Emerging Infectious Diseases • Vol. 19, No. 11, November 2013

Synopsis

Tropheryma whipplei Endocarditis ...................... 1721
F. Fenollar et al.
This emerging clinical entity occurs mostly in middle-aged and older men with arthralgia.

Research

Atypical Scrapie Prions from Sheep and Lack of Disease in Transgenic Mice Overexpressing Human Prion Protein....................... 1731
J.D.F. Wadsworth et al.
Humans are less likely to acquire prion disease from sheep with naturally occurring disease than from cattle with epizootic disease.

G. La Ruche et al.
A revised method of quantifying data shows increase in imported dengue fever cases.

Pseudorabies Virus Variant in Bartha-K61–Vaccinated Pigs, China, 2012................................. 1749
T.-Q. An et al.
A virulent variant can evade the protection induced by this vaccine.

Migration and Persistence of Human Influenza A Viruses, Vietnam, 2001–2008............................... 1756
M.Q. Le et al.
The virus possibly persists for >1 year and migrates to and from China, Hong Kong, Taiwan, Cambodia, Japan, South Korea, and the United States.

Severe Influenza-associated Respiratory Infection in High HIV Prevalence Setting, South Africa, 2009–2011................................. 1766
C. Cohen et al.
HIV-infected persons should be prioritized to receive influenza vaccination.

Common Epidemiology of Rickettsia felis Infection and Malaria, Africa ........................................... 1775
O. Mediannikov et al.
An increased risk for Rickettsia felis infection in malaria-endemic regions of Africa is suggested.

Use of National Pneumonia Surveillance to Describe Influenza A(H7N9) Virus Epidemiology, China, 2004–2013............................... 1784
N. Xiang et al.
Closure of live-poultry markets appeared to reduce A(H7N9) transmission to humans.
Possible Association between Obesity and Clostridium difficile Infection .......... 1791
J. Leung et al.
Obesity was found at higher frequency among patients with C. difficile infections who had few other traditional risk factors.

Transmissibility of Livestock-associated Methicillin-Resistant Staphylococcus aureus ............ 1797
D.J. Hetem et al.
Transmissibility associated with livestock is 4.4 times lower than that not associated with livestock.

G. Wang et al.
These isolates can be acquired in health care settings and exhibit high rates of antimicrobial drug resistance.

Mobile Phone–based Syndromic Surveillance System, Papua New Guinea ............................. 1811
A. Rosewell et al.
This technology has the capacity to improve the timeliness and efficiency of public health surveillance.

Dispatches

1819 Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia
Z.A. Memish et al.

1824 Hantavirus Pulmonary Syndrome Outbreak, Brazil, December 2009–January 2010
A.C.P. Terças et al.

1828 Increased Incidence of Campylobacter spp. Infection and High Rates among Children, Israel
M. Weinberger et al.

1832 Two Novel Arenaviruses Detected in Pygmy Mice, Ghana
K.C. Kronmann et al.

1836 West Nile Virus, Texas, USA, 2012
K.O. Murray et al.

1839 Mayaro Virus Infection, Amazon Basin Region, Peru, 2010–2013
E.S. Halsey et al.

1843 Evidence of Vaccine-related Reassortment of Rotavirus, Brazil, 2008–2010
T.L. Rose et al.

1847 Three Outbreak-causing Neisseria meningitidis Serogroup C Clones, Brazil
D.E. Barroso et al.

1851 Severe Plasmodium vivax Malaria in Pakistan, A.B.S. Zubairi et al.

1855 Infectious Shock and Toxic Shock Syndrome Diagnoses in Hospitals, Colorado, USA
M.A. Smit et al.

1859 Human Bocavirus in Patients with Encephalitis, Sri Lanka, 2009–2010
D. Mori et al.
1863 Building Influenza Surveillance Pyramids in Near Real Time, Australia
C.B. Dalton et al.

