Italy (*10*), and Spain (*11*). We investigated an outbreak of PED that occurred during the summer of 2014 in central Ukraine to determine the causative PEDV strain.

The Study

The outbreak occurred at a large, indoor, 5,000-sow farm in the Poltava region of Ukraine where 240 sows per week were kept to give birth, which is referred to as farrowing on porcine farms.

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DOI: http://dx.doi.org/10.3201/eid2112.150272

Clinical signs were first seen in a farrowing sow that was vomiting and had profuse diarrhea 10 days postfarrowing. Within hours, her piglets began to vomit and show profuse watery diarrhea. Vomiting and diarrhea then spread throughout the farrowing area. Disease was most severe among piglets <10 days of age; the case-fatality rate reached nearly 100%. The decision was made to euthanize piglets <10 days of age during a 3-week period from the start of the outbreak. Piglets >10 days old became sick, but most (95%) survived. Disease was less severe in adults, whose appetite returned and diarrhea ceased within 3 days.

Abortions occurred immediately following the outbreak in 38% of 1 batch of sows that were at 28–35 days' gestation; no diagnostic investigation was made to determine the cause. It was not established whether they were a result of the PED outbreak or control methods such as "back-feeding" fecal material to piglets. Abortions did not occur in other groups of sows and have not been reported as a feature of PED outbreaks in North America. The reproductive status of sows at other stages of gestation was not substantially affected by the initial PED outbreak, and no higher return rate was observed. Postpartum sows did not fail to produce milk, but those affected by PEDV had a reduced feed intake and associated reduced milk output.

The performance of the pig unit took 20 weeks to return to preoutbreak levels. During that time, a total of 30,000 piglets died, which equates to a loss of 6 weaned piglets per sow per year.

The outbreak was controlled by a combination of 2 methods. Lactogenic immunity enhancement was initiated by deliberate reexposure of pregnant sows to infected piglet feces 6 weeks before farrowing. The environmental viral load was reduced by cleaning and disinfection of the area, euthanizing of neonatal piglets, and reduction of transmission by humans and other vectors through enhanced internal biosecurity.

A diagnosis of PED was made after clinical and postmortem examination of affected piglets. We tested them onsite using a lateral flow device, Antigen Rapid PED/TGE Ag Test Kit (Bionote, Hwasung-si, Korea), which indicated the presence of PEDV antigen in the feces. We confirmed these findings at the UK Animal and Plant Health Agency using an in-house PEDV PCR and a commercially available PEDV/transmissible gastroenteritis virus quantitative reverse transcription-PCR kit (QIAGEN, Hilden, Germany). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the 160-nt PCR amplicon revealed the highest

similarity (99%) to PEDV strains from the United States and China. PEDV RNA was then subjected to deoxyribonuclease I digestion and converted to cDNA for preparation of sequencing libraries by using a Nextera XT kit (Illumina, San Diego, CA, USA). Paired-end sequencing was performed on an Illumina MiSeq. The consensus sequence was obtained by de novo assembly by using the Velvet 1.2.10 algorithm (*12*) of the sequence reads that mapped to the reference strain (GenBank accession no. NC003426).

The PEDV Ukraine/Poltava01/2014 strain genome (GenBank accession no. KP403954) is 27823 nt (excluding the 3′ poly A tail). Nucleotide analyses of the full genome of the virus showed the highest similarity to PEDV strains reported in 2013 from the United States; specifically, strains USA/Kansas29/2013 (GenBank accession no. KJ645637.1) and USA/Colorado30/2013 (GenBank accession no. KJ645638.1), with 99.8% nucleotide identity. The nucleotide identity was substantially lower (98.5%) when compared to those of the 2014 strains isolated in Germany, which are similar to another strain isolated in North America, Ohio851/2013 (*8*). Accordingly, the Ukraine virus clustered phylogenetically with PEDV strains from North America in genetic clade II (*6*) but was distinct from strains currently and previously found elsewhere in Europe, such as the prototype PEDV strain CV777, with which it shares only 96.5% homology (Figure [*13*]). However, the genetic analysis does not, at this stage, support drawing conclusions regarding the relative pathogenicity of this apparently virulent PEDV strain, similar to past instances of PEDV in Europe, of which very few have been characterized; parallel experimental infection of pigs would be required for further investigation.

The farm was found to be free from *Mycoplasma hyopneumoniae*, *Brachyspira hyodysenteriae*, mange, toxigenic *Pasteurella multocida,* porcine reproductive and respiratory syndrome virus, transmissible gastroenteritis virus, Aujeszky's Disease virus, and classical and African swine fever viruses. Persons on the farm practiced strict biosecurity measures to maintain a specific pathogen free status. No breach in biosecurity could be identified after a review of potential PEDV introduction routes involving pigs or humans or vehicles, equipment, and other fomites; the

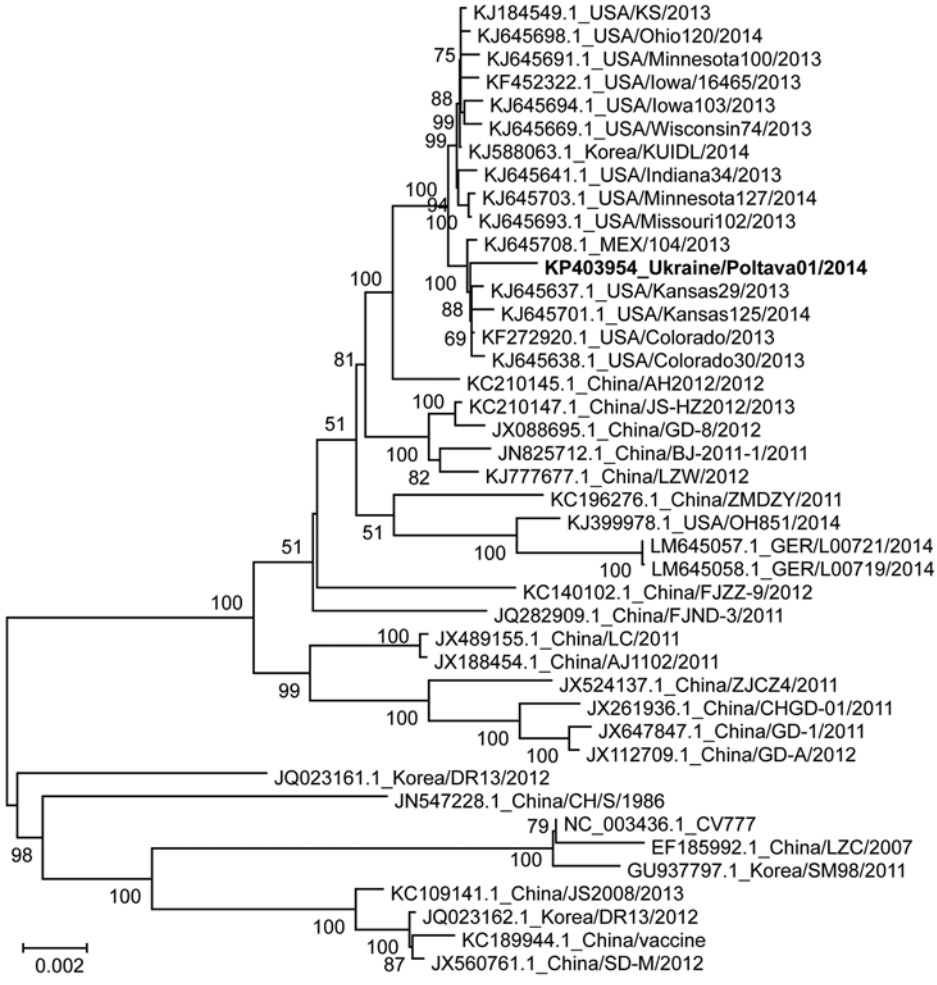

Figure. Phylogenetic analysis of the full-length genome of the porcine epidemic diarrhea virus (PEDV) Ukraine/Poltava01/2014 (bold text). The full-length genomes of PEDV were aligned by using the MegAlign software of the DNASTAR Lasergene Core Suite (DNASTAR, Inc., Madison, WI, USA) and phylogenetic analysis was done by using MEGA 5.2 software (*13*). The tree was constructed by using the neighbor-joining method and 1,000 bootstrap replications. Only bootstrap values of more than 50% are shown in the figure. Each virus on the tree is represented by accession number, strain, and year of sample collection. Ukraine/Poltava01/2014 clusters in close proximity to recent strains in the United States other than the Ohio851 variant, and both are substantially genetically different from the previous European variants, such as the prototype strain CV777, which is embedded in another cluster. Scale bar indicates nucleotide substitutions per site.

source of infection remains unknown. However, there were reports of a PED-affected pig farm 1.5 km from this unit, and the potential for unidentified fomite or other transmission from this herd, or another undisclosed infected herd, therefore existed. Windborne transmission has been suggested to explain some outbreaks in the United States (*14*) and is another possibility to consider in this case.

Conclusions

This PED outbreak in Ukraine showed clinical characteristics similar to outbreaks caused by virulent strains of PEDV reported from North America (*4*). The virus clusters phylogenetically with viruses from recent outbreaks in the United States and Mexico (*6*). The presence of such a PEDV strain in Ukraine highlights the threat to neighboring countries and those in the European Union where PEDV has not been detected (e.g., Scandinavia) or has not caused disease in recent decades (e.g., the United Kingdom) and where pig herds are considered largely naive to PEDV. Furthermore, pig farming has changed over recent decades, and the establishment of more large holdings would produce more virus following introduction of PEDV.

This outbreak emphasizes the need for decision-makers of countries, pig farms, and allied industries to implement and maintain biosecurity measures to minimize the risk for spread of PEDV to new areas. Early detection of suspected clinical signs on pig farms and, when these occur, prompt testing for PEDV by PCR are also vital (*15*). A coordinated approach is essential to prevent introduction of PEDV, promote early detection should introduction occur, control disease, and minimize spread of infection.

Acknowledgments

We thank S. Anna La Rocca and Christopher Finnegan for performing the confirmatory PCRs and preparing the sample for NGS sequencing.

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Isolation of Porcine Epidemic Diarrhea Virus during Outbreaks in South Korea, 2013–2014

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DOI: http://dx.doi.org/10.3201/eid2112.150437

To the Editor: Porcine epidemic diarrhea (PED) is an acute infectious diarrhea caused by the PED virus (PEDV), which belongs to the order *Nidovirales*, family *Coronaviridae*, genus *Alphacoronavirus* (*1*). The virus is transmitted mainly through fecal–oral routes and infects all age groups of pigs; the most severe form of disease occurs in suckling piglets (*1*). PEDV was first reported in South Korea in 1992 (*2*), with the occurrence of an outbreak, and has since circulated with considerable genetic diversity (*1*,*3*). During 2013, PED outbreaks reoccurred in South Korea; however, the emerging PEDVs in these outbreaks were not variants of previous Korean isolates or attenuated vaccine strains (*4*,*5*). We report on a field isolate of a novel emerging PEDV and the isolate's genetic relationship with other PEDV strains.

During October 2013–June 2014, dead piglets and fecal swabs from 9 provinces of South Korea were sent to the Department of Veterinary Medicine Virology Laboratory at Seoul National University to confirm diagnoses of enteric viral diseases. All samples (30 intestine samples of dead piglets and 16 fecal swabs) were found to be PEDV positive. Attempts to isolate the field strains of PEDV on Vero cell lines followed a previously described protocol with modifications (*6*). An overnight monolayer of Vero cells (80%–100% confluence) was washed twice with 1× phosphate-buffered saline before homogenized samples (0.02 μm filtered) were inoculated with 10% suspension. After 30 min absorption at 37°C with 5% CO_2, maintenance medium (Dulbecco's Modified Eagle Medium supplemented with trypsin [10 μg/mL]), yeast extract (0.04%), tryptose phosphate broth (0.6%), and Antibiotic-Antimycotic 100´ (4 μl/mL; Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) were added at a ratio of 1:10. The inoculated cells were cultured for 3–4 days at 37°C in 5% CO_2 atmosphere and were blindly passaged 5 times. One field strain of PEDV (named BM1) was successfully adapted for growth on Vero cells. This virus was isolated from a 60-sow farm (identified as BM farm) that had not vaccinated its animals against PEDV. Pigs of all ages from the farm showed clinical symptoms of diarrhea, and death occurred for 100% of suckling piglets and 10% of sows. Examination at necropsy revealed that the dead piglets from BM farm were covered with brown blotches of dried diarrheal feces and their stomachs were filled with undigested milk. Thin, translucent small intestines that contained yellow fluid were also observed (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0437-Techapp.pdf). The BM1 PEDV field isolate induced cytopathic effects of rounded shape (online Technical Appendix Figure 2, panel A) within 48 hours at passage 10. The presence of PEDV in the cell culture was confirmed by immunofluorescence assay (VDPro PEDV FA Reagent kit, MEDIAN Diagnostics, Gangwon-do, South Korea), which showed the specific fluorescence signal (online Technical Appendix Figure 2, panel B). In addition to evidence by microscopic observation, real-time reverse transcription PCR showed that the quantity of viral RNA increased incrementally as the number of passages increased: from 30,325 copies/μL (cycle threshold 16.11) at passage 2 to 418,000 copies/μL (cycle threshold 13.77) at passage 10. Infective titers of the BM1 isolate increased from $10^{4.7}$ 50% tissue culture infectious doses/mL at passage 2 to $10^{7.9}$ 50% tissue culture infectious doses/mL at passage 10 (online Technical Appendix; online Technical Appendix Table 2).

The complete S gene of BM1 (GenBank accession no. KP861982) was sequenced for genetic characterization; the gene was 4,161-nt long and encoded 1,386 aa. The spike protein of the BM1 isolate showed substitutions at neutralizing SS6 epitope from LQDGQVKI (*7*) to SQSGQVKI but identity at the SS2 (*7*) and 2C10 (*8*) neutralizing epitopes. The genetic relationship of the BM1 isolate with other PEDVs in the world was inferred from a codon-based alignment of 409 sequences of the complete S gene (online Technical Appendix Table 3). The maximum-likelihood phylogenetic tree was constructed by using the FastTree program (*9*), with the general time reversible nucleotide substitution model. The phylogeny constructed on the basis of the complete S gene (Figure) showed that the BM1 isolate belongs to subgroup 2a, genogroup 2 of PEDV. This isolate clustered closely with emergent PEDV strains in the United States (online Technical Appendix Figure 3), showing 99.2%–99.7% identity with PEDVs of North American

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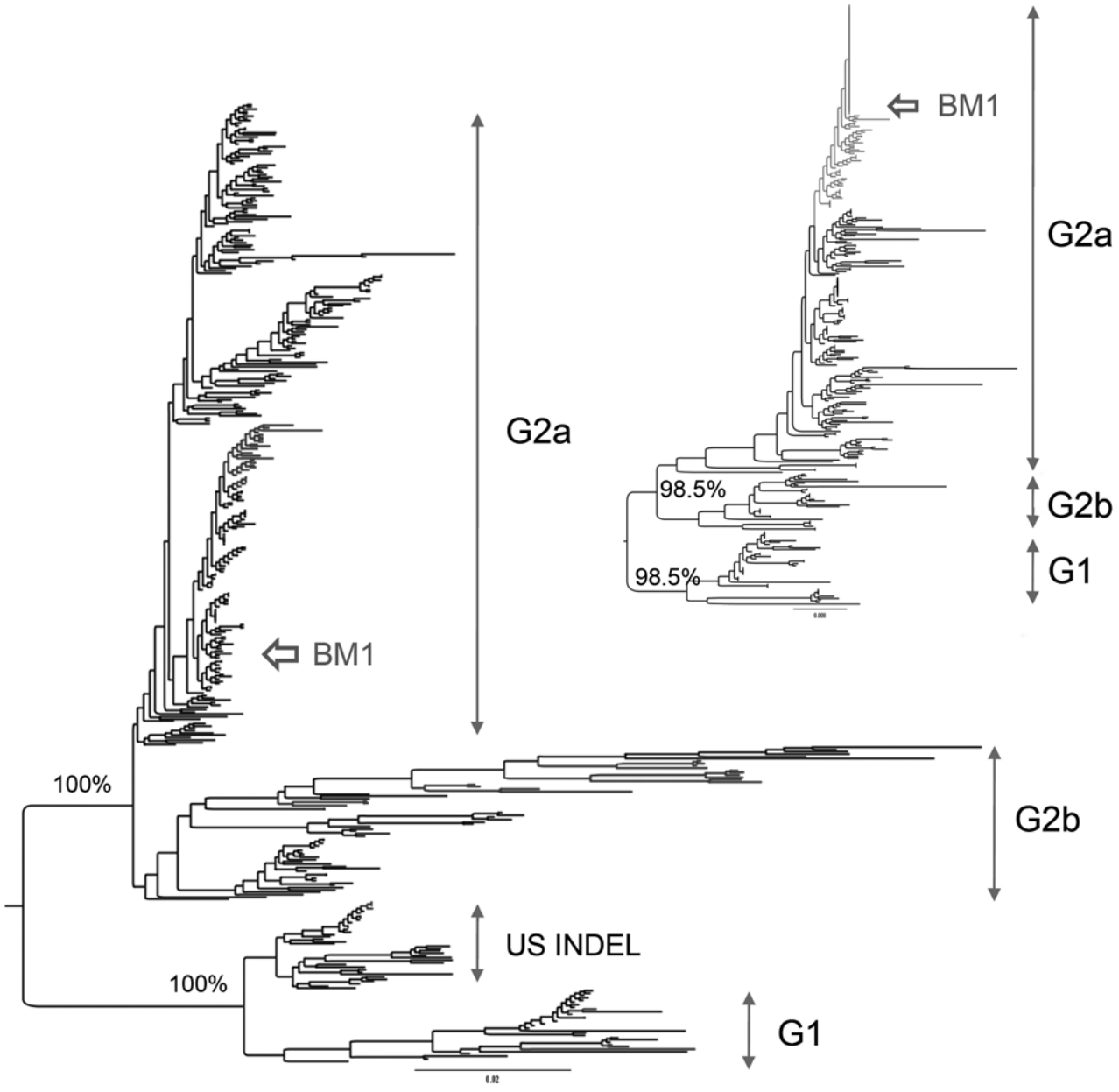

Figure. Maximum-likelihood phylogenetic tree of porcine epidemic diarrhea virus from piglet, South Korea, 2013–2014, constructed on the basis of codon alignment of complete S genes. Inset shows a phylogenetic tree inferred from the complete N genes. Genogroups are shown to the right of each tree. US INDEL is a prototype strain of porcine epidemic diarrhea virus that has insertions and deletions (INDELs) in the spike gene. Scale bars indicate nucleotide substitutions per site. A color version of this figure is available online (http://wwwnc.cdc.gov/eid/article/21/2/15-0437-F.htm).

strains (*10*). This observation was repeated by the phylogenetic inference of the complete N gene (Figure; online Technical Appendix Table 4 and Figure 4). The branching pattern (Figure) clearly showed that BM1 is genetically less related (92.9–93.4% identity) to the live vaccine strains that are derived from genogroup 1 and used currently to prevent PEDV infections in South Korea.

In summary, we isolated the BM1 strain (GenBank accession no. KP861982) in South Korea from a sample from a suckling pig with severe diarrhea; the pig came from a farm that had not vaccinated its pigs against PEDV. The strain was adapted and grew to high titers on Vero cells. The isolate belongs to genogroup 2 and genetically clustered with emerging PEDVs of North American

strains but was loosely related to genogroup 1, the basis of the vaccine used for inoculation against Korean PEDV strains. This isolate may need further evaluation as a candidate for a vaccine to prevent reemerging PEDVs in South Korea.

Acknowledgments

This study was supported by the BioGreen 21 Program, Rural Development Administration (grant no. PJ011184), and by the Bio-industry Technology Development Program (grant no. 114055031SB010), Ministry of Agriculture, Food and Rural Affairs, South Korea.

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Tickborne Lymphadenopathy Complicated by Acute Myopericarditis, Spain

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DOI: http://dx.doi.org/10.3201/eid2112.150672

To the Editor: *Dermacentor*-borne necrosis erythema lymphadenopathy/tickborne lymphadenopathy (DEBONEL/TIBOLA) is an apparently benign, self-limiting rickettsial disease transmitted by *Dermacentor* ticks (*1*,*2*). *Rickettsia slovaca* was the first etiologic agent isolated, but other species, such as *R. raoultii* and *Candidatus* R. rioja, also might be involved (*3*–*6*). If the scalp is affected, a larger number of agents (including *Francisella tularensis*, *Bartonella henselae*, *R. massiliae*, *R. sibirica mongolitimonae*, and *Borrelia burgdorferi*) should be considered within the differential diagnosis of a similar syndrome recently named scalp eschar associated with neck lymphadenopathy after a tick bite (SENLAT) (*7*). Nevertheless, in Spain, only *R. slovaca*, *Candidatus R. rioja*, and *F. tularensis* are known to cause DEBONEL/TIBOLA/SENLAT (*4*,*6*). This entity is considered an emerging rickettsiosis in Europe; cases have been reported from Italy, France, Hungary, Germany, and Portugal (*8*).

We recently saw a patient in whom acute myopericarditis developed after he was bitten by a large tick on the scalp and showed clinical signs of DEBONEL/TIBOLA/SENLAT, most likely attributable to *R. slovaca* or *Candidatus* R. rioja infection. The patient, a previously healthy 28-year-old man, went on a day-long hiking trip to the northern mountains of Madrid (central Spain; mean altitude 1,300 m) on November 2, 2014. Three days later, he noticed a mild ache on the occipital area of his scalp and found an attached tick that he removed with his fingers. A week later, he sought care from an infectious disease specialist because of itchy discomfort at the area of the tick bite.

Examination revealed an erythematous and elevated punctiform lesion with mild fluctuation in the occipital region accompanied by tender, small lymph node enlargement of both occipital lymphatic chains (Figure). No widespread rash was present. DEBONEL/TIBOLA/SENLAT was diagnosed, and doxycycline (100 mg every 12 hours) was initiated. IgG titer against spotted fever group *Rickettsia*

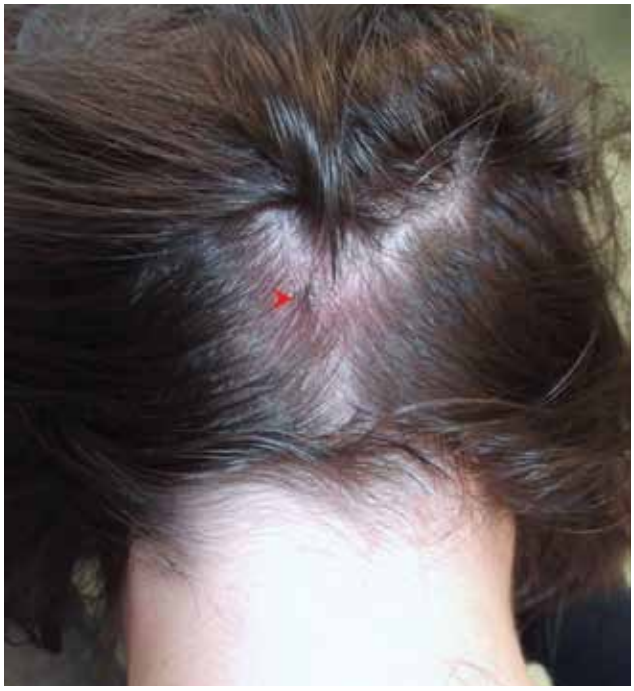

Figure. *Dermacentor*-borne necrosis erythema lymphadenopathy/tickborne lymphadenopathy/scalp eschar associated with neck lymphadenopathy after a tick bite. Shown is an erythematous, punctiform lesion in the scalp (arrow), accompanied by enlarged occipital lymph nodes. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/21/12/15-0672-F.htm).