1866 Incidence of Influenza A(H1N1) pdm09 Infection, United Kingdom, 2009–2011
S. Sridhar et al.

1870 Nontoxigenic Highly Pathogenic Clone of Corynebacterium diphtheriae, Poland, 2004–2012
A. A. Zasada

1873 Tula Hantavirus Infection in Immunocompromised Host, Czech Republic
H. Zelená et al.

1877 Human Bocavirus in Children with Acute Gastroenteritis, Chile, 1985–2010
J. Levican et al.

1881 Full Genome of Influenza A (H7N9) Virus Derived by Direct Sequencing without Culture
X. Ren et al.

1885 Mild Illness in Avian Influenza A(H7N9) Virus–Infected Poultry Worker, Huzhou, China, April 2013
H. Lv et al.

1889 Pulmonary Nontuberculous Mycobacterial Disease, Ontario, Canada, 1998–2010
T.K. Marras et al.

1892 Severe Fever with Thrombocytopenia Syndrome, South Korea, 2012
K.-H. Kim et al.

1895 Seoul Virus in Rats (Rattus norvegicus), Hyesan, North Korea, 2009–2011

1896 Schmallenberg Virus Infection in Dogs, France, 2012

1898 Geographic Co-distribution of Influenza Virus Subtypes H7N9 and H5N1 in Humans, China

1900 New Variant of Rabbit Hemorrhagic Disease Virus, Portugal, 2012–2013

1902 Mycobacterium yongonense in Pulmonary Disease, Italy

1904 Subcutaneous Infection with Dirofilaria spp. Nematode in Human, France

1906 Cytomegaloviruses: From Molecular Pathogenesis to Intervention

1908 My Name is Nobody

1838 Diphtheria

1908 Arbovirus Diseases, Southeastern United States
http://dx.doi.org/10.3201/eid1911.130650
This collection of 92 excerpts and covers from *Emerging Infectious Diseases* will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

**READY TO ORDER**

*Art in Science: Selections from EMERGING INFECTIOUS DISEASES*  
*Hardcover*

The journal’s highly popular fine-art covers are contextualized with essays that address how the featured art relates to science, and to us all.

Available at [http://amzn.to/16LNHtc](http://amzn.to/16LNHtc)
Tropheryma whipplei Endocarditis

Florence Fenollar, Marie Célard, Jean-Christophe Lagier, Hubert Lepidi, Pierre-Edouard Fournier, and Didier Raoult

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit.

This activity has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is accredited by the ACCME to provide continuing medical education for physicians.

Medscape, LLC designates this Journal-based CME activity for a maximum of 1 AMA PRA Category 1 Credit(s)™. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test with a 70% minimum passing score and complete the evaluation at www.medscape.org/journal/eid; (4) view/print certificate.

Release date: October 10, 2013; Expiration date: October 10, 2014

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

• Analyze the epidemiology of T. whipplei endocarditis
• Distinguish the most common symptom of T. whipplei endocarditis
• Assess laboratory findings associated with T. whipplei endocarditis
• Evaluate other findings among patients with T. whipplei endocarditis.

Tropheryma whipplei Endocarditis differs from classic Whipple disease, which primarily affects the gastrointestinal system. We diagnosed 28 cases of T. whipplei endocarditis in Marseille, France, and compared them with cases reported in the literature. Specimens were analyzed mostly by molecular and histologic techniques. Duke criteria were ineffective for diagnosis before heart valve analysis. The disease occurred in men 40–80 years of age, of whom 21 (75%) had arthralgia (75%); 9 (32%) had valvular disease and 11 (39%) had fever. Clinical manifestations were predominantly cardiologic. Treatment with doxycycline and hydroxychloroquine for at least 12 months was successful. The cases we diagnosed differed from those reported from Germany, in which arthralgias were less common and previous valve lesions more common. A strong geographic specificity for this disease is found mainly in eastern-central France, Switzerland, and Germany. T. whipplei endocarditis is an emerging clinical entity observed in middle-aged and older men with arthralgia.
Whipple disease was first described in 1907 (1). This chronic infection is characterized by histologic indication of gastrointestinal involvement, determined by a positive periodic acid–Schiff (PAS) reaction in macrophages from a small bowel biopsy sample (2). It is caused by *Tropheryma whipplei* and encompasses asymptomatic carriage of the organism to a wide spectrum of clinical pathologic conditions, including acute and chronic infections (1,2).

In 1997, *T. whipplei* was first implicated as an agent of blood culture–negative endocarditis in 1 patient by use of broad-range PCR amplification and direct sequencing of 16S rRNA applied to heart valves from patients in Switzerland (3). Two years later, 4 additional cases were reported in Switzerland (4). In 2000, the first strain of *T. whipplei* was obtained from the aortic valve of a patient with blood culture–negative endocarditis (5).

Blood culture–negative endocarditis accounts for 2.5%–31.0% of all cases of endocarditis. The incidence rate of *T. whipplei* endocarditis among blood culture–negative endocarditis cases has not been established; however, at our center (Assistance Publique Hôpitaux de Marseille, Marseille, France), this incidence rate was estimated to be 2.6% (6). In Germany, the reported incidence rate for *T. whipplei* endocarditis is 6.3%; *T. whipplei* was the fourth most frequent pathogen found among 255 cases of endocarditis with an etiologic diagnosis and was the most common pathogen associated with blood culture–negative endocarditis. This incidence rate exceeds rates of infections caused by *Bartonella quintana*; *Coxiella burnetii*; and members of the *Haemophilus, Actinobacillus, Cardiobacterium, Eikenella, Kingella* spp. group (7). Smaller studies found incidence rates of 3.5% in Denmark (8), 4.3% in Switzerland (9), 7.1% in the Czech Republic (10), 2.8% in Spain (11), and none in Algeria (12). We describe 28 cases of *T. whipplei* endocarditis and compare them with cases reported in the literature.

**Materials and Methods**

**Patient Recruitment and Case Definitions**

Our center in Marseille, France, has become a referral center for patients with *T. whipplei* infections and blood culture–negative endocarditis (2,5,6). We receive samples from France and other countries. Each sample is accompanied by a questionnaire, completed by the physician, covering clinico-epidemiologic, biological, and therapeutic data for each patient. We analyzed data from October 2001 through April 2013. Diagnosis of *T. whipplei* endocarditis was confirmed by positive results from PAS staining and/or specific immunohistochemical analysis and 2 positive results from specific PCRs of a heart valve specimen in addition to lack of histologic lesions in small bowel biopsy samples or lack of clinical involvement of the gastrointestinal tract.

**Laboratory Procedures**

DNA was extracted from heart valves, 200 µL of body fluid (blood in a tube containing EDTA, saliva, or cerebrospinal fluid), small bowel biopsy samples, and ≈1 gram of feces by using QIAGEN columns (QIAamp DNA kit; QIAGEN, Hilden, Germany) according to the manufacturer’s recommendations. Quantitative real-time PCR (qPCR) was performed by using a LightCycler instrument (Roche Diagnostics, Meylan, France) and the QuantiTect Probe PCR Kit (QIAGEN) according to the manufacturer’s guidelines. From October 2001 through September 2003, all specimens were tested by qPCR selective for the 16S–23S rRNA intergeneric spacer and the *rpoB* gene, as described (13); from October 2003 through March 2004, all specimens were tested by qPCR selective for *T. whipplei* repeated sequences (repeat PCR), as described (13). When an amplified product was detected, sequencing was systematically performed. Since April 2004, all specimens have been tested by a qPCR selective for *T. whipplei* repeated sequences, which used specific oligonucleotide Taqman probes for the identification (13). To validate the tests, we used positive and negative controls (13). For determination of DNA extract quality, the human actin gene was also detected. For positive specimens, *T. whipplei* genotyping was performed as described (14). In parallel, all heart valves and blood samples from patients with suspected endocarditis underwent systematic PCR screening for all bacteria (16S rRNA) and all fungi (18S rRNA) and underwent specific real-time PCR selective for *Streptococcus oralis* group, *Streptococcus gallowayticus* group, *Enterococcus faecium* and *E. faecalis*, *Staphylococcus aureus*, *Mycoplasma spp.*, *Coxiella burnetii*, *Bartonella spp.*, and *T. whipplei* as described (6).