(SFGR) was 1:128. Four days later, the patient sought care at an emergency department, reporting retrosternal chest pain. Electrocardiogram revealed a diffuse ST-segment elevation with PR-segment depression; serum creatine phosphokinase and troponin T levels were 327 IU/L (reference range 10–190 IU/L) and 420 ng/mL (reference <14 ng/mL), respectively. Myopericarditis was diagnosed. A transthoracic echocardiogram ruled out pericardial effusion, valve vegetations, and left ventricular dysfunction; cardiovascular magnetic resonance imaging performed 4 days later showed myocardial inflammation. Blood cultures were sterile, pneumococcal urinary antigen test result was negative, and IgM against coxsackievirus and *Mycoplasma pneumoniae* were not detected. Nonsteroidal antiinflammatory drugs were prescribed. The patient improved clinically, and electrocardiogram findings resolved. The patient received doxycycline for 4 weeks.

On a convalescent-phase serum specimen collected after 8 weeks, indirect immunofluorescence assays (IFA) for IgG against SFGR were performed in Spain's national reference center for rickettsioses (Hospital San Pedro–Centro de Investigación Biomédica de La Rioja [CIBIR], Logroño, Spain). Commercial (Focus Diagnostics, Cypress, CA, USA) and in-house *R. conorii*, *R. slovaca*, and *R. raoultii* antibody testing showed an IgG titer of 1:512 against the 3 species. A subsequent cross-adsorption assay using *R. slovaca*, *R. raoultii*, and *R. conorii* antigens prepared on the basis of strains from the collection at Hospital San Pedro-CIBIR showed a decrease in IgG titers against *R. conorii* and *R. raoultii* to 1:64 and 1:256, respectively, whereas titer against *R. slovaca* remained at 512. IFA against *Bartonella* spp. and *C. burnetii* (Focus Diagnostics), chemiluminescence immunoassay for *B. burgdorferi* (Liason, DiaSorin, Spain), and in-house microagglutination assay for *F. tularensis* were not reactive. The patient recovered, with only a residual scarring alopecia on the occipital region of the scalp and without cardiac dysfunction after 9-month follow-up.

Myopericarditis is a rare complication of rickettsiosis, usually associated with *R. rickettsii* and *R. conorii* (*9*). Although tetracycline-induced cardiac adverse reactions have been described (*10*) and the patient reported here had signs of myopericarditis shortly after the initiation of doxycycline, he completed a 4-week treatment without recurrence. Therefore, the clinical picture seems unlikely to be attributable to doxycycline-induced toxicity. Because the patient was bitten in November (when only *Dermacentor* spp. ticks are active in central Spain), we have further epidemiologic evidence for attributing the infection to SFGR causing DEBONEL/TIBOLA/SENLAT. After serum adsorption, IFA titer against *R. slovaca* was 3-fold higher than that against *R. conorii*. *R. slovaca* and *Candidatus* R. rioja are the species most commonly found in *D. marginatus* ticks and in cases of DEBONEL/TIBOLA/SENLAT in Spain (*8*).

In view of the seroconversion to *Rickettsia* spp. with negative test results for other possible causative agents and the clinical response to doxycycline, rickettsiosis caused by *R. slovaca* or *Candidatus* R. rioja remains the most probable diagnosis. Because DEBONEL/TIBOLA/SENLAT is an emerging disease, physicians should consider that this entity may be associated with systemic complications similar to those of other tickborne rickettsioses.

Acknowledgment

We thank P. Anda for providing the *F. tularensis* antigen.

M.F.R. holds a clinical research contract "Juan Rodés" (JR14/00036) from the Spanish Ministry of Economy and Competitiveness, Instituto de Salud Carlos III.

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Parainfluenza Virus 5 as Possible Cause of Severe Respiratory Disease in Calves, China

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DOI: http://dx.doi.org/10.3201/eid2112.141111

To the Editor: Parainfluenza virus 5 (PIV5), family *Paramyxoviridae*, genus *Rubulavirus*, was previously known as simian virus 5 because of its discovery in primary monkey kidney cells in 1954 (*1*). PIV5 was later isolated from various hosts, including humans, dogs, pigs, cats, and rodents. The neutralizing antibody for PIV5 is detectable in symptomatic and asymptomatic humans; thus, its association with human disease remains controversial (*2*). In addition, previous studies have not documented illness in infected animals, except kennel cough in dogs (*1,3*). Isolation of PIV5 from cattle has not previously been reported.

Since 2012, an infectious respiratory disease has been prevalent in weaning calves (≈10 d to 2 mo of age) in Baicheng City, Jilin Province, China. Initial clinical signs included secretion of clear nasal mucus, anorexia, sluggishness, and loss of body weight. After 10–20 d, ≈10% of the sick calves died of dyspnea and interstitial pneumonia. Farmers observed that 80%–90% of calves in the affected farms demonstrated clinical signs, but most recovered. All attempts of local veterinarians to treat the animals with various chemical compounds and antimicrobial drugs failed. The disease persists throughout the year but occurs mainly during spring (from February through March), which has resulted in substantial economic losses in the cattle industry.

To identify the causative agent of the disease, we tested 15 lung samples from calves that had died and 10 lung samples from healthy calves that were slaughtered for serum products (all from 1 farm). The samples were homogenized in phosphate-buffered saline and analyzed by using electron microscopy. Paramyxovirus-like particles were identified in the tissues of sick calves. Reverse transcription PCR with the generic primers for the paramyxovirus polymerase gene was performed (*4*).

Of the 25 calf specimens, the 15 samples from the sick calves were positive by reverse transcription PCR, and amplicons of the expected size were obtained and sequenced. The generated sequences were closely related to the PIV5 sequences available from GenBank, particularly to sequences of the recently identified KNU-11 and SER viruses in pigs (*5,6*).

The suspensions of lung tissue from sick calves were purified by centrifugation at 12,000 × *g* for 5 min, and 0.2 mL of the supernatant was added to a Vero cell monolayer in a 25-cm^2 cell culture flask (EasyFlasks; Thermo Fisher Scientific, Odense, Denmark). After virus adsorption for 1 h at 37°C, the cell monolayer was rinsed with phosphate-buffered saline (pH 7.4) and then incubated in Dulbecco minimal essential medium/2% newborn calf serum at 37°C in a 5% CO_2-humidified incubator. The infected cells were serially passaged every 3 days at 37°C and detected by using monoclonal antibody against SV5 (AbD Serotec; Bio-Rad, Kidlington, UK) by indirect fluorescent antibody test (*7*). A PIV5 strain was isolated in the cell culture and designated PIV5-BC14 (BC14 stands for Baicheng City 2014).

For amplification and analysis of the full-length viral genome, 13 pairs of primers covering overlapping

[1]These authors contributed equally to this article.

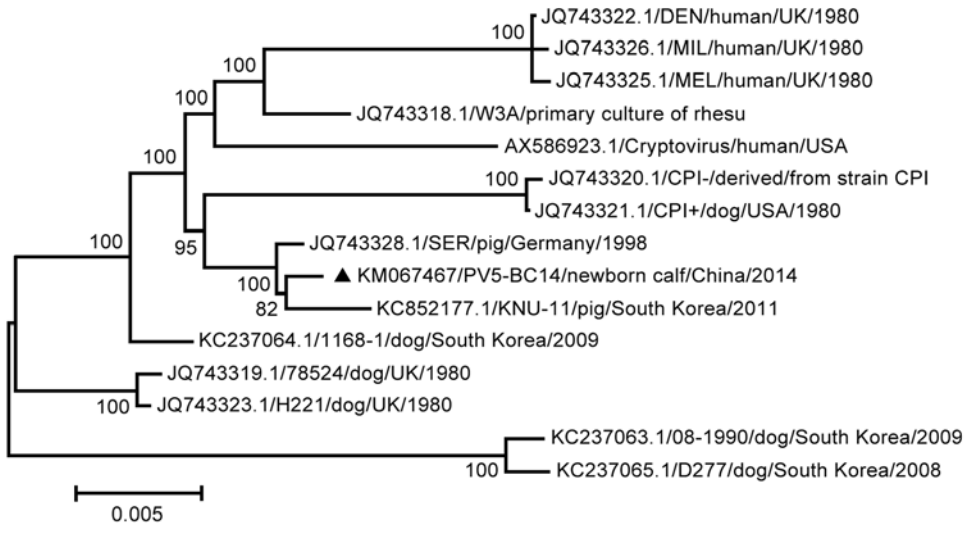

Figure. Maximum-likelihood phylogenetic tree based on the complete genome sequences of parainfluenza virus 5 (PIV5). The black triangle indicates isolate PIV5-BC14 (Baicheng City 2014). Scale bar indicates nucleotide substitutions per site.

fragments of the genome were designed on the basis of the sequence of the PIV5 isolate KNU-11 (*8*). The 3′ and 5′ termini of the genome were resolved by using the 3′ and 5′ Full RACE Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PIV5-BC14 genome (GenBank accession no. KM067467) was 15,246 nt with coding and untranslated regions at the same positions as in other PIV5 isolates (*9*). However, comparison of this genome with 15 available full-length genomes of PIVs revealed that 18 nt substitutions, resulting in 9 aa changes, are found only in PIV5-BC14. Among these 9 aa changes, 5 (at positions 303, 634, 1054, 1722, and 1773) are present in an RNA-dependent RNA polymerase protein, 2 in a phosphoprotein (at positions 43 and 332), 1 in a nucleoprotein (at position 75), and 1 in a hemagglutinin–neuraminidase (at position 322).

The highest nucleotide identity (99.72%/99.52%) was observed between PIV5-BC14 and porcine PIV, particularly with the SER virus isolate. This observation was confirmed after construction of a phylogenetic tree based on the 15 available nucleotide sequences of the full-length genomes (Figure). The analysis was carried out by using the maximum-likelihood method in MEGA 5.0 (*10*), and the reliability of tree topology was evaluated through bootstrapping with 1,000 replicates.

Although the pathogenic role of PIV5 infections in cattle remains unknown, no PIV5 RNA was found in any apparently healthy cattle from the same farm. This result suggests a strong relationship between the identified virus and the disease.

This research was funded by the China National "973" Program (approval no. 2011CB504700).

Drs. Liu and Li are both research fellows at the Academy of Military Medical Sciences (China). Their research interests include rabies epidemiology and vaccines. More recently, they are both working on emerging and reemerging infectious diseases in animals in China.

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Alternative Routes of Zoonotic Vaccinia Virus Transmission, Brazil

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DOI: http://dx.doi.org/10.3201/edit2112.141249

To the Editor: Vaccinia virus (VACV) causes exanthematous disease (bovine vaccinia) in Brazil. Outbreaks of this disease in humans have been reported since the late 1990s and have spread throughout Brazil (*1*). Natural human infections with VACV occur by close contact with infected cattle during milking. Lesions can spread to secondary body sites (forearms, arms, and face). Thus, person-to-person transmission occurs (*1*).

Moreover, virus can persist in household environments, remain infectious, and be transmitted by fomites (*2*). Although raw milk and cheese are potential sources of infection, no clinical cases have been associated with this transmission route (*3,4*). Data for person-to-person transmission in Brazil are scarce, but person-to-person transmission was recently reported (*5*). We report a possible case of person-to-person transmission of VACV.

This study was approved by the Research Ethics Committee of Universidade Federal de Minas Gerais (registration protocol FR-413704). In September 2012, during a serologic survey in a rural area of Serro City (18°36′17″S, 43°22′46″W), Minas Gerais, Brazil (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/21/12/14-1249-Techapp1.pdf), blood samples were obtained from a family of 5 persons (father, mother, and 3 daughters). The father and mother were 48 and 53 years of age, respectively, and had been vaccinated against smallpox. They reported contact with cows and horses (online Technical Appendix Table 1). Only the father had milked cows. The 3 daughters (13, 13, and 14 years of age) did not engage in any exposure activity. However, all family members had consumed raw milk and cheese.

Bovine vaccinia lesions were observed on the hand of the father (online Technical Appendix Figure, panel B). In 2011, he had vesicular disease (no laboratory diagnosis) with clinical and epidemiologic features (lesions) suggestive of bovine vaccinia on his hands and forearms and systemic symptoms (fever, headache, malaise, myalgia, lymphadenopathy, and abdominal pain). His symptoms were mild and without any systemic clinical features. Two lesions developed on his hands and dried swab samples were collected from both lesions. Swab samples were processed as described (*2*) and used for virus isolation and molecular diagnosis.

On the basis of previous studies that detected viral DNA in clinical samples from persons with bovine vaccinia (*1*), we used a quantitative PCR to amplify the *vgf* and *ha* genes of VACV (*3–5*), a standard PCR to detect the *ha* gene (*3–5*), and a seminested PCR to detect the *ati* gene (F.L. Assis, unpub. data). Serum samples were used for detection of virus-neutralizing antibodies (orthopoxvirus 50% plaque-reduction neutralization test) and molecular diagnostic studies (*1*). Virus isolation was attempted in Vero cells and chorioallantoic membrane. All results were negative.

The 50% plaque-reduction neutralization test showed that the father, mother, and 14-year-old daughter had neutralizing antibodies against orthopoxvirus (titers 800, 3,200, and 800 neutralizing units/mL, respectively). All family members had positive results by molecular diagnostic test for ≥1 virus gene (online Technical Appendix Table 1). To rule out infection with parapopoxvirus, a complementary PCR (*6*) was also performed, and all family members had negative results.

Quantitative PCR products for the *ha* gene from 3 virus-positive samples were sequenced in both directions in triplicate (Mega BACE Sequencer; GE Healthcare, Little Chalfont, UK). Sequences were aligned by using ClustalW (http://www.genome.jp/tools/clustalw/) and MEGA4.1 (http://www.megasoftware.net/) and showed 100% identity with each other (Figure). A phylogenetic tree was constructed by using the neighbor-joining method and 1,000-bootstrap replicates in the Tamura-3 parameter model (MEGA4.1). Sequences were grouped with VACV group 2 isolates. Sequences obtained were deposited in GenBank under accession nos. KP889223–5).

In Brazil, outbreaks of bovine vaccinia are associated with rural environments. However, some clinical and

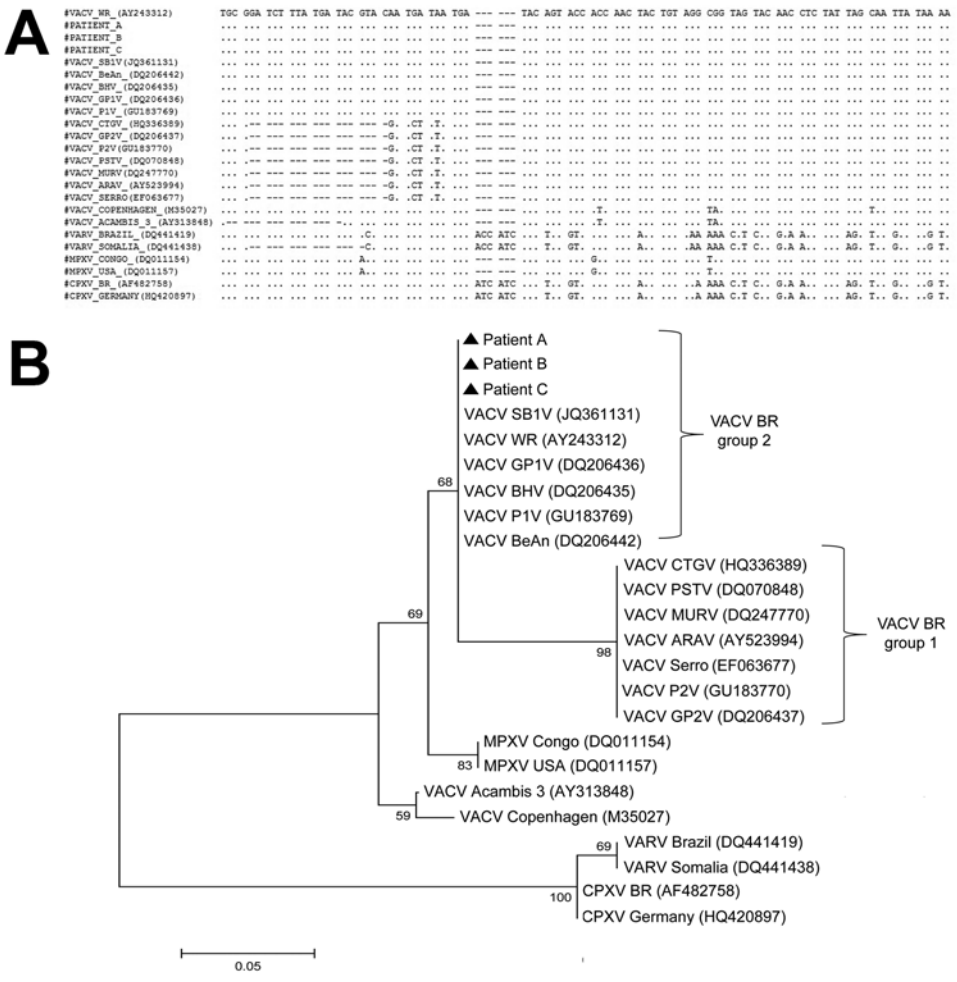

Figure. A) Nucleotide sequence of vaccinia virus (VACV) hemagglutinin gene and homologous sequences of several orthopoxviruses, Brazil. Dots indicate sequence identity; dashes indicate deletions. VARV, variola virus; MPXV, monkeypox virus; CPXV, cowpox virus. B) Consensus phylogenetic tree based on nucleotide sequences of orthopoxvirus hemagglutinin genes. Tree was constructed with hemagglutinin gene sequences by using the neighbor-joining method with 1,000 bootstrap replicates and the Tamura 3-parameter model in MEGA4 (http://www.megasoftware.net/). Strains had the deletion region conserved and were grouped with other VACV (group 2) isolated in Brazil. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

epidemiologic aspects remain unclear. The infection of the father was associated with direct contact with cattle. Immunity conferred by smallpox vaccination did not prevent infection; this lack of immune response has been demonstrated in other studies in Brazil (*7*). Long-term protection might require multiple virus exposures, and severity of poxvirus infections might be influenced by the immunologic state of the host and virulence of virus strains (*1*,*8*,*9*).

The mother and 2 daughters with virus DNA in blood samples and the 14-year-old daughter with high titers of virus-neutralizing antibodies suggest that alternative routes (other than milking) for VACV infection of humans should be considered. These alternative routes can include person-to-person or environmental transmission because the 2 daughters did not report any exposure activities related to milking or contact with cattle (online Technical Appendix Tables 1, 2). Persistence of VACV in household environments has been reported (*2*,*10*). The family also consumed raw milk and cheese, a common practice in the region. Therefore, infection with VACV through raw milk and cheese consumption should also be considered. The patients did not report oral lesions or a history of skin/mucosal lesions.

In conclusion, person-to-person transmission of VACV in these cases might have been caused by direct contact between the father and family members, contact with virus in the home, or consumption of unpasteurized milk and cheese. Additional studies are necessary to elucidate the role of these transmission pathways in spread of VACV in Brazil.

Acknowledgments

We thank João Rodrigues dos Santos and colleagues for excellent technical support and the Instituto Mineiro de Agropecuária for assistance.

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, and Pró-Reitoria de Pesquisa/Universidade Federal de Minas Gerais. G.S.T., P.C.P.F., and E.G.K. are researchers of the Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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Hunter Island Group Phlebovirus in Ticks, Australia

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DOI: http://dx.doi.org/10.3201/eid2112.141303

To the Editor: A recent article described the isolation and subsequent analysis of a tickborne phlebovirus: Hunter Island Group virus (HIGV), associated with an albatross disease event that occurred in 2002 on Albatross Island, 6 kilometers off the northwest coast of Tasmania, Australia (*1*). The authors present HIGV as a novel isolate; however, new data and historical records demonstrate that the virus was originally isolated in 1983. Provisionally named Albatross Island virus (ABIV), the virus was classified as unidentified because of its uniqueness and dissimilarity to any known virus in Australia. ABIV and HIGV were isolated from ticks of the same species, *Ixodes eudyptidis*, collected from the nests of shy albatross (*Thalassarche cauta*) on Albatross Island, the only island inhabited by albatross within the Hunter Island Group Important Bird Area. At the time of collections, many immature albatross were dying. Records from this time indicate that postmortem blood samples were collected from the birds, and subsequent virus neutralization studies conducted soon after demonstrated that 50% of these samples were ABIV positive. Ensuing testing of samples collected in the next 2 years also identified a positive sample from a black noddy in Queensland (Table). ABIV was subsequently sent for testing at the Arbovirus World Reference Laboratory and, more than a decade later, to the Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation, where it was identified as a bunyavirus but remained largely uncharacterized.

We recently sequenced the genome of ABIV by using high-throughput sequencing and have compiled near complete sequences for the large (L), medium (M), and small (S) segments (GenBank accession nos. KM198925–7). Overall, ABIV shares 99% nt identity with HIGV, and thus they can be considered isolates of the same virus. The translated nucleocapsid and S segment nonstructural proteins of both viruses are identical, and the polymerases and glycoproteins share 99% identity. There are 26 nt changes across the whole genome (1 in S, 8 in M, 17 in L), but only 7 of these translate into an amino acid change (3 in the Gn/Gc polyprotein, 4 in the polymerase protein). Predictive protein analysis indicates that at least 1 of the 3 aa changes occurs in the ectodomain of the Gn protein, which could affect virus–host interactions. Of the remaining changes, 14 are silent mutations and 5 occur in noncoding regions.