For histologic analysis, formalin-fixed paraffin-embedded heart valves and small bowel biopsy samples were cut in thin sections. Samples stained with hematoxylin-eosin-saffron and special stains were examined, and immunohistochemical investigations with a specific antibody were performed as reported (15). Cardiac valve and heparinized blood specimens were injected into cell and axenic cultures (5,16). Serologic assays were based on Western blot analyses (17).

**Statistical Analysis**

Statistical analyses were performed by using Epi Info 6 (www.cdc.gov/epiinfo/Epi6/El6dnjp.htm). A p value <0.05 was considered significant. Data for the population of France were extracted from the National Institute for Statistics and Economical Studies website (www.insee.fr/fr/).
Incidence rates of endocarditis in France referred to our center were significantly more affected than the rest of the population. Appendix Table 2), the patients from these 2 areas were living in the Rhône-Alpes area and 5 (18.5%) in the Pays de la Loire area. Although samples are sent from all over France, 66.6% of the patients were from only these 2 areas. If we focus on the 702 patients with blood culture-positive endocarditis, reported the disappearance of slight pain (21). All patients were male (Table 1), and mean age (± SD) was 58.6 (± 10) years (range 40–80 years). Among the 27 patients from France, 13 (48.1%) were living in the Rhône-Alpes area and 5 (18.5%) in the Pays de la Loire area. Although samples are sent from all over France, 66.6% of the patients were from only these 2 areas. If we focus on the 702 patients with blood culture–negative endocarditis in France referred to our center from May 2001 through September 2009 (online Technical Appendix Table 2), the patients from these 2 areas were significantly more affected than the rest of the population (Rhône-Alpes 9/106 [8.5%] vs. 7/596 [1.16%], p<0.001 and Pays de la Loire 4/26 [15.4%] vs. 12/676; p<0.001) (6). Incidence rates of T. whipplei endocarditis in these 2 areas are also significantly higher than those in the rest of France (Rhône-Alpes 0.25 cases/1 million inhabitants/year, p<0.001; Pays de la Loire (0.15 cases/1 million inhabitants/year, p<0.001) (Figure 1).

Immunosuppressive therapy had been given to 7 (29%) patients, of which 3 received a tumor necrosis factor inhibitor. Arthralgia was reported for 21 (75%) patients; mean delay between arthralgia onset and endocarditis diagnosis was 8.5 years. Among 12 patients who had been interviewed by 1 of the authors (D.R.), arthralgia was detected in 11. Among these 12, arthralgia was retrospectively noticed by 1 patient who, after beginning treatment for endocarditis, reported the disappearance of slight pain that had been present for many years. Previous heart valve disease was known for 9 (32%) patients. Heart failure occurred in 20 (71.4%) patients, acute ischemic stroke in 7 (29.2%), and peripheral arterial embolism in 4 (16.6%). Fever was detected in 11 (39%) patients, and weight loss was experienced by 4 (14.3%). Echocardiography was performed for all 28 patients: transthoracic echocardiography for 4 patients, transesophageal echocardiography for 9 patients, both procedures for 7 patients, and unspecified procedures for 8 patients. Cardiac vegetations were found in 22 (78.6%) patients, and aortic valve involvement was found in 18 (64.2%).

Laboratory Findings

At the time of T. whipplei endocarditis diagnosis, increased C-reactive protein levels were detected in 17 (81%) of 21 patients, anemia in 6 (37.5%) of 16, and leukocytosis in 5 (29.5%) of 17. T. whipplei endocarditis was diagnosed by heart valve analysis (either PCR for T. whipplei or histologic analysis) for 27 of the 28 patients (online Technical Appendix Table 3). Other molecular analyses were negative for other microorganisms on all heart valves and in blood samples (when available). Blood samples were positive for T. whipplei for 5 (31.2%) of 16 patients. For 1 patient (patient 12), at 4 days before heart valve replacement, a blood sample was positive for T. whipplei according to repeat PCR and negative according to 16S rRNA PCR. For another patient (patient 1), a pacemaker was positive for T. whipplei by PCR. T. whipplei was detected in 1 (6.7%) of 15 saliva samples, 2 (16.6%) of 12 fecal samples, and none of 8 cerebrospinal fluid samples.

T. whipplei–infected heart valves show the typical histologic features of infective endocarditis: vegetations, inflammatory infiltrates, and valvular destruction (15). They show fibrotic, scarred areas. Valvular inflammatory infiltrates mainly consisted of foamy macrophages and lymphocytes. The foamy histiocytes were filled with dense and granular material that was strongly positive on PAS staining and resistant to diastase or immunopositive with a specific antibody against T. whipplei. T. whipplei–infected macrophages were seen in the vegetations on the surface of the heart valves and more deeply in the valvular tissues (Figure 2). An arterial embolus surgically removed from the lower limb of patient 22 was positive by immunodetection (15). However, 1 year before the heart valve was removed, this embolus had been histologically analyzed but infection was not suspected. Only after the valve was found to be positive for T. whipplei did subsequent analyses show that the embolus was positive for T. whipplei. Small bowel biopsy samples were obtained for 19 patients; all samples were negative by PAS staining, probably ruling out asymptomatic involvement of the gastrointestinal tract.

Two strains of T. whipplei were isolated from blood specimens, and 7 strains (including the strain from the index case-patient) from heart valve culture (5). For patient 20, a strain was isolated from the blood and heart valve specimens. The delay in primary isolation was 2 weeks for the heart valve sample and 8 weeks for the blood sample.
No other microorganism was isolated. Serum was available for 18 patients. According to our previously established criteria, 10 (55.5%) patients had a negative or weakly positive serologic profile, as is observed for patients with classic Whipple disease, and 8 (44.5%) patients had a frankly positive profile, as is observed for chronic carriers. This finding suggests a potentially less decreased antibody-mediated immune response for these patients (17). Thus, the previously established serologic profile for patients with classic Whipple disease is observed significantly less frequently among patients with *T. whippelii* endocarditis (10/18) than among patients with classic Whipple disease (56/60; p<0.001). The serologic profile previously observed for chronic carriers also occurs significantly less frequently among patients with endocarditis (24/26 vs. 8/18; p = 0.01).

*T. whippelii* genotype was obtained for 19 heart valves. Genotype 3 was detected in 5 samples, and genotype 1 was detected in 2 samples. The other 12 samples...
Tropheryma whipplei harbored a unique genotype. Genotypes for 4 patients were those previously detected in other circumstances (genotypes 8, 11, 19, and 97). Only patients with T. whipplei endocarditis had genotypes 7, 24, 87, 90, 96, 99, 113, 117. For 1 of these patients (patient 13), genotype 7 was detected in a heart valve sample at the time of diagnosis in 2002, but genotype 101 was detected in saliva and fecal samples in 2011 (Table 2). The patient did not have characteristics that favor endocarditis relapse.

**Treatment and Outcomes**

We focused on 14 patients for whom the entire treatment was managed by our team, 13 of whom regularly consulted author D.R. (Table 2). Overall, 12 patients received a combination of doxycycline and hydroxychloroquine for 7–18 months, and 2 received trimethoprim–sulfamethoxazole. One patient who experienced relapse received treatment for 7 months. According to analysis of saliva and fecal samples, 2 patients had been colonized by T. whipplei at another time. Colonization of 1 of these patients was with a new strain, but neither had cardiac abnormalities. We prescribed treatment for these patients, including 1 who had been taking lifelong prophylactic doxycycline, as reported for a patient with classic Whipple disease (25).