In light of the genomic similarity of these 2 viruses, we suggest that the species name Albatross Island virus encompass both isolates, ABIV and HIGV, thereby representing the name of the original 1983 isolate and the location

Table. Virus neutralization assay results for Albatross Island virus, Australia

Source (species)	Location/year sample collected	No. positive/no. tested
Magpie goose (*Anseranas semipalmata*)	Northern Territory/1974	0/2
Masked lapwing (*Vanellus miles*)	Northern Territory/1975	0/1
Little corella (*Cacatua sanguinea*)	Northern Territory/1975	0/1
Whimbrel (*Numenius phaeopus*)	Northern Territory/1975	0/8
Cattle egret (*Ardea ibis*)	Gatton, Queensland/1981	0/5
Shy albatross (*Thalassarche cauta*)	Albatross Island/1983	18/36
Black noddy (*Anous minutus*)	Heron Island, Queensland /1985	1/115
Wedge-tailed shearwater (*Ardenna pacifica*)	Heron Island, Queensland /1985	0/46
Domestic chickens (*Gallus gallus*)	Armidale, New South Wales/1979	0/10
Little red flying fox (*Pteropus scapulatus*)	Unknown/unknown	0/5
Seals (unknown)	Macquarie Island/unknown	0/35
Maruspials (various wallabies, kangaroos, possums)	Queensland /Northern Territory/unknown	0/97
Cattle (*Bos taurus*)	Queensland /Northern Territory/1974–1985	0/160
Rusa deer (*Rusa timorensis*)	SE Queensland/1984	0/30

where both viruses were isolated. These 2 viruses are closely related to 2 tickborne phleboviruses: severe fever with thrombocytopenia syndrome virus, isolated in China (*2*), and Heartland virus, isolated in the United States (*3*). Each of these recently emerged viruses causes severe febrile illness with thrombocytopenia; deaths have been reported from 4 countries. In addition, Malsoor virus (*4*), a phlebovirus recently isolated from bats in India, has been shown to be closely related to severe fever with thrombocytopenia syndrome virus and Heartland virus. At the protein level, the similarity of ABIV to these 3 viruses is as follows: L, 66%–67%; M, 52%–56%; S, 58%–62%.

Deaths of albatross chicks in the Albatross Island colony occur every year; the intensity of these events varies from year to year (*5*). The cause of these events is multifaceted, but fowlpox is believed to be a major factor (*6*). No tests exist to quantify the extent and cause of the problem, although solutions are being pursued (R. Alderman, pers. comm, 2015). Wang et al. were unable to confirm infection of albatross with the HIGV isolate (*1*); however, the results presented here suggest that ABIV does infect albatross. Although infection is not direct evidence of disease, the fact that both isolates were collected from the same albatross colony during disease events almost 2 decades apart should not be neglected. Viral challenge studies would be useful for determining if and how ABIV contributes to disease in these birds.

Birds of the albatross family tend to fly long distances over open water. The geographic range of shy albatross extends from their breeding base in Tasmania to southern Africa (*5*). White-capped albatross (*T. steadi*) reportedly migrate from their breeding base in New Zealand as far as South America and eastward into shy albatross territory (*7*). It is possible to misidentify 1 of these albatross species as the other; indeed, the phylogenetic distinction between these species, once considered the same (*Diomedea cauta*), is controversial. The ease of albatross movement between vast geographic areas could provide an opportunity for intercontinental spread of emerging infectious diseases. Consequently, phleboviruses similar to ABIV may be present in bird populations in the southern areas of Africa and South America.

The need for intensified international investigations to identify genetically related tickborne phleboviruses with zoonotic potential is evident. The opportunity for the distribution of such viruses over a large global area is of concern to public health. Surveillance and investigation on an international level are needed.

Acknowledgments

We gratefully acknowledge the work of Nigel Brothers, Roy Mason, and colleagues who collected the ticks in 1983. Thanks also to Rachael Alderman for providing information about disease events on Albatross Island and the ongoing work to gain more understanding of such events. ABIV was isolated in 1983 by Toby St. George and colleagues.

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Toxoplasma gondii in Wild Red Squirrels, the Netherlands, 2014

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DOI: http://dx.doi.org/10.3201/eid2112.141711

To the Editor: *Toxoplasma gondii*, a zoonotic protozoan parasite for which felids are the only definitive hosts, can infect humans and other warm-blooded animals. Transmission usually occurs orally from oocysts shed by felids in water and on food, through tissue cysts in undercooked meat, or transplacentally. In particular, young cats shed oocysts that can sporulate and become infectious within a day, depending on temperature and humidity. Sporulated oocysts can survive in moist soil for months to years (*1*).

In September 2014, the number of dead squirrels reported to the Dutch Wildlife Health Centre and the Dutch Mammal Society increased suddenly. The red squirrel (*Sciurus vulgaris*) is the only species of squirrel endemic to the Netherlands. Members of the public claimed that squirrels were "dropping dead from trees." Subsequently, the public was encouraged to report and submit dead squirrels. A total of 187 animals were reported through October 2014, of which 37 were submitted for necropsy. Necropsy included macroscopic examination; cytologic analysis of liver, spleen, lungs, and intestinal contents stained with hemacolor (Merck, Darmstadt, Germany); and histologic examination of samples of various organs fixed in formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin.

For 8 adult animals, body condition (based on degree of fat storage and muscle development) was good; 12 juveniles were in poor condition. Typically, the trachea contained foam, and lungs were hyperemic and edematous. The liver was enlarged and pale, and the spleen was enlarged. In 13 animals, numerous small crescent-shaped organisms, with eccentrically placed nuclei consistent with tachyzoites of *T. gondii*, were identified by cytology in lung, liver, and spleen (*2*). Main histopathologic findings were pulmonary interstitial lymphoplasmocytic and neutrophilic infiltrates with edema and numerous intra-alveolar macrophages (17/20) and multifocal lymphoplasmocytic infiltrates with necrosis in the liver (13/20). Extensive splenic necrosis was occasionally observed (4/20). Intestines contained mild plasmacytic infiltrates. Numerous tachyzoites consistent with *T. gondii* were present in alveolar macrophages and epithelial cells, splenic macrophages, and hepatocytes. Duplicate slides were stained immunohistochemically by using polyclonal antibodies against *T. gondii* following a standard ABC protocol (*3*). Organisms stained for *T. gondii* in liver, spleen, lungs, and intestine. *Toxoplasma* was not detected in any brain. DNA was isolated (DNeasy Blood and Tissue Kit; QIAGEN, Hilden, Germany) from tissues of 14 squirrels and tested by quantitative PCR (*1*); *T. gondii* DNA was detected in 13. We successfully sequenced the *T. gondii* GRA6 gene for 11 squirrels and identified sequences to clonal type II *T. gondii* previously identified in sheep from the Netherlands (GenBank accession no. GU325790) (*4*). Incidental findings in the animals tested were encephalitis (2/20), coccidiosis (5/20), trauma (6/20), myocarditis (4/20), nephritis (1/20), lymphadenitis (1/20), and intestinal (3/20) and external (5/20) parasites.

The remaining 17 animals showed ≥1 of the following pathologic conditions: hemorrhages consistent with trauma (12/17), mild to severe intestinal coccidiosis (12/17), pneumonia (3/17), splenitis (1/17), *Taenia martis* cysticerci (1/17), and external parasites (8/17). Immunohistochemistry results for all 17 were negative for *T. gondii*.

On the basis of necropsy and molecular findings, we conclude that 20 of 37 examined squirrels died of disseminated *T. gondii* type II infection. These animals included adults and juveniles and were not restricted to specific geographic areas (Figure). The remaining animals died of trauma (12/17) or other causes (5/17).

Red squirrels are susceptible to *T. gondii*, and infection can lead to death. However, in our sample, the proportion of squirrels that died of toxoplasmosis (>50%) was higher than in other studies (≈16%) (*5*–*7*). The apparent increase in squirrel deaths and unexpectedly high proportion of fatal *T. gondii* infections suggests a toxoplasmosis outbreak among red squirrels. Possible explanations for this surge in cases include increased exposure to the parasite, increased susceptibility to infection, or increased virulence of the pathogen. Clonal *T. gondii* type II, the strain most frequently involved in human cases and endemic to Europe and North America, was identified. An increased virulence of the pathogen could not be proven (*8*). On the basis of lymphoid hyperplasia in

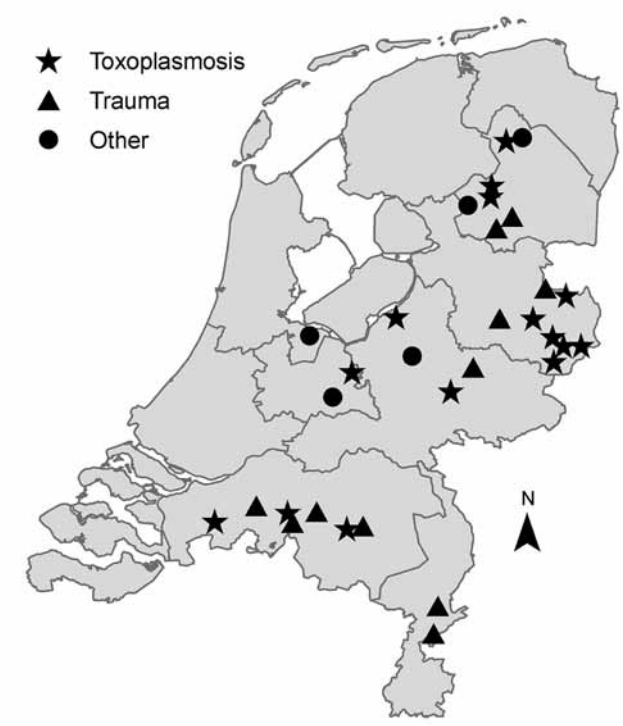

Figure. Spatial distribution of wild red squirrels (*Sciurus vulgaris*) investigated for *Toxoplasma gondii* and classified by cause of death, the Netherlands, 2014.

the spleen and lymph nodes, affected squirrels had no signs of immunosuppression. Thus, the most likely explanation is increased exposure to the parasite.

Sources of infection for red squirrels are not known; however, oocysts shed in cat feces may contaminate the nuts, fungi, shoots, and berries that constitute the diet of the squirrel. Stray, unspayed cats are common in the Dutch countryside. More than 3 million domestic cats (*Felis domesticus*) exist in the Netherlands, including several tens of thousands of free-roaming cats that reproduce (*9*). Determining the exact source of infection is important because humans also harvest wild fruits, nuts, and fungi from these areas. This outbreak highlights that contamination of the environment with *T. gondii* oocysts is of concern not only from a public health viewpoint but from a biodiversity perspective as well (*1*,*10*).

Acknowledgments

We thank R. Thomas for proofreading and J. van der Giessen for facilitating the molecular work.

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CTX-M-15–Producing *Escherichia coli* in Dolphin, Portugal

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DOI: http://dx.doi.org/10.3201/eid2112.141963

To the Editor: The global emergence and pandemic spread of sequence type (ST) 131 CTX-M-15–producing

[1]These authors contributed equally to this article.

Escherichia coli among humans and its detection in livestock, companion animals, and wildlife is a major cause for concern (*1*,*2*). Hence, it is imperative to identify and explore its dissemination traits. If CTX-M-15–producing *E. coli* continues to spread among different environments, therapeutic options in veterinary and human medicine will be greatly narrowed (*1*). *E. coli* is one of the gram-negative bacteria most frequently isolated from bottlenose dolphins (*3*). However, few studies about antimicrobial drug–resistant bacteria in dolphins have been published (*4*–*6*). We explored dissemination linkages between CTX-M-15–producing *E. coli* isolated from a marine dolphin (*Tursiops truncatus*) and clinical isolates collected during the same period from humans all over Portugal.

In 2009, *E. coli* strain LV143, isolated from respiratory exudate collected through the spiracle of a female dolphin from a zoo, was sent to the National Institute for Agricultural and Veterinary Research in Lisbon, Portugal, for bacteriological and mycological analysis and antimicrobial drug susceptibility testing. No clinical history for the animal was available. Mycologic examination detected no fungi or yeasts.

Drug susceptibility testing of the dolphin *E. coli* strain (LV143), performed by the agar dilution method and interpreted according to European Committee of Antimicrobial Susceptibility Testing (http://www.eucast.org/), revealed a non–wild-type phenotype to cefotaxime (MIC >8 μg/mL); it also showed a synergy toward clavulanic acid, suggesting production of extended-spectrum β-lactamase (ESBL). LV143 was also non–wild-type to ampicillin (MIC >64 μg/mL), nalidixic acid (MIC >512 μg/mL), ciprofloxacin (MIC >8 μg/mL), gentamicin (MIC >32 μg/mL), and tetracycline (MIC >64 m /mL). This isolate remained wild-type to chloramphenicol (MIC 4 μg/mL), florfenicol (MIC 8 μg/mL), sulfamethoxazole (MIC 32 μg/mL), trimethoprim (MIC ≤0.25 μg/mL), and streptomycin (MIC 4 μg/mL).

To analyze the zoonotic potential of the dolphin isolate, we selected 61 human clinical *E. coli* isolates, previously recovered from different specimens during 2004–2009 in 7 geographically separated hospitals in Portugal (Figure), from the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections collection. Inclusion criteria for the clinical isolates were 1) non–wild-type susceptibility to cefotaxime, 2) presumptive phenotypic ESBL production, and 3) genetic similarity by pulsed-field gel electrophoresis. Analysis of the genetic relatedness of human and dolphin isolates, determined by pulsed-field gel electrophoresis that used *Xba*I digested DNA (*7*), revealed 1 major cluster, which included 22 (35%) clinical isolates from 3 regions in Portugal and the isolate from the dolphin (Figure).

The genetic characterization of the 1 dolphin and 22 clinical isolates was performed by PCR and sequencing

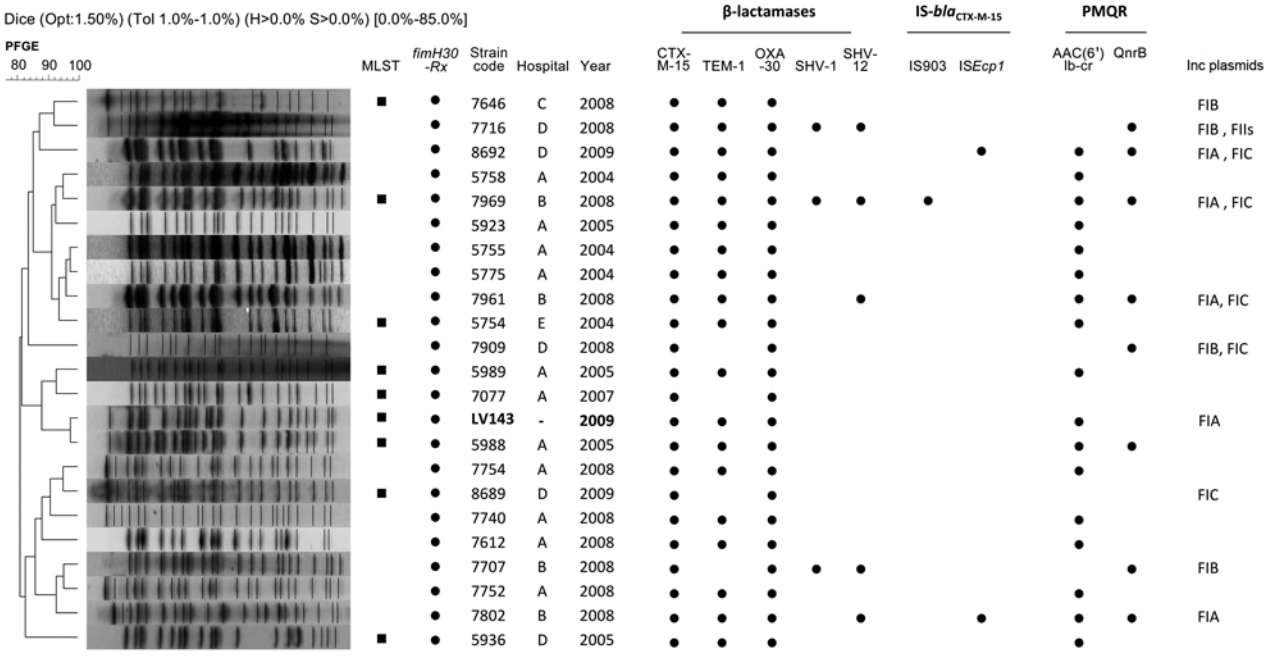

Figure. Dendogram of pulsed-field gel electrophoresis (PFGE) profiles showing the relationship between a clonal strain of *Escherichia coli* of animal origin (LV143, in boldface), and 22 *E. coli* isolates from humans. We used the unweighted pair group method and the Dice coefficient with 1.8% optimization (opt) and band position tolerance (tol) of 1%. Isolates with a Dice band–based similarity coefficient of ≥80% were considered to belong to the same cluster. Black squares under multilocus sequence typing (MLST) indicate sequence type (ST) 131 positivity. Year indicates year of isolation. Black circles indicate fimbral adhesin gene *fimH*, β-lactamase, IS-*bla*$_{CTX-M-15}$, and plasmid-mediated quinolone resistance (PMQR) positivity of indicated combinations. *E. coli* clinical isolates genetically unrelated to the dolphin isolate are not shown. Scale bar indicates percentage relatedness.

selective for the most prevalent ESBL-mediated genes (bla_{TEM}, bla_{SHV}, bla_{OXA-G1}, bla_{CTX-M}) and genes encoding plasmid-mediated quinolone resistance (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6')Ib-cr*), as previously described (*7*). Specifically, the strain recovered from the dolphin contained $bla_{CTX-M-15}$, bla_{TEM-1}, and bla_{OXA-30}, associated with a plasmid-mediated quinolone resistance gene, *aac(6')-Ib-cr* (Figure). All clinical isolates were also positive for $bla_{CTX-M-15}$ and bla_{OXA-30} genes; 18 isolates contained the bla_{TEM-1} gene and 3 bla_{SHV-1}, 5 bla_{SHV-12}, 8 *qnrB*, and 16 *aac(6')-Ib-cr* genes. The presence of class 1 integron, IS*Ecp1*, IS*26*, and IS*903* elements was also investigated, as has been done previously (*8*). The LV143 strain was positive for the insertion sequence IS*Ecp1*, associated with $bla_{CTX-M-15}$ (Figure), and was negative for the class 1 integron (data not shown). In 2 clinical isolates, we identified IS*Ecp1*, and in 1 isolate we identified IS*903*. PCR-based replicon typing (*9*) revealed the presence of IncF plasmid group in the 1 animal and 9 human isolates (a selected sample to evaluate PCR-based replicon typing) (Figure).

Multilocus sequence typing (MLST) was performed for 9 of 23 *E. coli* isolates. According to the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli), clones from the dolphin and from the humans exhibited the same combination of alleles across the 7 sequenced loci, corresponding to the epidemic ST131, associated with CTX-M-15 and widely disseminated among hospitals in Portugal (*2*,*7*). Within-ST subclones were analyzed on the basis of sequence variation of the *E. coli* fimbrial adhesin gene *fimH*, as previously described (*10*). The *fimH30-Rx* lineage was identified in all 23 *E. coli* isolates (fluoroquinolone-resistant and CTX-M-15–positive isolates), which clustered together on the dendrogram, regardless of MLST result (Figure). It is worth noting that the $bla_{CTX-M-type}$ gene has been detected in ESBL-positive *E. coli* isolates from healthy mammals (*1*).

Our study illustrated clonality among clinical isolates and a dolphin strain with common antimicrobial drug–resistance genes, specifically $bla_{CTX-M-15}$ and *aac(6')-Ib-cr*, and common plasmids, such as those from group IncF. These bacteria have gone through identical evolutionary genetic events, which ultimately led to the establishment of the same allelic diversity pattern (ST131 *fimH30-Rx*). The linkage between these 2 reservoirs highlights the zoonotic potential of this isolate from the dolphin.

Acknowledgments

We thank Fundação para a Ciência e a Tecnologia for project grant PEst-OE/AGR/UI0211/2011-2014, Strategic Project UI211-2011-2014.

V. M. and D.J.-D. were supported by grants SFRH/BPD/77486/2011 and SFRH/BD/80001/2011, respectively, from Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

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Onchocerca lupi Nematode in Cat, Portugal

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DOI: http://dx.doi.org/10.3201/eid2112.150061

To the Editor: *Onchocerca lupi* (Spirurida, Onchocercidae) is a nematode that infects the ocular tissues of dogs and humans. This filarioid remained almost unknown until recently, when it was reported in dogs from Europe and North America (*1*–*3*). *O. lupi* was also detected in 2 cats from the United States (*4*), which suggests that not only canids but also felids are suitable hosts for this little-known nematode. In addition, the zoonotic potential of *O. lupi* nematodes was demonstrated in human patients from Iran, Tunisia, Turkey, and the United States (*3*,*5*).

Clinical signs of canine onchocercosis include conjunctivitis, exophthalmos, periorbital swelling, photophobia, discomfort, lacrimation, ocular discharge, subconjunctival granuloma, ulcerative keratitis, and anterior and posterior uveitis (*1*). Signs in cats are similar to those in dogs (*4*).

After the first case of canine ocular onchocercosis was reported in the Algarve region in southern Portugal (*6*), a survey to detect microfilariae in apparently healthy dogs revealed an 8.3% prevalence of infection (*7*). Because no data regarding *O. lupi* nematode infection in cats from Europe are available, the aim of this study was to evaluate the infection's occurrence in cats in Portugal, where canine infection has been previously reported (*8*).

In October 2014, a total of 155 stray cats were sampled from Praia de Faro in the Algarve (37°0′29.4546″N, 7°59′41.265″W, altitude 9 meters). The sampling area is a small peninsula within an area characterized by a line of sand dunes formed by peninsulas and sandy islands that protect a vast area of marshland, canals, and islets from the Atlantic Ocean. All stray cats were captured under the scope of a trap, neuter, and return project. This study was

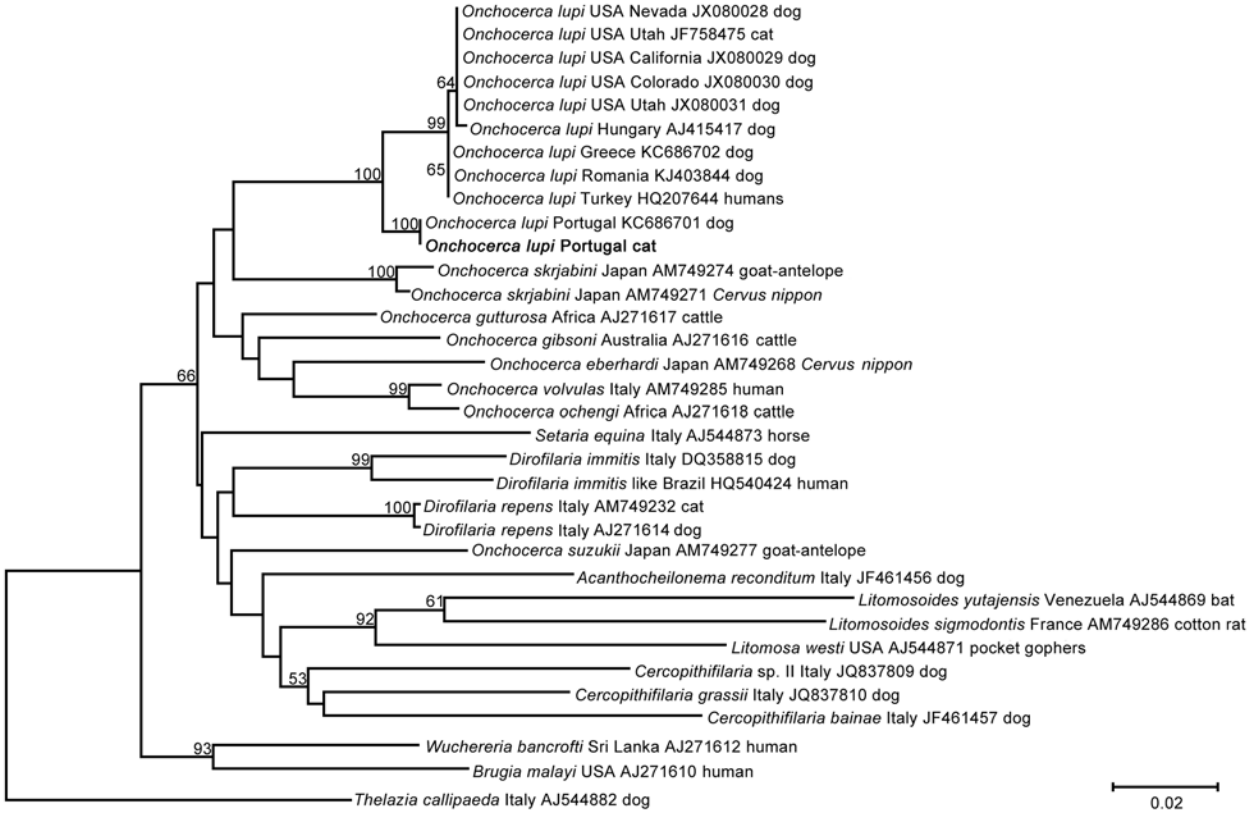

Figure. Phylogenetic analysis of partial cytochrome *c* oxidase subunit 1 gene segment (689 bp) of *Onchocerca lupi* isolated from a cat in Portugal (bold) compared with segments from other nematodes and roundworms retrieved from GenBank (accession numbers indicated). Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

approved by the ethical committee of the Faculty of Veterinary Medicine, Universidade Lusófona de Humanidades e Tecnologias.

Ear tipping is commonly done in trap, neuter, and return programs to identify cats that have been sterilized. These skin samples (0.5 cm^2) were soaked at room temperature in 1 mL of saline solution, and sediments were individually observed under light microscopy (*9*).

Of 155 cats, 1 (0.65%) with no clinical signs of ocular infection was positive for *O. lupi* microfilariae. Microfilariae were identified according to morphologic keys (*9*) and differentiated from those of other filarioid species infecting cats in the Mediterranean region. *O. lupi* microfilariae had a short, flattened, unsheathed body (mean length 110.1 ± 7.5 μm, width 6.8 ± 1.2 μm) with a rounded head bearing a tiny tooth on the cephalic edge. The body was blunt with a short bent tail of ≈11.7 μm.

After we made microscopic observations, skin samples were processed as described elsewhere (*10*). Partial cytochrome *c* oxidase subunit 1 (*cox*1) gene fragments (689 bp) were amplified (*10*). In accordance with the morphologic identification, BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of *cox*1 gene showed a high overall nucleotide homology with sequences of *O. lupi* available in GenBank. All *cox*1 sequences available in GenBank for *O. lupi* nematodes were analyzed by using MEGA6 (http://www.megasoftware.net) and showed a low intraspecific variability, ranging from 0% to 2.1% (mean 0.7%). Phylogenetic analysis of *cox*1 sequences with MEGA6 and the neighbor-joining method confirmed that the sequence obtained clustered with that of *O. lupi* nematodes from Portugal available in GenBank (Figure). The obtained sequence was deposited in GenBank (accession no. KP453715).

We describe detection of *O. lupi* nematodes in a cat from Europe. The complete life cycle of *O. lupi* nematodes remains unknown, although arthropods should act as a vector (*2,4,7*). Because most of the potential vectors (i.e., black flies, mosquitoes, and biting midges) increase their activity during spring and summer, we cannot rule out that skin sampling conducted in late October affected the chance to detect additional infected animals. In addition, sampling was performed during the day, instead of late afternoon or night, when the number of microfilariae is higher (*7*), which might account for the low prevalence of infection obtained in this study.

As previously reported for most infected dogs from the same area, the infected cat lacked apparent clinical signs of infection, suggesting that subclinically infected animals might be carriers and reservoirs of *O. lupi* nematodes (*7*). Further investigation such as population-based surveys should be performed to estimate the distribution of the infection in cats and dogs and to assess the risk to humans.

Detection of *O. lupi* nematodes in dogs and cats from Algarve confirms that this parasite is endemic to southern Portugal. Veterinarians, local pet owners, and tourists (particularly those from countries where the disease is not endemic and who bring their pets) should be alerted to the risk for infection by this filarioid and the need to implement measures to protect animals and persons. Physicians and ophthalmologists should include this zoonosis in the differential diagnosis for ocular nodular lesions, particularly in patients from areas where *O. lupi* nematodes have been reported.

Acknowledgments

We thank the Change for Animals Foundation, Associação Animais de Rua, and veterinary students (A. Radar, D. Gouveia, F. Costa, M. Caldas, M. Freitas, and N. França) from Faculdade de Medicina Veterinária—Universidade Lusófona de Humanidades e Tecnologias for their collaboration.

C.M. (SFRH/BPD/44082/2008) holds a scholarship from Fundação para a Ciência e a Tecnologia, Ministério da Educação e Ciência, Portugal. The work of C.M., A.G., and D.O. was done under the EurNegVec COST Action TD1303 program.

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Porcine Deltacoronavirus in Mainland China

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DOI: http://dx.doi.org/10.3201/eid2112.150283

To the Editor: Porcine deltacoronavirus (PDCoV) was discovered in 2012, during a study to identify new coronaviruses in mammals and birds in Hong Kong (*1*). In February 2014, this novel porcine coronavirus was detected in pigs in Ohio, United States (*2*), and has since been reported in at least 17 US states (*3–5*). Concern regarding the epidemiology, evolution, and pathogenicity of this emerging virus is increasing. Recently, PDCoV was identified in South Korea (*6*). We report PDCoV in mainland China.

Since December 2010, a large-scale outbreak of diarrhea in suckling piglets has occurred on swine farms in mainland China (*7*). The causative agent was considered to be a variant of porcine epidemic diarrhea virus (PEDV) (*8*), and the role of PDCoV in the outbreak was not investigated at that time.

Using 2 pairs of specific primers to detect PDCoV, as described by Wang et al. (*2*), we tested 215 intestinal or fecal samples collected at various times during 2004–2014 from piglets with clinical diarrhea in Anhui, Guangxi, Hubei, and Jiangsu provinces, mainland China (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0283-Techapp1.pdf). All samples were submitted from commercial pig farms to our laboratory for enteropathogen detection. Of these samples, 165 (124 from Hubei, 41 from Jiangsu) had been collected in 2014, and 50 (40 from Jiangsu, 6 from Anhui, 4 from Guangxi) had been collected during 2004–2013 and preserved in our laboratory. The 215 samples were simultaneously tested for PEDV and transmissible gastroenteritis virus (TGEV) by using the primers listed in online Technical Appendix Table 2. Of the samples tested, 14 (6.51%) were positive for PDCoV, 110 (51.2%) were positive for PEDV, and 5 (2.3%) were positive for TGEV. Of the 14 PDCoV-positive samples, 7 (50%) were also positive for PEDV; 2 of the 215 samples were co-infected with PEDV, TGEV, and PDCoV (online Technical Appendix Table 1). Previous studies have shown that prevalence of PDCoV in the midwestern United States in 2014 was high (30%) and that PDCoV co-infections with other pathogens (such as PEDV and rotavirus) are more common (78% of PDCoV infections) (*4*). At the same time in mainland China, the rate of PDCoV positivity was lower (7.27%), whereas that of PEDV was higher (52.73%), suggesting that PEDV remains the main causative agent of piglet diarrhea diseases in mainland China. Similarly, in South Korea in 2014, only 2 PDCoV-positive samples were detected in 113 samples of diarrhea from pigs (*6*).

We also examined the collection dates and geographic locations of the PDCoV-positive samples and found that PDCoV was detected in pigs in Hubei (8/124), Jiangsu (4/81), and Anhui (2/6) Provinces. However, all samples from pigs in Guangxi Province were negative for PDCoV. All PDCoV-positive samples from Hubei and Jiangsu Provinces had been collected in 2014, whereas the 2 PDCoV-positive samples from Anhui Province had been collected in 2004.

Among the PDCoV-positive samples, we selected 3 for complete genome sequencing with 16 pairs of overlapping primers, as described previously (*2*): one (CHN-AH-2004) collected from Anhui Province in 2004, one (CHN-HB-2014) from Hubei Province in 2014, and one (CHN-JS-2014) from Jiangsu Province in 2014. These complete genome sequences have been deposited in GenBank under accession nos. KP757890 (CHN-AH-2004), KP757891 (CHN-HB-2014), and KP757892 (CHN-JS-2014). The complete genome sequences of 3 PDCoV strains from pigs in mainland China shared high nucleotide identities (≥98.9%) with all previously reported PDCoV strains. Previous studies found that Hong Kong strain HKU 15–44 and all PDCoV strains from the United States and South Korea have a 3-nt insertion in the spike gene, which is not present in Hong Kong strain HKU 15-155 (*2–6*). This insertion is also present in CHN-AH-2004, whereas CHN-HB-2014 and CHN-JS-2014, like HKU 15-155, lack this insertion (online Technical Appendix Figure).

Although all reported PDCoV strains from China shared high similarity with each other, a phylogenetic tree based on all available complete PDCoV genome sequences showed that these PDCoV strains clearly cluster in different clades (Figure). Strain CHN-JS-2014 shares an ancestor with the strains from the United States and South Korea. CHN-AH-2004 and HKU15-44 share a common ancestor, and CHN-HB-2014 shares a common ancestor with CHN-AH-2004 and HKU15-44.

As an emerging virus, PDCoV has been poorly understood. Our data suggest that PDCoV has existed in

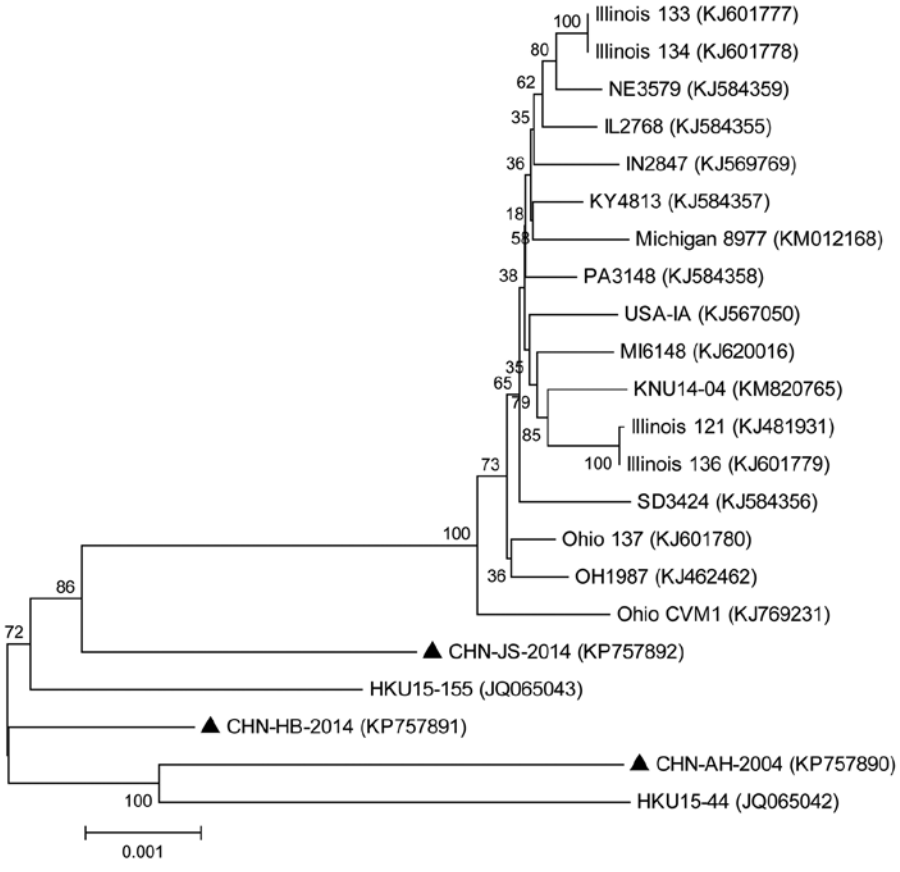

Figure. Phylogenetic tree of all complete porcine deltacoronavirus genome sequences available in February 2015. The phylogenetic tree was constructed by using the distance-based neighbor-joining method in MEGA 6.06 software (http://www.megasoftware.net/). Bootstrap values were calculated with 1,000 replicates. The number on each branch indicates bootstrap values. The reference sequences obtained from GenBank are indicated by strain abbreviations and GenBank accession numbers. Triangles indicate the 3 strains from mainland China. Scale bar indicates nucleotide substitutions per site.

mainland China for at least 11 years. Although the rate of PDCoV infection detected in mainland China in this study was relatively low, the results may not accurately reflect the prevalence of PDCoV in mainland China because the tested samples were collected from only 4 provinces. Extensive surveillance is required to define the epidemiology and evolution of PDCoV in mainland China. Recent confirmation that PDCoV is enteropathogenic in gnotobiotic pigs (*9*) highlights the need for effective vaccines against this emerging virus.

This work was supported by the Key Technology R&D Programme of China (2015BAD12B02), the Natural Science Foundation of Hubei Province (2014CFA009), and the Fundamental Research Funds for the Central Universities (2013PY043).

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NADC30-like Strain of Porcine Reproductive and Respiratory Syndrome Virus, China

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DOI: http://dx.doi.org/10.3201/eid1212.150360

To the Editor: Porcine reproductive and respiratory syndrome (PRRS), which is characterized by reproductive failure in sows and respiratory disease in pigs of all ages, is a viral disease with serious economic consequences for the global pork industry (*1*). PRRS virus (PRRSV), the causative agent of this disease, was identified in Europe in 1991 and the United States in 1992 (*2,3*). PRRSV is an enveloped, positive-strand RNA virus of the family *Arteriviridae*. This virus is divided into European genotype 1 and North American genotype 2. Emerging novel PRRSV strains have caused many outbreaks of severe PRRS (*4–7*). We report emergence of a novel PRRSV (NADC30-like) in China that is genetically similar to the NADC30 strain isolated in the United States in 2008 (*8*).

During August–December 2014, severe outbreaks of PRRS were observed on 7 intensive pig farms in Beijing, Tianjing, Shanxi, Henan, and Zhejing, China. Pregnant sows had abortions and stillbirth and piglets had respiratory disorders (case-fatality rate 30%–50%).

A total of 58 tissue samples from stillborn piglets, serum samples from diseased sows and piglets, and lungs and lymph nodes of dead piglets were tested for viral RNA by using reverse transcription PCR and primers specific for PRRSV open reading frame (ORF) 7, which encodes nucleocapsid protein, as described (*9*). Viral RNA was detected in 63.8% (4/7, 7/13, 5/5, 4/4, 5/10, 6/6, and 6/13 for the 7 farms, respectively) of samples tested.

All virus-positive lung samples were then used to amplify the entire ORF5 gene, which encodes major envelope glycoprotein 5 and is one of the most variable regions in the PRRSV genome. Amplified fragments were sequenced to analyze variation of PRRSV as described (*10*).

Comparative analyses of sequences showed that amplified ORF5s of viruses isolated on an individual farm had 100% identities and amplified ORF5s of viruses from 7 farms had 89.7%–97.7% nucleotide identities (88.6%–98.0% for deduced amino acids) with each other (GenBank accession nos. KP861625–31) and higher nucleotide (92.2%–97.0%) and amino acid (91.5%–96.5%) identities with NADC30. The ≈10% amino acid divergence among ORF5s from the 7 farms suggests possible variation of NADC30 during its transmission. These viruses had lower nucleotide (84.9%–87.6%) and amino acid (84.1%–88.6%) identities with representative PRRSV strains from China, including CH1a, HB-1(sh)/2002, HB-2(sh)/2002, and JXwn06, and lower nucleotide (85.1%–86.7%) and amino acid (82.1%–86.1%) identities with VR-2332.

A strain of PRRSV (CHsx1401) was isolated from a lung sample by using porcine pulmonary alveolar macrophages. Third-passage viral cultures were used for genomic sequencing as described (*9*). Genomic fragment amplification was conducted by using reverse transcription PCR and 14 pairs of primers (*10*), which had minor modifications made on the basis of the genomic sequence of NADC30 available in GenBank. Comparative analyses of all coding regions and their deduced amino acid sequences of the virus were performed with representative PRRSV strains from China and the United States. Similar to the genome of NADC30, the genome of CHsx1401 (GenBank accession no. KP861625) was 15,020 nt, excluding its poly A tail.

Amino acid alignment of the nonstructural protein 2 (NSP2) highly variable region of CHsx1401 with those other strains showed that this virus had amino acid deletions that were identical to that in NADC30 (*8*) and MN184 isolated in the United States (*4*). These deletions were identified as a 111-aa deletion at position 323–433, a 1-aa deletion at position 481, and a 19-aa deletion at position 533–551 (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/12/15-0360-Techapp1.pdf) when compared with sequence of prototype strain VR-2332.

Two recent virus isolates from China (HENAN-XINX and HENAN-HEB), whose sequences were submitted to GenBank in 2013, also had these deletions. Genome sequence of CHsx1401 had 95.7% nucleotide identity with NADC30, 93.0% identity with HENAN-XINX, and 93.2% identity with HENAN-HEB, but only 85.8% identity with VR-2332 and 83.8% identity with JXwn06, a highly pathogenic strain from China. Phylogenetic analysis of the whole genome of PRRSV was performed by using a distance-based neighbor-joining method with 1,000 bootstrap replicates in MEGA6 (http://www.megasoftware.net/). CHsx1401 was shown to be genetically more closely related to NADC30 and clustered into a specific branch (Figure).

Additional comparative analyses of viral protein amino acid sequences of CHsx1401 with those of NADC30, MN184A, MN184B, MN184C, JXwn06, and VR-2332 indicated that CHsx1401 had higher similarity with NADC30 (91.2%–99.1%) than with MN184 serial strains (78%–98.2%) and lower similarity with HP-PRRSV (JXwn06) from China and VR-2332 strains, except for NSP1α and NSP11 (online Technical Appendix Table). These data also indicate that CHsx1401 is genetically similar to NADC30.

[1]These authors contributed equally to this article.

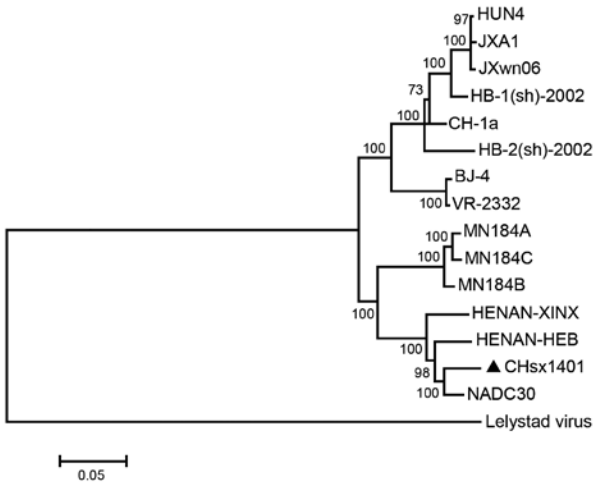

Figure. Phylogenetic analysis of whole genomes of porcine reproductive and respiratory syndrome virus (PRRSV) CHsx1401 (triangle) (GenBank accession no. KP861625); representative prototype strain VR-2332 (U87392); isolates BJ-4 (AF331831), CH-1a (AY032626), HB-1(sh)/2002 (AY150312), and HB-2(sh)/2002 (AY262352) from China; highly pathogenic strains JXA1 (EF112445), JXwn06 (EF641008), and HUN4 (EF635006); strains MN184A (DQ176019), MN184B (DQ176020), MN184C (EF488739), and NADC30 (JN654459) from the United States; and recent strains HENAN-HEB (KJ143621) and HENAN-XINX (KF611905) from China. Prototype Lelystad virus (M96262) was used as the outgroup. The phylogenetic tree was constructed by using the distance-based neighbor-joining method with 1,000 bootstrap replicates in MEGA6 (http://www.megasoftware.net/). Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

Recent widespread outbreaks of PPRS in China were associated with a novel NADC30-like strain of PPRSV. Whole genomic analysis showed that the strain differed from previously identified PRRSV strains in China, but had an overall genetic similarity and a unique deletion in the NSP2-coding region that was identical to that of NADC30, which originated in the United States. We propose that the NADC30 strain was introduced into China in recent years by importing of breeding pigs and has since undergone mutations, resulting in variant viruses.

This study was supported by the Major Program of National Natural Science Foundation of China (31490603) and the earmarked fund for Modern Agro-industry Technology Research System of China (CARS-36) from the Ministry of Agriculture of the People's Republic of China.

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Serologic Evidence of Influenza A (H14) Virus Introduction into North America

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DOI: http://dx.doi.org/10.3201/eid2112.150413

To the Editor: Although a diverse population of influenza A viruses (IAVs) is maintained among ducks, geese, shorebirds, and gulls, not all of the 16 avian

hemagglutinin (HA) subtypes are equally represented (*1*). The 14th HA subtype, commonly known as the H14 subtype, was historically limited to isolates from the former Soviet Union in the 1980s (*2*) and was not subsequently detected until 2010, when isolated in Wisconsin, USA from long-tailed ducks and a white-winged scoter (*3*–*5*). In the United States, the H14 subtype has since been isolated in California (*6*), Mississippi, and Texas (*7*); and has been reported in waterfowl in Guatemala (*7*). In this study, we examined whether there was serologic evidence of H14 spread among ducks in North America before (2006–2010) and after (2011–2014) the initial detection of the H14 subtype virus on this continent.

This report was reviewed and approved by United States Geological Survey under the Fundamental Science Practices policy (http://www.usgs.gov/fsp/). Serum samples from blue-winged teal, American green-winged teal, and mallard ducks were screened by using blocking ELISA (FlockCheck AI MultiS-Screen antibody test kit; IDEXX Laboratories, Westbrook, ME, USA) to detect antibodies against the influenza virus nucleoprotein. Positive samples were tested by microneutralization assays as described (*7*) against viruses representing H14 and H3 subtypes. H3 is commonly detected in ducks found in North America (*8*) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0413-Techapp1.pdf).

Antibodies against H3 were detected during 2006–2014 in Michigan, Minnesota, New Jersey, Texas, and Louisiana (Table); titers ranged from 20 to 320. Antibodies against H14 were detected in 1 duck in 2007 and in 24 ducks sampled in 2012 after August. H14 antibodies were detected in all years and most locations studied after 2012; antibody titers ranged from 20 to 160. Thus, antibody prevalence was consistent with the relative prevalence of H3 reported among ducks in North America (*1*,*4*,*8*) and the timing of initial detection of H14 viruses.