**Literature Review**

After checking for repeated reporting, we found 49 patients who met our criteria for T. whipplei endocarditis reported in the literature (online Technical Appendix Table 4); 7 (14.5%) were female (3,4,7–11,22–39). The patients were predominantly from Germany (15 [30.6%]) and Switzerland (12 [24.5%]). Figure 3 shows the number of reported cases of T. whipplei endocarditis per million inhabitants in Europe. The number of cases reported in the literature since 2010 has dramatically increased (Figure 4).

Among the cases reported in the literature, fever was scarcely observed (8/33, 24.2%), but vegetations (28/33, 84.8%) and involvement of the aortic valve (29/48, 60.4%) were frequent. The clinical manifestations were mainly heart failure (25/35, 71.4%), acute ischemic stroke (9/35, 25.7%), and peripheral arterial embolism (4/35, 11.4%). Arthralgia was observed significantly less frequently among patients reported in the literature (15/37, 40.5%) than among the patients we report (75%, p = 0.01). However, if the 14 patients

Figure 1. Number of reported cases of *Tropheryma whipplei* endocarditis per 1 million inhabitants in each area of France over 10 years. Data from this series and the literature (22–24) were included. Among the metropolitan areas in France, the incidence of T. whipplei endocarditis is significantly more frequent in the Rhône-Alpes area than in 11 others areas (Alsace, Aquitaine, Basse-Normandie, Bourgogne, Centre, Champagne-Ardenne, Haute-Normandie, Ile de France, Languedoc-Roussillon, Midi-Pyrénées, and Nord Pas-de-Calais; p = 0.04, p = 0.004, p = 0.048, p = 0.04, p = 0.01, p = 0.04, p = 0.02, p<0.001, p = 0.04, p = 0.007, p = 0.006, respectively). The incidence rate is also significantly more frequent in the Pays de la Loire area than in 6 other areas (Aquitaine, Bretagne, Centre, Ile-de France, Lorraine, Midi-Pyrénées, Nord Pas de Calais; p = 0.04, p = 0.04, p = 0.04, p = 0.03, p = 0.02, respectively).

Figure 2. Aortic valve from patient with *Tropheryma whipplei* endocarditis. A) Hematoxylin–eosin–safron stain (original magnification ×100). B) Foamy macrophages containing characteristic inclusion bodies (periodic acid–Schiff stain; original magnification ×200). C) Immunostaining of *T. whipplei* with polyclonal rabbit antibody against *T. whipplei* and Mayer’s hemalum counterstain (original magnification ×100). No destruction of this valve is visible.
from the recently published series from Germany (7) are excluded from the analysis, this difference is not significant (14/23, 60.9%; p = 0.4). The percentage of patients with a history of valvular heart disease was similar among the patients reported here (32%) and the patients reported in the literature (12/33, 36.4%; p = 0.9), but this analysis excludes the series from Germany. The patients in the Germany study experienced significantly more valvular heart disease before diagnosis with endocarditis (13/15, 87%; p = 0.002). The diagnosis was performed by analyzing the removed heart valve for all but 2 of these patients (patients 25 and 33). The clinical manifestations for 2 patients were mainly weight loss, not cardiac disease. The diagnosis for patient 25 was made by a positive PCR on blood and pleural effusion and for patient 39 by a positive PCR from a duodenal biopsy sample. According to the current modified Duke criteria (20), before the examination of the heart valve specimens, only 2 (4.25%) patients met the criteria for definite endocarditis (online Technical Appendix Table 1).

Data regarding treatment were available for 45 patients (online Technical Appendix Table 4). A total of 43 patients received antimicrobial drugs; at least 15 compounds were used. The most common treatment was trimethoprim–sulfoximethoxazole (34 patients); 10 of these patients had previously received ceftriaxone for 2 weeks. The maximum duration of treatment was 2 years; 24 patients received treatment for 1 year. Death was reported for 8 (21%) of 38 patients.