To address the possibility of cross-neutralizations between HA subtypes, we tested the 2007 H14-positive serum samples and 22 of the H14-positive serum samples from 2012–2014 against HA subtypes 1–12 (online Technical Appendix Table 1) by virus neutralization (online Technical Appendix Table 2). Among humans, broadly neutralizing antibodies within HA groups targeting conserved regions in the HA stalk have been described (*9*), and if present in samples from mallards, these could contribute to cross-neutralizations. The H14-positive serum samples from 2007 reacted to subtypes H3, H4, H7, and H11, and high titers were identified for H3 and H4, which are within the same clade. Samples from 17 of these birds tested antibody-positive for additional HA subtypes and 5 tested positive only to H14. An H14 virus was recovered by virus isolation from the same blue-winged teal population sampled in March 2013, from which serum samples were obtained (*7*); however, although H14 antibodies have been detected in Minnesota, an H14 virus has not yet been isolated in that state.

Our serologic results are temporally consistent with H14 isolation reports and suggest that H14 subtype viruses were not circulating among ducks in North America before initial virus isolation. However, there are potential challenges with serologic-based investigations. For

Table. H3 and H14 microneutralization assay data from ducks sampled during 2006–2014, North America*

Year	Month of sampling	State	Species	No.	H3N8, no. (%)	H14N5, no. (%)
2006	Aug	Michigan	Mallard	29	6 (21)	0
	Aug/Sep	Minnesota	Mallard	39	3 (8)	0
2007	Aug/Sep	Minnesota	Mallard	46	8 (17)	1 (2)
2008	Aug/Sep	Minnesota	Mallard	44	8 (18)	0
2009	Aug/Sep	Minnesota	Mallard	29	10 (34)	0
	Aug	New Jersey	Domestic and wild mallard	36	1 (3)	0
2010	Aug/Sep	Minnesota	Mallard	29	6 (21)	0
	Aug	New Jersey	Domestic and wild mallard	20	5 (25)	0
2011	Aug/Sep	Minnesota	Mallard	124	37(30)	0
2012	Feb/Mar	Texas	Blue-winged teal	19	3 (16)	0
	Aug/Sep	Minnesota	Mallard	188	11 (6)	2 (1)
2013	Feb/Mar	Texas/Louisiana	Blue-winged teal	120	13 (11)	12 (10)
	Feb/Mar	Texas/Louisiana	American green-winged teal	91	5 (5)	2 (2)
	Aug/Sep	Minnesota	Mallard	65	8 (12)	7 (11)
2014	Feb/Mar	Texas	Blue-winged teal	22	1 (5)	1 (5)
	Sep	Minnesota	Mallard	41	4 (10)	0
Totals						
2006–2010	NA	NA	All ducks	272	47 (17)	1 (0.3)
	NA	NA	Mallards only	272	47 (17)	1 (0.3)
2011–2014	NA	NA	All ducks	670	82 (12)	24 (3.5)
	NA	NA	Mallards only	418	60 (14)	9 (2.1)
	NA	NA	Blue-winged teal and American green-winged teal	252	22 (9)	15 (6)

*NA, not applicable.

example, the overall prevalence of H14 antibodies after the initial detection of H14 viruses (2011–2014) was low (3.5% of blocking ELISA positive samples), thus requiring a large sample size (n = 670) for H14 antibody detection. However, an even lower prevalence was observed by using virus isolation; we isolated only 1 H14 IAV during parallel sampling of these sites (n = 8,875) during 2011–2014.

Differences in pre- and post-H14 detection also varied between species, location, and season. Differences in H14 antibody prevalence were observed in all ducks sampled pre- and post- (0.3%–3.5%, p = 0.0103) H14 detection, but not in the mallard-only subset (0.3%–2.1%, p = 0.0963). A significant difference in seroprevalence also was detected between species (mallard [2%] vs teal [6%]) in the 2011–2014 samples (p = 0.0104). IAV show strong seasonal patterns in prevalence, and the observed differences in antibodies may be associated with the probability of IAV infection before sampling and the persistence of antibody responses in these species. Mallards (primarily hatch-year birds) were sampled at the beginning of fall migration (≈3–4 months of potential IAV exposure for hatch-year birds), whereas teal were sampled later, during spring migration (≈9–10 months of potential IAV exposure for birds hatched the previous spring or summer). It is apparent that the sampling approach used can affect results.

Interpretation of subtype-specific serologic data can be complex, especially in birds that are normally infected with several IAV subtypes during their lives. Nevertheless, this study demonstrates the value of a subtype-specific serologic approach to detect even relatively minor changes in subtype diversity and clearly shows that new viruses can establish in duck populations in North America. Serologic techniques also can be optimized to detect incursions of novel viruses such as the highly pathogenic Eurasian H5 viruses (*10*) among wild birds.

Acknowledgments

We thank B. Wilcox, D. Carter, J. Slusher, N. Davis-Fields, C. Kienzle, G. Knutsen, R. Poulson, P. Link, P. Walther, and G. Newsome for assistance acquiring the serum samples used in this study. We also thank M. Wille and 2 anonymous persons for providing reviews. Positive control antisera were provided by the National Veterinary Services Laboratories, Veterinary Services, United States Department of Agriculture.

The trapping, handling, and sampling of birds was approved through the University of Georgia Institutional Animal Care and Use Committee Animal Use Proposal A2013 05-021.

The work was funded through the National Institute of Allergy and Infectious Diseases, Centers of Excellence for Influenza Research and Surveillance (contract number: HHSN272201400006C) and by the United States Geological Survey through the Wildlife Program of the Ecosystem Mission Area. N.L.-M. was supported by postdoc grants from the Wenner-Gren Foundations, Stockholm, Sweden and the Swedish Research Council.

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Disseminated Infection Caused by *Francisella philomiragia*, France, 2014

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DOI: http://dx.doi.org/10.3201/eid2112.150615

To the Editor: *Francisella philomiragia* is a rare opportunistic pathogen. Only 17 cases of infection in humans have been reported over a 40-year period; 15 of these occurred in North America, mainly in persons who had near-drowning experiences and in patients with chronic granulomatous disease (*1*,*2*). We describe a case of *F. philomiragia* infection in a man in France who had the skin lesions of Sweet syndrome, characterized by nodules and pustules with dermal neutrophilic infiltration.

In 2014, a 58-year-old diabetic man with myeloproliferative disorders associated with Sweet syndrome (diagnosed in 2012), was hospitalized in the teaching hospital of Lille, France,with a 1-week history of fever (39°C) and cough. Physical examination showed inflammation around a central venous catheter, which was then removed. Blood, urine, and sputum samples that had been collected on admission were analyzed; leukocyte count was 5.83 × 10^9 cells/L (neutrophils 79%) (reference range 4–10 × 10^9 cells/L. Empirical treatment with piperacillin/tazobactam, vancomycin, and gentamicin was initiated. No pathogens were recovered from the samples, although the catheter was positive for *Staphylococcus epidermidis* and *Stenotrophomonas maltophilia*. These findings prompted us to replace piperacillin/tazobactam with ticarcillin/clavulanic acid in the treatment regimen. Despite a brief clinical improvement, the patient was highly febrile (40°C) 1 week after admission, and new blood samples were collected. A computed tomography scan of the thorax and abdomen revealed small nodules in the right lung and a single, large, hypodense lesion in the liver. Although histopathologic examination of a percutaneous liver biopsy specimen revealed multiple abscesses, no bacteria were observed after staining and culturing. Thus, the patient's respiratory and hepatic symptoms were considered to be extracutaneous manifestations of Sweet syndrome.

Two aerobic cultures (BactAlert FAN medium; bioMérieux, Lyon, France) of blood samples drawn >48 hours after ticarcillin/clavulanic acid therapy was begun yielded oxidase-positive gram-negative rods after 4 days' incubation at 37°C. These bacteria were identified as *F. philomiragia* in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (Bruker Daltonic, Bremen, Germany). Identification was confirmed by amplification and DNA sequencing of a portion of the 16S and 23S rRNA encoding genes as well as the intergenic region (*3*). On the basis of antimicrobial drug susceptibility testing results (performed 2 days after the positive culture), a combination of cefotaxime plus gentamicin was initiated (followed 3 days later by a regimen that included cefotaxime and ciprofloxacin). The patient's symptoms resolved after 14 days of this treatment. Serum samples collected at admission and 2 weeks later were positive for IgM and IgG against *F. philomiragia* (titer 1:640 and 1:160, respectively, cutoff >1:20).

F. philomiragia is halophilic and appears to be ubiquitous in marine habitats in northern Europe (*4*). Thus, the patient may have been inoculated cutaneously with *F. philomiragia* through saltwater exposure because he lived near the coast of the North Sea and had bought and prepared locally caught fish and shellfish for his own consumption. The neutrophilic dermatosis lesions on the patient's hands may have provided a portal of entry for *F. philomiragia*. Indeed, cutaneous inoculation of *F. philomiragia* has been reported in a patient after he was scratched by a crab (*2*). However, the patient we report could not recall ever having any local inflammation, skin ulcers, or subsequently enlarged lymph nodes after handling fish and shellfish. Although human cases of *F. philomiragia* infection are predominantly associated with saltwater, this organism also has been isolated from freshwater ponds, marshes, and warm springs in the United States (*5*,*6*). However, this patient did not recall contact with any of these aquatic environments.

In the few reported human cases of *F. philomiragia* infection, pneumonia was the most common clinical manifestation, as in the case we describe. Thus, inhalation of aerosols from a contaminated environment might constitute a noncutaneous route of *Francisella* spp. transmission. It is noteworthy that *Francisella*-like organisms were recovered from urban aerosol samples in Texas (*7*) and *F. guangzhouensis* (which displays 95% nucleotide sequence identity with the 16S and 23S rRNA genes of *F. philomiragia*) was isolated from water in air-conditioning cooling towers (*8*). However, this patient did not recall ever having lived near sources of potential contaminated aerosols.

The patient could have been inoculated with *F. philomiragia* by arthropod bite (analogous to the tick bites that transmit the virulent species *F. tularensis*). To date,

arthropod-based transmission *F. philomiragia* has not been suspected. However, *F. philomiragia* DNA was found in 19% of a sample of dog ticks (*Dermacentor reticulatus*) in France (*9*). This finding suggests that *D. reticulatus*, which is now broadly distributed across Europe because of global warming and increased travel with pets, may have a role in the life cycle and transmission of *F. philomiragia* (*10*). The patient did not own a dog and did not recall having had contact with dogs. However, his job (a municipal gardener) constituted a risk factor for tick bites in urban green spaces.

Although multiple points for *F. philomiragia* to enter this patient were suspected, none were laboratory confirmed. Further investigation is needed to better define the natural life cycle of this organism, especially the role of tick species in its transmission.

Acknowledgments

We thank Michel Simonet for critically reading the manuscript.

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Severe Ocular Cowpox in a Human, Finland

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DOI: http://dx.doi.org/10.3201/eid2112.150621

To the Editor: We describe cowpox with corneal involvement in a 31-year-old atopic woman who lived in southern Finland and was unvaccinated for smallpox. In August 2009, she noticed irritation and edema in her right eye and sought care from a local physician; she started topical antimicrobial drug therapy and oral cephalexin 2 days later. Over the following week, fever developed (37.6°C –39.0°C), edema developed on half her face, the eye became increasingly painful, and visual acuity decreased. The conjunctiva was severely chemotic and hyperemic, but the cornea was clear and the other eye unaffected.

Microbiologic samples taken from the eye 11 days after onset showed neither bacteria nor respiratory viruses. Orbital tomography results were normal. The patient was hospitalized, and broad-spectrum intravenous antimicrobial treatment (meropenem, vancomycin, valacyclovir, and fluconazole) was started, combined with topical corticosteroids and antimicrobial drugs. Within 2 weeks, the conjunctiva showed necrosis, and epithelial erosions appeared in the lower cornea, but visual acuity normalized (online Technical Appendix Figure, panels A, B, http://wwwnc.cdc.gov/EID/article/21/12/15-0621-Techapp.pdf).

A strong cytopathic effect was observed in Vero cells infected with conjunctival swab (online Technical Appendix

Table 1), but the virus was unidentifiable by routine methods. In electron microscopy, cell culture and tear fluid samples contained particles with typical orthopoxvirus (OPV) morphology. PCRs for hemagglutinin (*1*) and 14-kDa genes (*2*) verified OPV infection. Additional PCRs and sequencing confirmed zoonotic cowpox virus (CPXV) with strain designation FIN/K2009. Nucleotide sequences of the hemagglutinin, thymidine kinase, and A-type inclusion body protein genes were identical to those of CPXV strains T2000 and E1989 previously identified in Finland (*3*). In phylogenetic analysis (Figure), CPXV/FIN/K2009 clustered with strains from Austria and shared ancestry with vaccinia virus. OPV IgG and IgM were detected by immunofluorescence assay (*3*) in serum samples up to 5 months after symptom onset (online Technical Appendix Table 1).

The patient was started on intravenous polyclonal gammaglobulin and topical trifluorothymidine with in vitro anti-OPV effects; nevertheless, corneal erosions enlarged, corneal stromal edema ensued, and intraocular pressure increased (online Technical Appendix Figure, panel C), suggesting trabeculitis. Topical autologous serum drops had no effect. Periorbital edema slowly resolved, but corneal erosions persisted. Amniotic membrane transplantation (AMT) (*4*) was performed 2.5 months after onset. The inferior cornea melted, and the cornea lost transparency (online Technical Appendix Figure, panel D). AMT was repeated twice at 1-month intervals because of corneal thinning.

At 3.5 months after symptom onset, tecovirimat (400 mg 2×/d) was given orally for 14 days. Despite treatment, ocular OPV PCR test results remained positive until 9 months after onset (online Technical Appendix Table 1), and corneal melting progressed (online Technical Appendix Figure, panel E). Corneal collagen cross-linking and a fourth AMT were performed at 5 months after onset with partial success (online Technical Appendix Figure, panel F).

At 1 year after symptom onset, corneal limbal stem cell deficiency with deep corneal neovascularization was evident. Autologous limbal stem cell transplantation from the patient's other eye and another AMT were performed, resulting in stable corneal surface 2 months later (online Technical Appendix Figure, panel G). Neovascularization regressed, the cornea cleared, and vision improved (online Technical Appendix Figure, panels H, I).

Cowpox is transmitted to humans sporadically from rodents or cats (*5*). We snap-trapped 23 wild rodents from the yard of the patient's home and from an adjacent meadow and trapped 136 rodents from 3 other regions 30–100 km from the patient's home (online Technical Appendix Table 2). We also collected 8 environmental samples from the patient's storehouse. In accordance with the Finnish Act on Use of Animals for Experimental Purposes (62/2006) and the Finnish Animal Experiment Board's later decision (May 16, 2007), the animal capture technique used is not an animal experiment and requires no ethics license.

Diluted blood for IFA was collected from all rodents (*6*), and DNA was extracted from rodent liver and lungs and from environmental samples. One vole and 1 mouse from the meadow were seropositive for OPV; however,

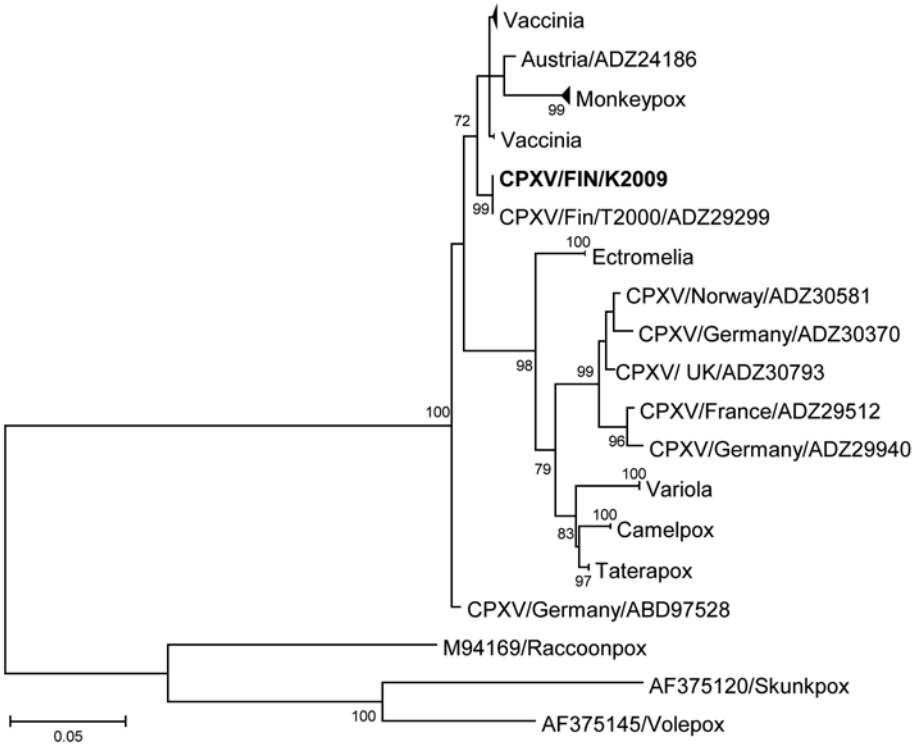

Figure. Phylogenetic tree of orthopoxviruses constructed on the basis of the hemagglutinin gene; boldface indicates the CPXV strain infecting the patient described in this article. The phylogeny shows that the sequence derived from this patient represents a locally circulating strain that shares ancestry with a few other CPXV strains and vaccinia virus. A maximum-likelihood tree was built with 1,000 bootstraps in MEGA 6.06 software (http://www.megasoftware.net/). MEGA was used to estimate the best nucleotide substitution model (general time reversible plus invariable sites). The sequence dataset was compiled from the Virus Pathogen Resource database (http://www.viprbrc.org) and aligned by using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Scale bar indicates nucleotide submissions per site. CPXV, cowpox virus.

no CPXV DNA was amplifiable in the samples from the liver, lungs, or environment (online Technical Appendix Table 2).

CPXV infection may manifest in severe ocular forms along with self-limiting cutaneous pocks (*5*). Our patient had keratitis with no other identifiable cause but CPXV. Culture and PCR from early conjunctival samples and serology confirmed the etiologic diagnosis.

Our case and that of another report (*7*) highlight the challenges of treating cowpox keratitis. Topical and systemic antiviral drugs and AMT appear ineffective during the acute phase. Corneal melting and scarring continued as long as CPXV was observed and until combined limbal stem cell and AMT treatment had favorable outcomes. Anamnesis of therapy-resistant keratitis should include information on rodent contacts.

We dated the infection to mid-August (incubation 7–21 days). Catching OPV-IgG–positive rodents close to the patient's home 2 months after onset showed that OPVs were circulating in the local rodent population and indicated the putative role of CPXV-infected voles as the source of infection.

The latest cowpox outbreak in Central Europe involved several humans and pets (*8*). This patient was born in 1977, after Finland ceased smallpox vaccinations. Declining cross-reactive smallpox-vaccination immunity enables emergence of unusual cowpox infections in humans (*9*).

Acknowledgments

We thank Katja Koskela with the Finnish Defence Forces for help with the sequence analysis program.

This research was partially funded by EU grant GOCE-2003-010284 EDEN.

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Human Alveolar Echinococcosis, Czech Republic, 2007–2014

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DOI: http://dx.doi.org/10.3201/eid2112.150743

To the Editor: Human alveolar echinococcosis (AE) is a zoonotic parasitic disease caused by larvae of *Echinococcus multilocularis* tapeworms that manifests most often in the host's liver, although it can infect the lungs, brain, and other organs. Clinical symptoms usually develop after a long incubation period (5–15 years), causing considerable diagnostic difficulties and delay in treatment. The disease is reported in all countries neighboring the Czech Republic: Slovakia, Poland, Austria, and Germany (*1*,*2*). To complete data from central Europe, we present results on the occurrence of AE in the Czech Republic collected by the National Reference Laboratory for Tissue Helminthoses during 2007–2014.

In the Czech Republic, the occurrence of *E. multilocularis* in definitive (red fox, dog, cat, raccoon dog) and intermediate (bank vole) hosts was reported (*3*). After the first reports on detection of the parasites in foxes during 1995 (*4*; Figure), physicians started to request laboratory examinations for AE in persons with liver lesions, suspicious clinical symptoms, or both. During 1998–2014, examinations of 1,892 patients revealed 20 AE cases (12 women, 8 men); the first 2 cases were diagnosed during 2007 (*5,6*). In all cases, the diagnosis was based on AE characteristic imaging by using ultrasonography, computed tomography, magnetic resonance imaging, or a combination of these methods; in 19 (95%) cases, the results were confirmed by *E. multilocularis*–specific serology. In-house *E. multilocularis* crude–antigen was used for ELISA and Western blot testing and for ELISA IgG for detection of *E. multilocularis*. Em2–Em18 antibodies (Bordier Affinity Products SA, Crissier, Switzerland) were used for some laboratory examinations. Since 2009, in-house Western blot has been done by using a commercial set (LDBIO, Lyon, France).

In 18 cases, AE was also confirmed by characteristic histopathologic findings, species–specific molecular analysis of tissue biopsies, or both. PCR assay according to Schneider et al. (*7*) has been used in the National Reference Laboratory since 2011. In 17 (85%) patients, the liver was the only affected organ, and the infection was classified according to Brunetti et al. (*8*) as PN0M0; in 3 patients, liver and brain (PN0M1), retroperitoneum (PN1M0), or kidneys (PN1M0) were also affected.

Analysis of gender and age at the time of initial AE diagnosis showed that the youngest and the oldest patients were 21 and 82 years old, respectively. To examine differences in non–Gaussian distributed variables between male and female patients, we used the 2-way Mann–Whitney nonparametric test (GraphPad, San Diego, CA, USA) to analyze age data. The mean and median age of patients were lower among women (mean 45, median 36.5 years) than in men (mean 53, median 60 years), but these differences were not statistically significant ($p>0.05$).

According to the site of residence, the patients originated from different parts of the Czech Republic (Figure). The disease was diagnosed in 18 native Czech citizens and in 2 citizens from Slovakia.

Physicians interviewed 17 of 20 patients in whom AE was diagnosed and completed questionnaires with patient data including clinical signs and symptoms, laboratory findings, and medical history (e.g., job, hobbies, travels abroad, ownership of animals) at the time of the first medical visit before diagnosis. Ten patients reported a prickling sensation and abdominal discomfort or pain. Three patients palpated a solid mass in the right hypochondrium before physical examination. Another 3 patients reported fever, fatigue, or malaise; 1 patient reported weight loss. The first clinical examinations by physicians revealed hepatomegaly in 16 patients; in addition, 3 of these patients had anemia and 1 had jaundice.

In the medical history, dog or cat ownership, gardening, farming, or hunting were recorded in some cases, which is similar to what was reported by Kern et al. (*10*). Of 15 persons interviewed who were native to the Czech Republic, 6 reported no travel outside the country (Figure). Because of the occurrence of *E. multilocularis* in animals (Figure), we assume that AE may have a characteristic of autochthonous infection in the Czech Republic.

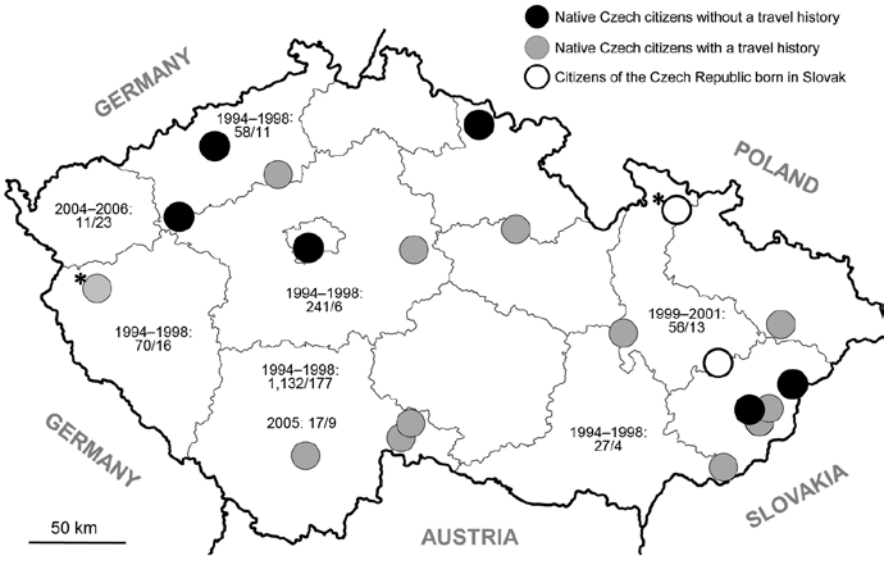

Figure. Distribution of human alveolar echinococcosis (AE) in the Czech Republic during 2007–2014, according to the site of residence of 20 case-patients, including their travel history. Asterisks (*) indicate AE cases already published (*6*,*7*). Six patients reported no travel outside the country; 2 patients were born in Slovakia and lived in the Czech Republic for 5 and 14 years before the time of initial AE diagnosis; the remaining patients traveled from the Czech Republic to various countries, including those to which AE is endemic. Nonperiodic examinations of red foxes (*4*,*9*) revealed the presence of *Echinococcus multilocularis* in the country. Date ranges indicate the period of examination; numbers separated by virgules indicate the number of foxes examined and those that tested positive, respectively.

The 2 patients from Slovakia lived in the Czech Republic for 5 (*5*) and 14 years, respectively, before the diagnosis of AE. Considering the long incubation period of the disease, these patients were likely infected in Slovakia, where occurrence of AE is also reported (*1*).

In summary, we report 20 cases of human AE in the Czech Republic during 1998–2014. However, because asymptomatic patients with only mild liver involvement are unlikely to seek clinical investigation, the actual number of patients in the Czech Republic who have AE is expected to be even higher than that reported here.

Acknowledgments

We thank Markéta Leissová and Petra Kotíková for technical assistance during the examination.

The study was supported by the Charles University in Prague (Research Programs PRVOUK No. P25/LF1/2 and UNCE–Grant No. 204017), and Grant Agency of the Ministry of Health IGA MZCR NT 13108-4/2012.

L.K. conceived and wrote the paper. J.M., J.H., H.K., L.H., V.Z., H.A., and F.S. participated in the design of the analysis, commented on the first draft of the paper, and approved the final version.

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Use of Capture–Recapture to Estimate Underreporting of Ebola Virus Disease, Montserrado County, Liberia

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DOI: http://dx.doi.org/10.3201/eid2112.150756

To the Editor: Underreporting of cases during a large outbreak of disease is not without precedent (*1–5*). Health systems in West Africa were ill-prepared for the arrival of Ebola virus disease (Ebola) (*6*). The Ebola outbreak in Liberia was declared on March 31, 2014, and peaked in September 2014. However, by mid-June, the outbreak had reached Montserrado County, where the capital, Monrovia, is located. In response, the Liberia Ministry of Health and Social Welfare (MOHSW) created a National Ebola Hotline: upon receipt of a call, a MOHSW case investigation team was dispatched to the site of the possible case. Additionally, persons could seek care at an Ebola Treatment Unit (ETU) or be referred to an ETU by another health care facility. During June 1–August 14, 2014, MOHSW, Médecins Sans Frontières, and the US nongovernment organization Samaritan's Purse managed 3 ETUs in Montserrado County, including 2 in Monrovia operated by Eternal Love Winning Africa (ELWA).

In August 2014, to assess the extent of underreporting in the midst of the Ebola outbreak, we analyzed 2 sources of data collected during June 1–August 14. The first comprised data collected by MOHSW case investigation teams. These data were collected on MOHSW case forms and entered into a database emulating these forms using Epi Info version 7 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). The second data source

(designed on Excel 2003; Microsoft, Redmond, WA, USA) comprised data on all patients admitted to the 2 ELWA ETUs (ELWA1 and ELWA2). We used a capture–recapture (CRC) approach.

CRC can evaluate the completeness of reporting and thereby be used to correct for underreporting (*7*). CRC methods use data from overlapping databases to estimate the number of unreported cases and thus more closely derive the true number of Ebola cases. Both databases were populated and managed separately, although the included Ebola cases are assumed to reflect the same patient population in Montserrado County. These 2 databases enabled us to use CRC to estimate the true number of Ebola cases in Montserrado County.

To be included in either database, a case must have been classified as suspected, probable, or confirmed Ebola. The case definitions, following the official MOHSW definition for Ebola, were identical in both databases. Eventually, after laboratory confirmation, cases could be reclassified as "not a case" and thus be excluded from the analysis.

To estimate the total number of Ebola cases during the study period, we used Chapman's 2-sample CRC population estimate (*7*); we calculated the 95% CI as proposed by Wittes et al. (*8*). We performed a sensitivity analysis measuring impact of error in matching cases during record linkage.

A total of 227 Ebola cases were recorded in the MOHSW database and 99 Ebola cases in the Montserrado County ETUs database (Table). Of these, 25 were found in both databases, 202 in the MOHSW database only, and 74 in the Montserrado County ETU database only. We estimated that the cumulative number of Ebola cases for Montserrado County during the study period was 876 (95% CI 608–1,143).

A sensitivity analysis performed with ±5 cases showed that, with 5 additional cases in common between databases, the cumulative number of cases would decrease to 734 (95% CI 537–931); with 5 additional discordant cases, the estimate would increase to 1,085 (95% CI 700–1,469). Our analysis shows that the number of cases in Montserrado Country was at least 3-fold higher than that reported during the study period.

Our study had several limitations. According to the doctor in charge of data collection up to August 4, some forms (<10) completed at the beginning of June 2014 might have been misplaced. Additionally, some patients who entered the ETU were not recorded in the registry book (<5). CRC assumes a closed population. In Montserrado County, persons can move freely. In both databases, we included only cases that occurred in or were reported in Montserrado County.

CRC assumes that links between the 2 sources based on identifying case information are error free. The sensitivity analysis suggested that even if up to 5 case matches were not detected, our conclusion was relatively robust.

CRC assumes homogeneity in the likelihood of being captured and recaptured and that data sources are independent. In our analysis, homogeneity is unlikely. For example, the MOHSW database was more likely to capture cases in persons more likely to seek care; the ETU database was more likely to detect cases in persons referred by health workers. Similar behaviors might have resulted in positive dependency in each data source. Both heterogeneity and positive dependency with data sources leads to underestimation.

Despite these limitations, we estimated more Ebola cases than were reported through official channels during the beginning of the outbreak in Montserrado County. Routine studies similar to ours can rapidly provide public health officials managing the outbreak response with estimates of underreporting and enable timely mobilization of appropriate resources. However, we believe that further exploration of this technique to better understand the possible difference of capture preference of each source may help improve the technique and benefit future outbreaks.

Acknowledgments

We thank Ian Alley for his great help in writing and structuring this article. We also thank the Ministry of Health and Social Welfare for its support and commitment. We are grateful to the data encoders, the investigation team, and the health care workers who collected data in very difficult circumstances.

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Malformations Caused by Shuni Virus in Ruminants, Israel, 2014–2015

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DOI: http://dx.doi.org/10.3201/eid2112.150804

To the Editor: Viruses in the Simbu serogroup are arboviruses that cause abortion, stillbirth, and congenital abnormalities in domestic ruminants. Akabane virus (AKAV), Aino virus (AINV), and Schmallenberg virus are the most studied in this serogroup; Shuni, Sabo, Shamonda, and Sango viruses (*1*,*2*) are examined less frequently. Until 2012, only AKAV had been associated with congenital abnormalities in Israel, although AINV had been identified serologically in dairy cow herds with no clinical signs in 2003 (*3*). Moreover, of 15 brain samples collected during February–October 2012 from adult cows with central nervous system manifestations, 6 were positive for AKAV by PCR.

In late December 2014, the Israeli Veterinary Field Services was notified of the appearance of arthrogryposis-hydranencephaly syndrome (*1*) in 2 herds of sheep in the villages of Yokneam and Sde Ya'akov, respectively; both villages are located in the Izre'el Valley, in Israel's northern valleys (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0804-Techapp.pdf), where several arboviral infections have occurred in recent decades. From our past experience (*3*), ≥1 virus of the Simbu serogroup was suspected to have infected the ruminants, probably during August–October 2014.

We collected 27 samples of brain, placenta, spleen, lung, and blood (mixed with EDTA to prevent coagulation) from 15 sheep, goats, and cattle. Most samples were from the 2 affected flocks in the northern valley; a few were from ruminants in additional locations: Avadon, near Israel's border with Lebanon; Ein Hachoresh, near central Israel; and Hura, close to the Negev desert (online Technical Appendix Figure 1).

Of the 27 samples, 23 (85%) were positive for Shuni virus (SHUV) by PCR (Table). SHUV, which had not been reported in Israel, was isolated from the brain and placenta of 1 malformed lamb (strain 2504/3/14; sample 11 in the Table). Moreover, partial nucleotide sequences of the small, medium, and large DNA segments (580/850, 4,320/4,326, and 285/6,880 bp, respectively) were identified from 3 samples (strains Yokneam 2417/2/14 and 2504/3/14 and Hura 273/14 from samples 2, 11, and 9, respectively, in the Table; online Technical Appendix Figure 2). Sequence data obtained by conventional PCR in this study have been deposited into GenBank (accession nos. KP900863–5, KP900873–5, KP900879–80, and KP900884). Phylogenetic analysis of the samples showed that they were isolates of SHUV (online Technical Appendix Figure 2). Additional SHUV RNA-specific fragments were detected in pathologic samples from kids, lambs, and calves (Table). Full-genome sequences were not performed, although sequencing should be done when possible to determine precise origin of isolates.

For further testing, we inoculated homogenate material from 7 distinct malformations (samples 1, 2, 6, 8, 11, 12, and 15 in the Table) into baby mice; only 1 family of baby mice inoculated intracerebrally with the SHUV isolate (sample 11 in the Table) exhibited characteristic neurologic signs of nervousness. PCR confirmed that SHUV caused the cerebral infections in these mice. The isolate was also suitable for further propagation in the Vero cell line (Table).

Our results showed the presence of SHUV in sheep in Israel during the winter of 2014–15 and suggest a northward expansion of SHUV from sub-Saharan Africa. Although SHUV was first isolated in the 1960s (*2*), its role as a pathogen has been shown only recently in its involvement in encephalitis in horses (*4*). We isolated SHUV from the pathologic fetal brain of a malformed lamb, an unusual laboratory finding because, although Simbu viruses are readily isolable from vectors or exposed animals during the 3 or 4 days of viremia, they are seldom isolable from pathologic specimens collected for study of congenital malformations. We deduce from the clinical evidence that malformations appear up to 6 months after infection with SHUV and after the virus has been eliminated from the host after immune

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Table. Summary of diagnostic and laboratory findings, animal species, sample materials, and region where samples were collected in the study of Shuni virus infection in ruminants, Israel, 2014–15*

Animal no.	Laboratory no.	Species	Clinical manifestation	Region	PCR-positive sample	Vero isolation	Virus isolation in mice
1	2417/1/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
2	2417/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Negative	Negative
3	267/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
4	267/3/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
5	267/4/14	Sheep	Malformed, aborted fetus	Northern valley	Brain	Not done	Not done
6	2498/1/14	Sheep	Weak lamb syndrome	Northern valley	Brain, EDTA-blood	Negative	Negative
7	2504/1/14	Sheep	Malformed aborted fetus	Northern valley	Brain	Not done	Not done
8	2504/2/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
9	273/14	Sheep†	Malformed, aborted fetus	Negev	Brain	Not done	Not done
10	274/14	Sheep	Aborted fetus	Northern valley	Brain, placenta	Not done	Not done
11	2504/3/14	Sheep†	Malformed, aborted fetus	Northern valley	Brain, placenta	Positive	Positive
12	275/1/14	Sheep	Malformed, aborted fetus	Northern valley	Brain, placenta	Negative	Negative
13	275/2/14	Sheep	Malformed aborted fetus	Northern valley	Brain, placenta	Not done	Not done
14	263/14	Goat	Malformed, aborted fetus	Northern valley	Brain, placenta	Not done	Not done
15	215/14	Cattle	Aborted fetus	Upper Galilee	Brain	Negative	Negative

*Not done, not performed if insufficient brain material was available for cerebral inoculation or if the infected brain failed to propagate in the cell line. For some animals, >1 sample was collected.
†Sequences used to build the phylogenetic trees in online Technical Appendix Figure 2 (http://wwwnc.cdc.gov/EID/21/12/15-0804-Techapp1.pdf).

activity. Thus, isolation of SHUV from malformed brains may indicate strong neurotropism of this putative pathogen. The possibility of its replication in the fetal nervous system should also be considered because an affected fetus that is born alive is likely a reservoir. Indeed, AKAV was identified in the hippocampus (only) of adult lactating cows (data not shown), and similar epidemiologic evidence might result from other Simbu virus infections.

A serologic survey conducted in Israel during the 2001–2003 outbreaks of AHS showed reactivity of AINV to serum samples of ruminants in Israel's southern regions (*3*). Because AINV and SHUV are known to have a strong serologic cross-reaction, SHUV has likely previously infiltrated Israel. However, whether the seroreactivity results from AINV or SHUV remains unresolved.

The emergence and reemergence of arboviruses should interest medical practitioners, particularly epidemiologists. The appearance of exotic viruses in unexpected locations might result in more severe pathology in newly invaded regions than in the original arbovirus-endemic areas. Furthermore, SHUV has been detected in a child with febrile illness (*2*), a finding that suggests a potential zoonotic problem.

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Genetic Characterization of Highly Pathogenic Avian Influenza A(H5N6) Virus, Guangdong, China

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DOI: http://dx.doi.org/10.3201/eid2112.150809

To the Editor: Since the first detection of the influenza A(H5N1) virus in geese in China during 1996 (http://www.cdc.gov/flu/avianflu/h5n1-virus.htm), H5 subtype viruses have continued to reassort and evolve, giving rise to multiple virus clades and gene constellations. Recently, clade 2.3.4.4 viruses have shown a predilection for genetic reassortment, giving rise to H5N2, H5N5, H5N6, and H5N8 virus subtypes, and have become globally widespread, causing infections in wild birds or poultry elsewhere in Asia, and in Europe and North America (*1*–*3*). The H5N6 subtype viruses have circulated in China since 2013 and have been mainly identified in ducks or chickens in the southern (Jiangxi, Guangdong) or western (Sichuan) areas (*4*,*5*). Two lineages of H5N6 viruses with distant genetic background were found among the H5N6 viruses isolated in China (*5*).

In China, there have been 3 cases of H5N6 virus infection among humans, causing 2 deaths. We recently reported the clinical characteristics and progression of a patient infected by the H5N6 virus in Guangzhou City, China, who was the second reported case-patient infected with this subtype (*6*). After having contact with poultry, he began to manifest an influenza-like illness on December 3, 2014, and progressed to a primary viral pneumonia. The H5N6 virus A/Guangzhou/39715/2014 (GenBank accession nos. KP765785–KP765792) was isolated from a throat swab specimen collected on day 8 of his illness by inoculation into 9–11-day-old, specific pathogen-free embryonated chicken eggs. He recovered from his infection and was discharged from the hospital on day 58.

Multiple sequence alignments showed that the hemagglutinin (HA) and neuraminidase (NA) genes of A/Guangzhou/39715/2014 shared the highest nucleotide identity with A/chicken/Dongguan/2690/2013 (H5N6) (99.4% and 98.3%, respectively) (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp1.pdf). All internal genes were also closely related to A/chicken/Dongguan/2690/2013 (H5N6), ranging from 98.5% nucleotide identity for the polymerase acidic (PA) gene and 100.0% for the matrix (M) gene. The genome segments were also 98.2%–99.7% identical to A/duck/Guangdong/GD012014 and 98.3%–99.4% identical to A/chicken/Laos/LPQ001/2014, which caused outbreaks in domestic ducks and poultry, respectively, indicating that these viruses have the same genotype.

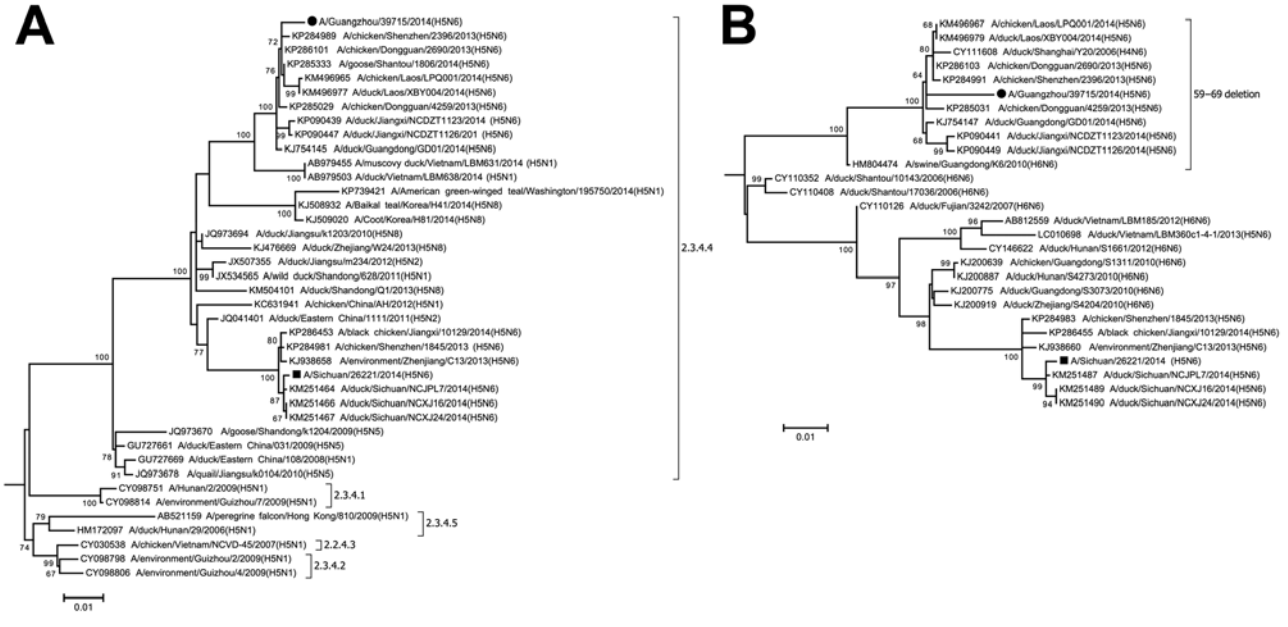

Figure. Phylogenetic trees of influenza A(H5N6) virus isolate A/Guangzhou/39715/2014 compared with other influenza viruses based on the A) hemagglutinin (HA) and B) neuraminidase (NA) genes, China. Maximum-likelihood trees were constructed by using the the general time reversible plus gamma distribution plus invariant sites (GTR+G+I) model in MEGA 6.06 (http://www.megasoftware.net). Bootstrap values were calculated on 1,000 replicates; only values >60% are shown. A/Guangzhou/39715/2014 and A/Sichuan/26221/2014 are indicated by a circle and a square, respectively. Brackets denote H5 subtype virus clades. Scale bars indicate nucleotide substitutions per site. Full HA and NA trees are provided in online Technical Appendix 1 (http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp1.pdf).

HA gene phylogeny confirmed that this virus belonged to clade 2.3.4.4 (online Technical Appendix 1). Notably, the HA genes of the H5N1, H5N2, and H5N8 viruses that were recently detected in wild birds in North America also belong to this clade, indicating that viruses from this clade are becoming globally widespread. More specifically, this isolate clustered within a sublineage that includes H5N6 isolates from poultry from Guangdong and Jiangxi provinces, China, and from Laos (*5,7*). The A/Sichuan/26221/2014 (H5N6) virus that recently caused a fatal human infection in Sichuan Province, China is also within clade 2.3.4.4, but clusters in a distinct sub-lineage (Figure, panel A).

The HA cleavage site of both human isolates contained multiple basic amino acids, suggesting that that they are highly pathogenic avian influenza viruses. Amino acid substitutions E190D, Q226L, or G228S (H3 numbering) in the HA gene that are known to enhance binding to mammalian receptors were not found. The NA gene phylogeny showed that A/Guangzhou/39715/2014 is likely originated from group II lineage influenza A(H6N6) viruses that circulate among domestic ducks in China (*8*) (Figure, panel B). An 11-aa deletion at the residue 59–69 position of the NA protein was identified in the isolate of this study, in the other H5N6 viruses of the same cluster, and in an H4N6 virus isolate from a duck in Shanghai, China. This deletion was monophyletic and likely originated from A/swine/Guangdong/K6/2010 (H6N6)–like viruses (Figure, panel B). However, it was not observed in other 2.3.4.4 viruses, such as A/Sichuan/26221/2014.

No mutations associated with oseltamivir or amantadine resistance was found in NA or M2 genes. The internal genes of the current H5N6 isolate were similar to 2.3.2.1b H5N1 subtype viruses found in domestic ducks from south-central and eastern China (*5,7–10*; online Technical Appendix 1). The 6 internal genes are 97%–99% homologous to another isolate from a human, A/Sichuan/26221/2014, suggesting that the internal genes of the viruses may reassort from a common origin.

The phylogenetic clustering observed for the HA gene was also conserved for the internal genes. In contrast with all avian viruses within this clade, the current human isolate contains the mammalian adaptation mutation PB2-E627K, and A/Sichuan/26221/2014 has acquired PB2-D701N, suggesting a rapid acquisition of mammalian adaptation changes that likely arose after human infection.

There is still limited information on human disease caused by the emerging H5 lineage. Our genetic analysis suggests that the H5N6 virus isolated from the patient is originated from the avian host. Although the genetic background of H5N6 virus isolated from the third case in Yunnan Province, China, on January 2015 is still not known, the isolates from the human cases of H5N6 infection reported to date show distant genetic diversity, indicating that viruses from both clusters may pose a threat to humans. This rapidly evolving and globally spreading virus lineage thus provides a threat to global public health.

Acknowledgments

We thank the authors and originating and submitting laboratories of the sequences in the EpiFlu Database from Global Initiative on Sharing Avian Influenza Data, on which this research is based (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/21/12/15-0809-Techapp2.xlsx).

This study was supported by Municipal Science and Technology Bureau Foundation of Guangzhou (2014Y2-00031), National Science and Technology Major Project of the Ministry of Science and Technology of China (2014ZX10004006), Science research project of the Guangdong Province (2013B020224006), the Area of Excellence Scheme of the Hong Kong University Grants Committee (AoE/M-12/06) of the Government of Hong Kong Special Administrative Region, China and Contract HHSN272201400006C from the National Institute of Allergy and Infectious Disease, National Institutes of Health, USA.