<table>
<thead>
<tr>
<th>Patient no. †</th>
<th>First drug (duration)</th>
<th>Second drug (duration) †</th>
<th>Outcome</th>
<th>Length of follow-up at the end of the last treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMX + GEN (15 d)</td>
<td>DOX + HCQ (ongoing)</td>
<td>Well, including arthralgia disappearance</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>2</td>
<td>AMX + GEN (15 d)</td>
<td>DOX + HCQ (ongoing)</td>
<td>Well, including arthralgia disappearance</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>5</td>
<td>CEF + GEN (15 d)</td>
<td>DOX + HCQ (ongoing)</td>
<td>Well</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>DOX + HCQ (1 yr)</td>
<td>Relapse 4 mo after the end of treatment; prosthetic dehiscence without fever; heart valve positive by PAS and immunohistochemical staining; negative by PCR</td>
<td>1 yr Ongoing new treatment</td>
</tr>
<tr>
<td>7</td>
<td>AMX + GEN (NA)</td>
<td>DOX + HCQ (7 mo)</td>
<td>Well</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>9</td>
<td>AMC + GEN (11 d)</td>
<td>DOX + HCQ (ongoing)</td>
<td>Well</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>12</td>
<td>CEF (5 d)</td>
<td>DOX + HCQ (ongoing)</td>
<td>Well</td>
<td>Ongoing treatment</td>
</tr>
<tr>
<td>14</td>
<td>CEF + GEN (15 d)</td>
<td>DOX + HCQ (1 yr)</td>
<td>Well</td>
<td>2.5 yr</td>
</tr>
<tr>
<td>17</td>
<td>CEF + GEN (15 d)</td>
<td>DOX + HCQ (1.5 yr)</td>
<td>Well</td>
<td>3.5 yr</td>
</tr>
<tr>
<td>20</td>
<td>NA</td>
<td>DOX + HCQ (1 yr)</td>
<td>Well</td>
<td>6 mo</td>
</tr>
<tr>
<td>21</td>
<td>AMX + GEN (18 d)</td>
<td>SXT (1.5 yr)</td>
<td>Well</td>
<td>5 yr</td>
</tr>
<tr>
<td>23</td>
<td>VAN + DOX + OFX (19 d)</td>
<td>DOX + HCQ (1.5 yr)</td>
<td>1 yr after end of treatment, saliva sample positive for T. whipplei by PCR (genotype NA); SXT started and continued for 12 mo</td>
<td>9 mo after onset of lifelong prophylaxis</td>
</tr>
<tr>
<td>24</td>
<td>AMX + GEN (4 wk)</td>
<td>DOX + HCQ (1.5 yr)</td>
<td>Well</td>
<td>5.5 yr after end of treatment, saliva and fecal samples positive for T. whipplei by PCR (new genotype: 101); no cardiac abnormalities observed; started lifelong prophylaxis with DOX at 100 mg 2×/d; well</td>
</tr>
<tr>
<td>25</td>
<td>AMX + GEN (15 d)</td>
<td>SXT (14 mo)</td>
<td>12 mo after the end of the treatment; saliva specimen positive for T. whipplei by PCR (genotype NA); SXT replaced DOX + HCQ after a perforated sigmoid diverticulitis with spreading peritonitis for 18 mo; well</td>
<td>6 yr</td>
</tr>
</tbody>
</table>

*Team from Assistance Publique Hôpitaux de Marseille, Marseille, France. All patients had undergone heart valve surgery. AMX, amoxicillin; GEN, gentamicin; HCQ, hydroxychloroquine; PAS, periodic acid–Schiff; CEF, ceftriaxone; AMC, amoxicillin–clavulanate; VAN, vancomycin; DOX, doxycycline; OFX, ofloxacin.