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Surveillance for Ebola Virus in Wildlife, Thailand

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DOI: http://dx.doi.org/10.3201/eid2112.150860

To the Editor: Active surveillance for zoonotic pathogens in wildlife is particularly critical when the pathogen has the potential to cause a large-scale outbreak. The recent outbreak of Ebola virus (EBOV) disease in West Africa in 2014 was initiated by a single spillover event, followed by human-to-human transmission (*1*). Projection of filovirus ecologic niches suggests possible areas of distribution in Southeast Asia (*2*). Reston virus was discovered in macaques exported from the Philippines to the United States in 1989 and in sick domestic pigs in the Philippines in 2008 (with asymptomatic infection in humans) (*3*). Dead insectivorous bats in Europe were found to be infected by a filovirus, similar to other members of the genus *Ebolavirus* (*4*).

Although EBOV has historically been viewed as a virus from Africa, recent studies found that bat populations in Bangladesh and China contain antibodies against EBOV and Reston virus recombinant proteins, which suggests that EBOVs are widely distributed throughout Asia (*5*,*6*). Thus, an outbreak in Asian countries free of EBOV diseases may not only be caused by importation of infected humans and/or wildlife from Africa but may arise from in-country filovirus–infected wildlife. Serologic and molecular evidence for filoviruses suggests that members of the order Chiroptera (bats) may be their natural reservoir (*7*).

As part of a proactive biosurveillance program, we conducted a cross-sectional study for EBOV infection in bats and macaques in Thailand. We screened 500 *Pteropus lylei* bats collected from 10 roosting sites during March–June 2014 (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/12/15-0860-Techapp1.pdf) for antibodies against EBOV antigen by using an ELISA validated by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*8*).

Bats and macaques were captured with permission from the Department of National Parks, Wildlife and Plant Conservation. The Institutional Animal Care and Use Committee at the University of California, Davis (protocol #16048) approved the capture and sample collection protocols.

To further screen a wide range of wildlife species in Thailand for active EBOV infection, we sampled and tested 699 healthy bats, representing 26 species, and 50 long-tailed macaques (*Macaca fascicularis*). Additional bat species were randomly captured (≥50/site) in 6 provinces in Thailand during 2011–2013 and identified by morphologic traits. Macaques were captured and sampled in March 2013 from 1 site at Khao Chakan, Sa Kaeo Province, and released at the same site. Blood, saliva, urine, and feces were collected from anesthetized macaques or nonanesthetized bats. All animals were released after sample collection. Details on species screened, sample sizes, and trapping localities are provided in the Table.

All nonblood specimens were collected in nucleic acid extraction buffer (lysis buffer) and transported on ice to the World Health Organization Collaborating Centre for Research and Training on Viral Zoonoses laboratory (Bangkok, Thailand) for storage and testing. Three types of specimen (saliva, urine, and serum) were collected from individual animals and pooled.

Nucleic acid was then extracted with NucliSENS easyMAG (bioMérieux, Boxtel, the Netherlands) and analyzed by reverse transcription PCR (RT-PCR). A consensus RT-PCR was used to screen for all known species of Ebola virus and Marburg virus, including EBOV (*9*). In total, 5 RT-PCRs were performed on each specimen, a regimen that included 4 sets of primers specific to known filoviruses and 1 degenerate primer set to detect novel viruses in this family. The sensitivity of RT-PCR on synthetic standard was 50–500 copies/reaction (*9*). We ran 3,745 PCRs, covering a range of assays, to increase detection sensitivity. All specimens examined were negative for filoviruses by EBOV ELISA and PCR (Table). For *P. lylei* ELISA screening, optical density values for all 500 bats ranged from 0.000 to 0.095, well below the potential positive cutoff value of 0.2.

Assuming a population size of ≈5,000 bats/roost and a sample size of 50 bats/site, we have 95% confidence that

Table. Overview of bats and macaques tested by Ebola virus IgG ELISA or PCR for filoviruses, Thailand, 2011–2014

Species	Host family	No. tested (no. positive)	Test method*	Specimen type†	Location‡
Chiroptera					
Pteropus lylei	Pteropodidae	500 (0)	ELISA	Serum	a
Cynopterus brachyotis	Pteropodidae	10 (0)	PCR	Pooled	b
C. sphinx	Pteropodidae	4 (0)	PCR	Pooled	b
Eonycteris spelaea	Pteropodidae	12 (0)	PCR	Pooled	b
Macroglossus sobrinus	Pteropodidae	2 (0)	PCR	Pooled	b
Megaerops niphanae	Pteropodidae	1 (0)	PCR	Pooled	b
Rousettus amplexicaudatus	Pteropodidae	3 (0)	PCR	Pooled	b
Hipposideros armiger	Hipposideridae	113 (0)	PCR	Pooled	b
H. cineraceus	Hipposideridae	4 (0)	PCR	Pooled	b
H. larvatus	Hipposideridae	33 (0)	PCR	Pooled	b, c
H. lekaguli	Hipposideridae	158 (0)	PCR	Pooled	b
Megaderma lyra	Megadermatidae	1 (0)	PCR	Pooled	b
Miniopterus magnate	Vespertilionidae	132 (0)	PCR	Pooled	b, c
M. pusillus	Vespertilionidae	1 (0)	PCR	Pooled	b
M. schreibersii	Vespertilionidae	22 (0)	PCR	Pooled	b
Myotis horsfieldi	Vespertilionidae	6 (0)	PCR	Pooled	b
M. muricola	Vespertilionidae	1 (0)	PCR	Pooled	b
Rhinolophus shameli	Rhinolophidae	44 (0)	PCR	Pooled	b
R. coelophyllus	Rhinolophidae	7 (0)	PCR	Pooled	c
R. luctus	Rhinolophidae	1 (0)	PCR	Pooled	b
R. malayanus	Rhinolophidae	4 (0)	PCR	Pooled	c
R. microglobosus	Rhinolophidae	1 (0)	PCR	Pooled	b
R. pusillus	Rhinolophidae	1 (0)	PCR	Pooled	b
Scotophilus kuhlii	Vespertilionidae	1 (0)	PCR	Pooled	b
Taphozous longimanus	Emballonuridae	27 (0)	PCR	Pooled	b
T. melanopogon	Emballonuridae	110 (0)	PCR	Pooled	b
Total		699 (0)			
Primate					
Macaca fascicularis	Cercopithecidae	50 (0)	PCR	Pooled	d

*ELISA for IgG against Ebola virus.
†Nucleic acid extraction from Pooled saliva, serum, and urine.
‡a, Central Thailand; b, Eastern Thailand; c, Chaing Mai Province; d, Kao Chakan, Sa Kaeo Province.

if >6% of the population had antibodies against EBOV antigen, we would have detected it. If we assume that all 500 animals are part of 1 large panmictic population, and we have 95% confidence that if EBOV were circulating in >0.5% of the population, we would have detected it. Therefore, although we cannot rule out infection of this species with 100% confidence, *P. lylei* bats, the most abundant species of large pteropid bats in Thailand, are highly unlikely to be reservoirs for EBOV.

Our sample sizes for PCR screening of other bat species in this study were much smaller, and we had no supported serologic data, but these negative results could add to the knowledge of filovirus infection in nontissue specimens from healthy bats. Previous studies have detected Ebola virus–like filovirus RNA in lung tissue of healthy *Rousettus leschenaultia* bats in China (*10*) and from organs and throat and rectal swab specimens from a die-off of *Miniopterus schreibersii* bats in Spain (4). In our study, which included 22 *M. schreibersii* and 132 *M. magnate* bats, none of the bats tested positive for filoviruses.One limitation of the cross-sectional sampling strategy used here, however, is that PCR-negative findings do not necessarily mean that the bats were not infected in the past. Although we found no evidence of filovirus infection in wildlife species tested in Thailand, we believe that continuing targeted surveillance in wildlife should enable early detection and preparedness to preempt emerging zoonoses.

This study was supported by a research grant from Department of National Parks, Wildlife and Plant Conservation, the Thailand Research Fund (RDG5420089), the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (RES560530148-HR), Health and Biomedical Science Research Program by National Research Council of Thailand and Health System Research Institute, the Research Chair Grant, the National Science and Technology Development Agency, Thailand, and the Naval Health Research Center (BAA-10-93) under the Cooperative Agreement no. W911NF-11-2-0041, and the United States Agency for International Development Emerging Pandemic Threats PREDICT project.

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Probability of Spirochete *Borrelia miyamotoi* Transmission from Ticks to Humans

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DOI: http://dx.doi.org/10.3201/eid2112.151097

To the Editor: Borreliosis caused by *Borrelia miyamotoi* is an emerging disease transmitted by *Ixodes* ticks (*1*). Each year in the Netherlands during 2007–2009, ≈70,000 bites by hard ticks occurred per 1 million inhabitants (*2*). In the Republic of Udmurtia, Russia, ≈10,000 hard tick bites per 1 million inhabitants are reported annually among persons seeking medical help. Recent studies indicate that almost 3% of *I. ricinus* ticks in the Netherlands and 2%–6% of *I. persulcatus* ticks in Russia are infected with *B. miyamotoi* (*1*,*3*,*4*). Human exposure is substantial, and comparable exposure to *B. miyamotoi* is expected in many Eurasian countries and in North America (*4*,*5*). The probability of *B. miyamotoi* transmission from ticks to humans requires examination to estimate the true risk for human disease.

In Izhevsk (population 650,000), a city in European Russia (Republic of Udmurtia), we identified 95 human cases of *B. miyamotoi* infection during 2010–2014 (*6*). In this city, primarily because of concern about tickborne encephalitis (TBE), all patients with suspected tickborne infection are hospitalized in the Republican Hospital of Infectious Diseases (RHID). A service also enables tick-bitten persons to bring the removed tick for PCR for TBE virus (TBEV) and *B. burgdorferi* sensu lato. We supplemented that with PCR testing for *B. miyamotoi* (*3*).

In June 2014, twenty-four persons (≈5% of those bitten by ticks subjected to PCR-based investigation for TBEV, *B. burgdorferi* sensu lato, and *B. miyamotoi*) were bitten by adult *I. persulcatus* ticks infected with *B. miyamotoi* only. We informed these persons of their results and about the clinical features of *B. miyamotoi* infection and recommended self-observation during 2 months (twice the maximum incubation period for *B. miyamotoi* infection [*3*,*6*]). These persons were advised to contact their medical supervisor at RHID (D.S. Sarksyan) if fever, fatigue, erythema migrans, or any other possible symptom of a tickborne infection developed. In 2 patients, such symptoms developed: one 12 days (patient 1), the other 16 days (patient 2), after the tick bite. *B. miyamotoi* DNA was detected by PCR in their blood on admission to RHID. Neither IgM nor IgG were found by a nonspecific ELISA (Omnix, St. Petersburg, Russia [*7*]) that reacts with serum from *B. burgdorferi* sensu lato–infected and *B. miyamotoi*–infected persons. However, *Borrelia* IgM and IgG were detected in serum obtained 12 and 45 days after illness onset from patient 1 and 6 and 39 days later from patient 2. Clinical characteristics were typical of *B. miyamotoi* infection: chills, sweating, headache, dizziness, fatigue, thirst, nausea, vomiting, fever (axillar temperature 38.8°C in patient 1 and 39.0°C in patient 2). Erythema migrans was absent. Both patients had modest thrombocytopenia (134 [patient 1] and 118 [patient 2] $\times 10^9$ platelets/mL [reference range 150–400 $\times 10^9$ platelets/mL]) and increased band neutrophils (10% [patient 1] and 8% [patient 2] of leukocytes [reference range 1%–5%]). Patient 2 had clinical and laboratory signs of kidney dysfunction (oliguria, leukocytes, erythrocytes, and epithelial cells in a urine sample), a complication observed in 18% of 95 patients with *B. miyamotoi* disease (*4*). Both patients were

treated with doxycycline (100 mg 2×/d) for 10 days; they clinically recovered, and laboratory abnormalities returned to reference ranges at discharge 12 days after admission.

The remaining 22 persons did not report any malaise and were examined 1 month after tick bite. They appeared healthy at that time, and PCR and ELISA gave negative results, arguing against possible asymptomatic *B. miyamotoi* infection.

We estimated the probability of *B. miyamotoi* transmission to humans to be 8.3% (95% CI 4%–18% using a Bayesian approach [*8*] or 95% CI 0%–21% using an SPSS bootstrapping procedure [SPSS Inc., Chicago, IL, USA]). For comparison, among 68 persons bitten by *B. burgdorferi* sensu lato–infected ticks in the Netherlands, erythema migrans developed in 4.4% (95% C.I. 2.1%–8.3%) persons; 3 (4.4%) others seroconverted without clinical symptoms (*9*).

This pilot study has several limitations. We did not follow up persons bitten by *B. burgdorferi* sensu lato– or TBEV-infected ticks because they received either antimicrobial drugs or anti-TBE immunoglobulin as a preventive measure. Because of labor constrains, we did not study persons bitten by "PCR-uninfected" ticks; however, they were not hospitalized at RHID, the only hospital in the region for patients with evident tick-borne diseases. We did not use any serologic techniques specific for relapsing fever *Borreli* (e.g., GlpQ ELISA). Although we did not test for *Rickettsia* or *Babesia* spp., we did not find TBEV RNA, *B. burgdorferi* sensu lato 16S RNA, pathogenic *Ehrlichia* 16S RNA, or pathogenic *Anaplasma* DNA in the 2 *B. miyamotoi*–positive patients' blood samples.

We demonstrated that the transmission rate of *B. miyamotoi* appears to be equal to, or higher, than that of *B. burgdorferi* sensu lato (*1*,*9*,*10*). Our data indicate that, annually, clinical *B. miyamotoi* infection might develop in at least 0.005% of persons living in regions to which *Ixodes* spp. ticks and *B. miyamotoi* are endemic. This estimate corresponds to ≈33 cases annually in Izhevsk, which is similar to the previously published results of hospital-based surveillance for *B. miyamotoi* (*3*,*6*).

This study was supported by the grant of Russian Scientific Foundation (project no. 15-15-00072) to A.E.P., D.S.S., and L.S.K.

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Outbreak of Exanthematous Illness Associated with Zika, Chikungunya, and Dengue Viruses, Salvador, Brazil

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DOI: http://dx.doi.org/10.3201/eid2112.151167

To the Editor: Zika virus (ZIKV) has been recognized as an emerging mosquito-borne flavivirus since

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outbreaks were reported from Yap Island in 2007 (*1*), French Polynesia in 2013 (*2*), and Cook Island and New Caledonia in 2014 (*3*). It has joined dengue virus (DENV) and chikungunya virus (CHIKV) as global public health threats (*4*). ZIKV infection typically causes a self-limited dengue-like illness characterized by exanthema, low-grade fever, conjunctivitis, and arthralgia, and an increase in rates of Guillain-Barré syndrome have been observed during ZIKV outbreaks (*5*).

In Brazil, clusters of cases of acute exanthematous illness have been reported from various regions since late 2014, and in April 2015, ZIKV was identified as the etiologic agent (*6*). In May 2015, the Brazilian Ministry of Health recognized circulation of ZIKV in Brazil. We report epidemiologic findings for an ongoing outbreak of acute exanthematous illness in the population of Salvador, the third largest city in Brazil.

The Salvador Epidemiologic Surveillance Office (ESO) was first alerted to cases of an acute exanthematous illness early in 2015. Reporting of cases increased during March, and in April the ESO established 10 public emergency health centers in Salvador as sentinel units for systematic surveillance of patients with acute exanthematous illness of unknown cause. The units searched retrospectively for suspected cases by review of medical charts of patients treated since February 15, continued with prospective case detection, and submitted weekly reports of identified cases to the ESO.

During February 15-June 25, a total of 14,835 cases of an indeterminate acute exanthematous illness were reported from the 12 sanitary districts in Salvador. The overall attack rate was 5.5 cases/1,000 persons (4.6 cases/1,000 men and 6.3 cases/1,000 women, 8.2 cases/1,000 children <15 years of age, 5.4 cases/1,000 persons 15–39 years of age, and 3.8 cases/1,000 adults ≥40 years of age).

The epidemic curve peaked in the first week of May, which was 1 week after molecular diagnosis of ZIKV in 8 patients residing ≈50 km from Salvador and during a period of intense media coverage of the outbreak (Figure) (*6*). Reporting of suspected dengue cases in Salvador did not vary substantially from that in other years and was >5 times lower: 2,630 cases, of which 165/366 (45.1%) were positive for dengue IgM, 20/590 (3.4%) positive for dengue virus nonstructural protein 1, and 1/11 (9.1%) positive for dengue virus by reverse transcription PCR (Figure). During the same period, 58 cases of suspected chikungunya were reported and 24 patients with suspected Guillain-Barré syndrome were hospitalized.

The median age of case-patients was 26 years (interquartile range 11–39 years), but all age groups were affected, which is a pattern typical of spread of new microorganisms (or subtypes) in a susceptible population. Median duration of symptoms at time of medical attention was 1 day (interquartile range 0–3 days). All patients had exanthema and most (12,711/14,093 [90.2%]) had pruritus. Fever (4,841/13,786, 35.1%), arthralgia (278/1,048 [26.5%]), headache (3,446/13,503 [25.6%]), and myalgia (223/1,033 [21.6%]) were less common.

Serum samples from some patients were examined for rubella IgM (2/200, 1.0% positive), rubella IgG (15/18, 83.3% positive), measles IgM (0/11, 0% positive), dengue nonstructural protein 1 (3/185, 1.6% positive), dengue IgM (17/80, 21.3% positive), parvovirus B19 IgM (0/1, 0% positive), and parvovirus B19 IgG (1/1, 100% positive). Reverse transcription PCR was performed on 58 serum samples stored at –20°C and confirmed ZIKV in 3 (5.2%) samples, CHIKV in 3 (5.2%) samples, DENV type 3 in 1 (1.7%) sample, and DENV type 4 in 1 (1.7%) sample.

Identification of ZIKV, CHIKV and DENV as etiologic agents of acute exanthematous illness suggests that these 3 *Aedes* spp. mosquito-transmitted viruses were co-circulating in Salvador and highlights the challenge in clinically differentiating these infections during outbreaks. Although we were not able to determine the specific incidence of each virus, the low frequency of fever and arthralgia, which

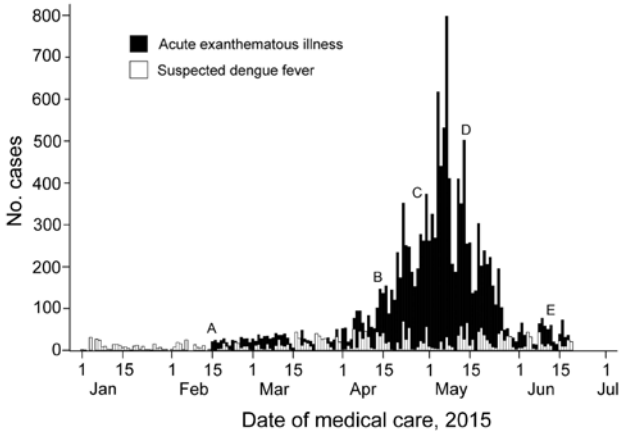

Figure. Reported cases of indeterminate acute exanthematous illness and suspected dengue fever in Salvador, Brazil, by date of medical care, February 15−June 25, 2015. Letters indicate specific events. A) February 15: systematic reporting of cases of acute exanthematous illness of unknown cause begins in Salvador. B) April 13: Salvador Epidemiologic Surveillance Office releases its first epidemiologic alert about the outbreak in Salvador. C) April 29: Zika virus is confirmed in 8 samples from patients residing ≈50 km from Salvador (http://portalsaude.saude.gov.br/index.php/situacao-epidemiologica-dados-dengue-2) and media coverage of the outbreak intensifies (http://www.correio24horas.com.br/detalhe/noticia/doenca-misteriosa-que-atinge-cidades-baianas-e-identificada-como-zika-virus/?cHash = 74792c41f3128395ba0ffa5e1ed9dbbe). D) May 14: Brazilian Ministry of Health announces circulation of Zika virus in Brazil (http://portalsaude.saude.gov.br/index.php/o-ministerio/principal/secretarias/svs/noticias-svs/17702-confirmacao-do-zika-virus-no-brasil). E) June 11: Brazilian press announces that cases of Zika virus infection have been confirmed in 8 states in Brazil (http://www1.folha.uol.com.br/cotidiano/2015/06/1640752-virus-primo-da-dengue-zika-ja-tem-casos-confirmados-em-oito-estados.shtml).

are indicators of dengue and chikungunya, point to ZIKV as the probable cause of several of the reported cases. Furthermore, laboratory-confirmed cases of infection with ZIKV were simultaneously identified in other cities within metropolitan Salvador (*6,7*) and in other states in Brazil (*8*). Low diagnosis of ZIKV infection is likely because viremia levels among infected patients appear to be low (*9*).

The spread of ZIKV represents an additional challenge for public health systems, particularly because of the risk for concurrent transmission of DENV and CHIKV by the same vectors, *Ae. aegypti* and *Ae. albopictus* mosquitoes, which are abundant throughout tropical and subtropical regions. To date, the largest outbreak of chikungunya in Brazil occurred in 2014 in Feira de Santana, Bahia, ≈100 km from Salvador, where dengue is also prevalent (*10*).

This report illustrates the potential for explosive simultaneous outbreaks of ZIKV, CHIKV, and DENV in the Western Hemisphere and the increasing public health effects of *Aedes* spp. mosquitoes as vectors. The apparent increase in reports of Guillain-Barré syndrome during the outbreak deserves further investigation to elucidate whether this syndrome is associated with ZIKV infection. Public health authorities in Brazil and neighboring countries should plan accordingly.

Acknowledgments

We thank the health professionals of the Municipal Health Secretariat of Salvador, particularly those working in surveillance activities, for invaluable assistance in case reporting and outbreak investigation; and the municipal laboratory staff for assistance.

This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq grant 400830/2013-2 and CNPq scholarships to I.A.D.P., M.S.R., U.K., M.G.R., and G.S.R.), the Bahia Foundation for Research Support (grant PNX0010/2011), and the Coordination for the Improvement of Higher Education Personnel, Brazilian Ministry of Education (CAPES scholarship to M.K.).

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Emerging Rabbit Hemorrhagic Disease Virus 2 (RHDVb), Australia

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DOI: http://dx.doi.org/10.3201/eid2112.151210

To the Editor: In May 2015 an isolate of the recently emerged variant of rabbit hemorrhagic disease virus (RHDV), RHDV2, was identified in an Australian wild rabbit (*Oryctolagus cuniculus*). RHDV2 (also called RHDVb) was first described in outbreaks in France in 2010 (*1*), then Italy and Spain in 2011 (*2*,*3*) and in Portugal from 2012 onwards (*4*). The virus is a genetically and antigenically distinct variant of RHDV that is able to partially overcome immunity to classical strains of RHDV (*1*,*2*). In contrast

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to case-fatality rates for other strains of RHDV, those for RHDV2 infection have been reported to be lower in mature rabbits (0%–75% in 1 study, compared with >90% for classic RHDV infection) (*3*) but higher (50% in 1 study) in rabbit kittens as young as 30 days of age, which are normally highly resistant to lethal RHDV infection (*2*). RHDV2 has been reported to spread effectively in domestic rabbits in Europe (*3*); it may be replacing existing strains of RHDV that infect wild rabbits on the Iberian Peninsula (*5*), possibly because of its ability to partially overcome immunity to these strains.

As part of ongoing opportunistic surveillance of RHDV field outbreaks, we analyzed 3 isolates from dead adult wild rabbits found in the wider Canberra region of Australia. The first virus isolate (BlMt-1) came from a rabbit found in Australian Capital Territory on May 13, 2015. The second isolate (BlueGums-2) was taken 3 days later from a rabbit in New South Wales, 50 km north of Canberra. On June 9, another dead rabbit, from which the third isolate (BlMt-2) was obtained, was found in the same location as the first. The isolates were initially typed by amplifying and sequencing the capsid gene (*6*), and the results were confirmed independently in 2 laboratories. Subsequently, full-length genome sequencing of the 3 virus isolates was performed by amplifying the viral genomes in overlapping fragments (*6*); the fragments were then sequenced by using Illumina MiSeq technology (*7*).

Phylogenetic analysis revealed that 2 isolates, BlMt-2 and BlueGums-2, were closely related to field strains currently circulating in Australia (*7*) (Figure). Strikingly, the third isolate (BlMt-1) was most closely related to an RHDV2 variant generated by recombination of the RHDV2 capsid gene (Figure, panel B) and the RHDV genogroup 1 nonstructural genes (Figure, panel A), which has recently been reported to be circulating in Portugal and the Azores (*8*,*9*). How the virus variant arrived in Australia is unclear, although our analysis indicates that it likely originated in southern Europe.

In 1991, CSIRO imported the Czech351 strain of RHDV to assess its potential as a biocontrol tool for controlling the European rabbit, which causes massive economic and ecologic damage and is declared a pest species in Australia. In 1995, after initial testing in quarantine, the virus escaped during field trials being conducted on a coastal island through passive fly transmission and subsequently spread across the continent. The RHDV2 variant reported here has not previously been investigated by CSIRO, and the organization did not possess it.

Rabbits are found in ≈70% of the 6.7 million km^2 Australian continent and Tasmania. However, natural outbreaks of RHDV infection are monitored in comparatively few locations, and their detection largely relies on opportunistic sampling. To follow the spread of this new variant and determine its current range, increased surveillance of outbreaks of RHDV infection in both wild and domestic rabbits in Australia is urgently required. The unique traits of strain RHDV2, particularly its ability to overcome immunity to classical RHDV strains (including vaccine strains) (*3*) and to infect rabbits at a younger age (*2*), may have wide-ranging implications for rabbit biocontrol in Australia. In

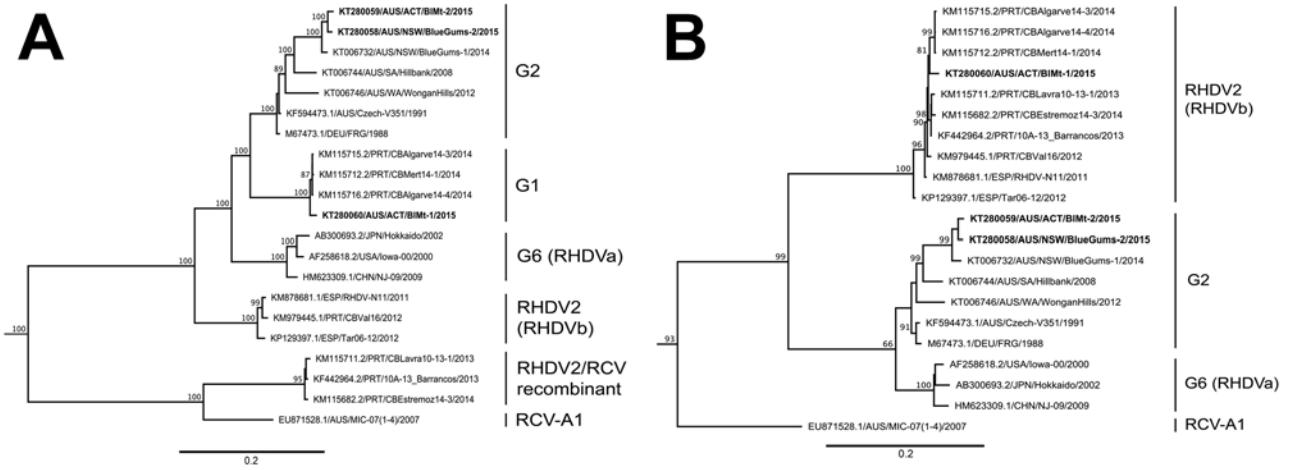

Figure. Maximum-likelihood phylogenetic analysis of the nonstructural protein genes (A) and the capsid gene (B) of rabbit hemorrhagic disease virus (RHDV) sequences. The 3 recent Australian field isolates sequenced for this study (indicated in bold) were aligned with representative RHDV and Australian rabbit calicivirus (RCV-A1) sequences from GenBank (accession numbers indicated in taxa names). Phylogenetic analysis was conducted separately for both the nonstructural genes (panel A) and the capsid gene (panel B). Phylogenies were rooted by using an early European brown hare syndrome virus strain (not shown). Statistical support for individual nodes was estimated from 1,000 bootstrap replicates with values shown for only those nodes where the bootstrap support was ≥70% (and all major nodes). Phylogenies were constructed by using the general time reversible plus gamma model of nucleotide substitution, as determined in jModelTest, by using PhyML(as available in Geneious version 8.1.5; Biomatters Limited, Auckland, New Zealand). Scale bars indicate nucleotide substitutions per site.

parallel with similar efforts in Europe, strategies need to be developed to protect commercial and pet rabbits.

Tracking the spread of RHDV2 in Australia, in competition with existing field strains, highlights the value of Australia's rabbits and their diseases as a model system for emerging infectious diseases. The point releases of both myxoma virus and RHDV into large naive host populations represent a grand experiment in disease emergence and evolution (*10*), which provides a unique opportunity to study the virulence evolution of emerging pathogens as well as their complex interactions with each other. It is notable that since the release of RHDV in Australia in 1995, strains of 1 viral lineage dominate the viral population nationwide despite hundreds of deliberate rereleases of the original virus strain for local rabbit control, which strongly suggests it has a major selective advantage (*7*). That RHDV2 appeared in a wild rabbit is therefore remarkable, particularly because Australian field strains were spreading simultaneously in the same area. Comparing the epidemiology of this strain in Australia to the epidemiology of its well-documented spread in Europe will provide valuable insights into RHDV epidemiology relevant to both continents.

Acknowledgments

We thank Roslyn Mourant and James Biglia for assistance with sample processing.

J.E.M. is supported by grant DP140103362 from the Australian Research Council. E.C.H. is supported by a National Health and Medical Research Council Australia fellowship (AF30).

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Characteristics of Traveler with Middle East Respiratory Syndrome, China, 2015

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DOI: http://dx.doi.org/10.3201/eid2112.151232

To the Editor: A traveler returning from the Middle East initiated an outbreak of Middle East respiratory syndrome (MERS) in South Korea in 2015, which resulted in 186 cases and 36 deaths (*1–3*). We report a case of

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MERS in a 43-year-old man from South Korea who acquired this disease during this outbreak (online Technical Appendix Figure 1, panel A, http://wwwnc.cdc.gov/EID/article/21/12/15-1232-Techapp1.pdf) (*4*).

The National Health and Family Planning Commission of China determined that collection of data for this patient was part of a public health investigation of an emerging outbreak. Therefore, informed consent was not required. This study was approved by the ethical committee of the First Affiliated Hospital of Guangzhou Medical University.

The patient had been receiving thiamazole for 7 years for hyperthyroidism. He had contact with the index case-patient during the outbreak in South Korea on May 16, 2015. On May 25, the patient traveled to Hong Kong and then to Huizhou, China. He was hospitalized in China on May 28 (day 7 of illness). At admission, he had a high fever (temperature 39.5°C) and a dry cough. Chest radiography on day 7 showed mild bilateral ground glass opacities in the lower lung (online Technical Appendix Figure 1, panel B).

The patient was given oseltamivir (150 mg, 2×/day for 2 days) until identified as being infected with Middle East respiratory syndrome coronavirus (MERS-CoV) on day 8 by real-time reverse transcription PCR. He was given ribavirin (2.0 mg on day 8; 0.6 mg 3×/d on days 9–16; and 0.6 mg 2×/d on days 17–19) and 135 μg of peginterferon αa-2a by intravenous injection on day 8 (online Technical Appendix Table 2). Thrombocytopenia and a decrease in the hemoglobin level developed, which might have been related to use of ribavirin (online Technical Appendix Table 1).

Chest radiography on June 1 (day 11) showed increased bilateral consolidation of the patient's lower lung (online Technical Appendix Figure 1, panel C). He was given intravenous immunoglobulin, antimicrobial drugs, and thymosin α1. His body temperature returned to normal on day 14 (online Technical Appendix Figure 2). Chest radiography on day 35 showed resolution of bilateral lung infiltrations (online Technical Appendix Figure 1, panel D). He was discharged on day 36.

Viral RNA was detected in sputum and fecal specimens up to day 26 of illness. Virus load in sputum specimens collected on days 11–15 were lower than in specimens obtained on days 16–18 (online Technical Appendix Figure 3, panel A). Swab samples collected on days 13 and 15 from the patient's palm, mobile telephone, blanket, and bed railings, and from his hospital room floor were negative for viral RNA.

Concentrations of proinflammatory cytokines and chemokines (interferon-α, interferon-inducible protein 10, monocyte chemoattractant protein-1, interleukin 6 [IL-6], IL-10, tumor necrosis factor-α, IL-8, macrophage inflammatory protein-α [MIP-1α], MIP-1β, and IL-1β) were determined for serial serum samples. Interferon-α, interferon-inducible protein 10, monokine induced by interferon-γ, IL-6, monocyte chemoattractant protein-1, and IL-8 were detected on day 11 of illness but levels decreased as the patient clinically improved (online Technical Appendix Figure 3, panel B).

The peginterferon αA2 the patient was given on day 8 might have influenced his plasma interferon-α levels (*6*). However, a previous study also showed increased levels of interferon-α in a patient who survived MERS-CoV infection but not in a person who died of MERS (*7*). Although MERS-CoV evades induction of innate immune responses by cell types, the virus elicits interferon responses in plasmacytoid dendritic cells in vitro (*8*). Levels of tumor necrosis factor-α, MIP-1α, MIP-1β, IL-10, and IL-1β did not increase in any of these specimens.

Peripheral blood mononuclear cells (PBMCs) obtained on day 24 of illness showed a strong specific T-cell response against MERS-CoV spike protein but not against severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein (online Technical Appendix Figure 3, panel C). PBMCs from persons who were infected with SARS-CoV in 2003, as well as healthy persons, showed low-level T-cell responses against MERS-CoV spike protein, although some persons with a history of SARS still had detectable responses to SARS-CoV spike protein. It was reported that T-cell responses to SARS-CoV were directed against spike and nucleocapsid proteins (*9*). We did not have sufficient PBMCs to test T-cell responses against nucleocapsid protein.

Results for MERS-CoV antibody were negative at day 11 of illness by MERS-CoV spike pseudotype assay (MERS-S ppNT), microneutralization, 50% plaque reduction neutralization test ($PRNT_{50}$), and S1 ELISA (EUROIMMUN AG, Lübeck, Germany). The patient showed seroconversion by day 14. MERS-S ppNT and $PRNT_{50}$ provided earlier evidence of seroconversion (day 15) and higher antibody titers than the microneutralization, (day 18) (online Technical Appendix Figure 3, panel D). Potent T-cell responses were elicited to MERS-CoV spike protein. These responses did not show cross-reactivity with SARS-CoV spike protein.

The MERS-S ppNT, which does not require Biosafety Level 3 containment, had sensitivity equivalent with that of $PRNT_{50}$, which requires containment. Thus, MERS-S ppNT is a sensitive and specific assay for detecting neutralizing antibody against MERS-CoV. The sensitivity and specificity of this assay have been well-documented with serum samples from dromedary camels and other animals (*10*).

This study was supported by the Science Research Project of the Guangdong Province (2013B020224006); the Municipal Science and Technology Bureau Foundation of Guangzhou (2014Y2-00031); the National Key Project of Clinical Faculty and Facility Construction on Infectious Diseases (2013–2014); and the National Institute of Allergy and Infectious Diseases, National Institutes of Health (HHSN272201400006C).

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The Politics and Crisis Management of Animal Health Security

By John Connolly. 216 pages. Farnham, United Kingdom: Ashgate Publishing, 2015. Hardback: $109.95, ISBN: 978-1-4724-3774-7; ebook (PDF): $109.95, ISBN: 978-1-4724-3775-4; ebook (ePUB): $109.95, ISBN: 978-1-4724-3776-1.

Even as the lessons learned from the response to the 2014–2015 Ebola outbreak in West Africa begin to be analyzed, history provides other key examples of health crises that challenge traditional thinking and, in some cases, government structures themselves. In his new book The Politics and Crisis Management of Animal Health Security, author John Connolly examines the events and aftermath of the 2001 foot and mouth disease (FMD) outbreak in the United Kingdom, an event that cost >£8 billion (US ≈$11.6 billion), resulted in the death or slaughter of 6.5 million animals, and led to the country's first delay in a national election since World War II.

Caused by a picornavirus, FMD affects cloven-hoofed animals including sheep, cattle, and pigs. Because of the disease's highly infectious nature, FMD-free countries like the UK must respond quickly to outbreaks because of economic trade consequences; a quick and well-coordinated response is key to success. However, in 2001, some early and notable missteps in UK government decisions, including a 3-day lag in imposing national animal movement bans, led to viral spread via animal movements and a mushrooming of the national crisis. The atmosphere of the outbreak response was highly charged, and battles between government and stakeholders played out daily in the media with political cartoons and images of burning carcasses. The crisis led to the dissolution of the country's Ministry of Agriculture, Forestry and Fisheries and the creation of the new Department for Environment, Food & Rural Affairs (DEFRA). In his book, Connelly asks, have the lessons learned during the aftermath of the 2001 FMD outbreak been adequately applied, and is the UK better prepared to deal with a substantial animal health crisis in the future?

Written in multiple parts, this book provides an initial primer in crisis management and defines theoretical drivers of change. Connelly describes the FMD outbreak in the United Kingdom as a "critical juncture" situation: that is, a public crisis combined with the political climate to drive necessary organizational change. Next, the book takes the reader through a description of the acute crisis, including technical details of the outbreak's spread and the primary features of the government response. The book then moves the reader into an understanding of DEFRA's post-FMD crisis management and how the new agency tackled a "fix" to the problem through contingency planning, improved communications, science advocacy, and stakeholder engagement. Finally, the book reviews the efficacy of DEFRA's new plans, as executed during a 2006–2007 avian influenza A(H5N1) crisis and a 2007 outbreak of FMD associated with an accidental laboratory release. The author's conclusions are that many of the lessons learned from the 2001 outbreak have resulted in positive change and a stronger capacity for the government to respond to recent and future outbreaks.

Reviewing this book carries personal significance for me, as I spent a month in England in 2001 helping the United Kingdom as part of the response. I talked directly with the farmers at the epicenter of the outbreak and saw firsthand the public distrust fueled by challenges in communicating government decisions. Now, working in public affairs and scientific communications myself, I found it very informative to review this book and look back at that time through a new professional lens. The book lacks, perhaps, a robust discussion of the role of media and public outcry in shaping government policy decisions, but nonetheless proves an excellent reference for anyone who wishes to understand organizational change in response to crises, particularly in the health and veterinary sectors. The positive changes that have taken place since 2001 provide me with hope that the United Kingdom—and other countries that choose to pay close attention—may indeed be better prepared for future outbreak responses.

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DOI: http://dx.doi.org/10.3201/eid2112.151507

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ABOUT THE COVER

Walter Crane (1845–1915), *Beauty and the Beast*, 1875. Book illustration, wood engraving, printed in color, 10 9/16 × 9 3/16 × 1/8 in/26.8 × 23.3 × 0.3 cm. Metropolitan Museum of Art, Open Access Collection, The Elisha Whittelsey Collection, The Elisha Whittelsey Fund, 1972

Anthropomorphism to Zoonoses: Two Inevitable Consequences of Human–Animal Relationships

Byron Breedlove and Paul M. Arguin

Many tales portray animals that mimic human behavior and characteristics by conversing, walking erectly, dressing in clothing, and inhabiting houses. Across myriad cultures and throughout history, stories and myths that feature anthropomorphism have helped us understand and relate to the natural world. This device is especially common, even expected, in children's literature.

Among the most enduring anthropomorphic stories is *Beauty and the Beast,* retold and reimagined in various printed versions, films, and plays. French author Gabrielle-Suzanne Barbot de Villeneuve (c. 1695–1755) is credited with creating the first version of the tale we know as Beauty and the Beast (*La Belle et la Bête*). Her 100-plus page story features a savage beast and various subplots, setting it apart from most subsequent versions; those derive from the 1767 abridged narrative by Jeanne-Marie Leprince de Beaumont, which depicts a kinder, gentler beast.

This month's cover image comes from a popular version of *Beauty and the Beast* by Walter Crane (1845–1915), published by John Lane in 1875. Crane's wood engravings used for the illustrations in this edition incorporated design elements from Japanese prints, classical sculpture, and tapestry. Crane has been recognized as "the most prolific and influential children's book creator of his generation" and because of his later success in other artistic endeavors as "one of the most ambitious British artists of the later nineteenth century."

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DOI: http://dx.doi.org/10.3201/eid2112.AC2112

This colorful, meticulously detailed illustration depicts a young woman, Beauty, dressed in prim Victorian clothing, administering to the dying Beast, a boar that has an elongated snout and tusks, but is dressed in a handsome red suit, lying in a field of flowers and surrounded by a dense forest. Two smartly dressed monkeys toting a fiasco of wine, "who always waited upon her with all the attention and respect that officers of a royal household are accustomed to pay to queens," watch with concern as the Beast's life hangs in the balance. Beauty, of course, saves the Beast and breaks the spell, restoring him to a handsome prince and leading to the "happily ever after" ending.

Beauty and the Beast offers a lens for viewing humankind's relationship and interactions with the other animals on the planet. Anthropomorphism—which requires the suspension of disbelief to pretend the impossible is real—is inevitable, considering that people have lived in close proximity to domesticated and wild animals for millennia. We rely on "beasts" for sustenance, transportation, and labor; as surrogates in our scientific and medical experiments; and as companions, protectors, and entertainers. We have a protracted history of both legal and illegal hunting and trading of wildlife, and we have long practiced and animal idolatry and worship.

Zoonoses are also an inevitable consequence of human–animal relationships and interactions. Zoonotic diseases can be caused by organisms such as viruses, bacteria, parasites, and fungi, and it is estimated that more than 60% of infectious diseases of humans are spread from animals. Knowing which animals could have zoonotic diseases proves challenging because both domesticated animals and wildlife may appear and act healthy and yet be carrying lethal pathogens.

With this knowledge, we can view the painting as a study in potential zoonotic exposures to poor Beauty. Is Beast really dying for want of the love of a beautiful maiden? Or is he gasping for breath due to his Nipah virus or swine influenza infection? One can almost hear Beast's rumbling cough as Beauty becomes infected by droplet transmission at such close proximity. And what about the healthy-appearing monkey attendants poised to pour a cup of wine? Southeast Asian macaques may be asymptomatically infected with herpes B virus or *Plasmodium knowlesi* parasites. Utensils shared with a monkey would be a convenient fomite for herpes B virus, which can be transmitted through monkey saliva. The woodland setting is also the ideal habitat for arboreal *Anopheles* mosquitoes that have been implicated in the transmission of malaria from monkeys to humans.

The One Health concept recognizes that the health of humans is connected to the health of animals and the environment. Interaction with animals is an integral part of our lives that works when we are mindful of the risks and take appropriate precautions when necessary.

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December 6–9, 2015
2015 National HIV Prevention Conference
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http://www.cdc.gov/nhpc/index.html

February 8–10, 2016
ASM Biodefense and Emerging Diseases Research Meeting
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biodefense@asmusa.org
http://www.asmbiodefense.org/

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ISID
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http://www.isid.org/icid/

May 18–21, 2016
The Society for Heathcare Epidemiology of America
Atlanta, GA USA
http://www.shea-online.org/Education/SHEASpring2016Conference.aspx

June 16–20, 2016
American Society for Microbiology
Boston, MA USA
http://www.asmmicrobe.org/

July 18–22, 2016
21st International AIDS Conference
Durban, South Africa
http://www.aids2016.org/

October 29–November 2, 2016
American Public Health Association
Denver, Colorado, USA
https://www.apha.org/events-and-meetings/annual/past-and-future-annual-meetings

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Article Title

Life-Threatening Sochi Virus Infections, Russia

CME Questions

1. You are seeing a 33-year-old man in the emergency department for a 2-day history of fever, malaise, and headache. His temperature is 39.4°C, and his pulse is 120 bpm. He is admitted, and a thorough workup is initiated, along with intravenous antibiotics and supportive care. Infection with hantavirus is in the differential diagnosis. Which one of the following statements regarding the demographics of patients infected with the Sochi virus in the current study is most accurate?

A. Patients were generally younger women
B. Patients were generally older men
C. Patients were generally older women
D. Patients were generally younger men

2. Which one of the following statements regarding laboratory testing of patients with Sochi virus in the current study is most accurate?

A. Only half of the cases demonstrated clear DOBV immunoglobulin G (IgG) positivity
B. 100% of patients developed anti-DOBV IgM in the acute phase
C. Patients' serologic reaction was stronger against other types of hantavirus compared with the Sochi virus
D. The genetic signature of the Sochi virus could not be distinguished from that of other hantaviruses

3. The patient is diagnosed with infection with the Sochi virus. Which one of the following anatomic sites had the highest concentration of Sochi virus among patients in the current study?

A. Lung
B. Kidney
C. Liver
D. Brain

4. Which one of the statements regarding the prognosis of patients infected with Sochi virus in the current study is most accurate?

A. The case-fatality rate was 1.5%
B. The case-fatality rate was 14.5%
C. Approximately half of the patients developed mild disease only
D. Disease was more severe among women vs men

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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.				
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1	2	3	4	5

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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.

Summary of Authors' Instructions

Author's Instructions. For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. 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 editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg 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.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

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.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

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.

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.

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

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.

In This Issue

Synopses

Research

Historical Review

Dispatches

ISSN 1080-6040

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Center for Disease Control and Prevention (CDC)
Mailstop D 61, Atlanta, GA 30329-4027

Vol 21, No 12, December 2015

EMERGING INFECTIOUS DISEASES

Pages 2117–2292

MEDIA MAIL
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