†DOX at 100 mg 2×/d and HCQ at 200 mg 3×/d; SXT at 320 mg trimethoprim and 1,600 mg sulfamethoxazole 3×/d.
Although the first description of *T. whipplei* endocarditis was made 15 years ago, diagnosing this disease remains difficult because clinical signs are often those of cardiac disease rather than infection. The first case was detected by chance when a broad-spectrum PCR was systematically applied to heart valve specimens (3). Although the efforts of that team might explain the high number of reported cases in Switzerland, studies in Marseille, France, that used the same technique did not detect *T. whipplei* in heart valves (40). In Germany, several physicians have been interested in Whipple disease for a long time, resulting in the development of new tools (1,32). In the Rhone-Alpes area of France, physicians have been interested in Whipple disease for several years, resulting in increased attention to *T. whipplei* (26).

With regard to the global effects of *T. whipplei* endocarditis, there seems to be a geographic gradient with higher incidence in eastern-central France, Switzerland, and Germany. Because 16S rRNA PCR is used in many areas to test for blood culture–negative endocarditis, which would enable detection of *T. whipplei* in heart valves, and significant differences in incidence rates exist, a potential bias seems unlikely (6,12). Whipple disease reportedly occurs mainly in white persons (1). Genotyping shows that a same strain of *T. whipplei* can be involved in chronic infections, acute infections, and chronic carriage. In addition, *T. whipplei* strains are heterogenic; thus, a patient could be colonized multiple times by a new strain (14). These data argue for the presence of specific host defects in patients with chronic infections. These defects could be linked to genetic factors that could explain the geographic distribution.

Even in the absence of diagnostic criteria, the reports of ≈50 cases in the literature enabled us to propose several characteristics that might help clinicians recognize potential *T. whipplei* endocarditis. This disease occurs mainly in white people.
men who are ≥50 years of age with cardiac manifestations including heart failure, acute ischemic stroke, and peripheral arterial embolism. These patients might have complained about arthralgia for several years and might have recently received immunosuppressants (4,7,28,33,34). Arthralgia was not frequently reported among patients in the Germany series (7) but was reported as a more prominent symptom by others (4,9,26,29,31,33,37,38). Arthralgia is sometimes subtle and noticed only after a careful clinical investigation (37). Because we have never received articular specimens from these patients, we do not know whether the joints are reactive or correspond to a second localization of T. whipplei. Of note, however, these arthralgias are highly sensitive to antimicrobial drugs. Overall, middle-age and older men with subacute endocarditis and no fever or low-grade fever should be asked about the presence of arthralgia because the combination of endocarditis and arthralgia suggests T. whipplei infection.

For now, diagnosis of T. whipplei endocarditis is made late, performed by molecular analysis of surgically obtained heart valves; specific repeat PCR is used because broad-spectrum PCR might lack sensitivity (40). Serologic assays only distinguish between classic Whipple disease and gastrointestinal carriage (17). Screening of saliva and fecal specimen has poor predictive value for diagnosis. The diagnostic situation is not satisfactory, but diagnostic improvements are challenging. Only optimization of molecular techniques and culture will enable diagnosis before heart valve analysis. Currently, 16S RNA amplification performed on blood specimens lacks sensitivity (6). Specific repeat PCR is more sensitive (13), enabling diagnosis of 31.2% of the patients reported here. In Marseille, for cases of blood culture–negative endocarditis, we systematically apply specific repeat PCR on blood specimens; this protocol enables us to make the diagnosis before heart valve removal (6). We suggest adding performance of repeat PCR for T. whipplei on blood specimens as a major criterion in the Duke classification for endocarditis, as PCR or serologic testing for C. burnetii have been added (20). The application of this criterion for patients who have benefited from molecular analysis of blood specimens significantly increases the definitive diagnosis of endocarditis (1/18 vs 6/18; p = 0.03) before the heart valve analysis. In the future, blood specimens from patients with blood culture–negative endocarditis should be also inoculated systematically on specific media. For patients for whom echocardiography is not informative, preliminary data have shown that positron emission tomography and computed tomography show promise, mainly for the detection of silent peripheral embolic events and infectious metastases (21).

There is no standard treatment for T. whipplei endocarditis. Our series represents a large study with a standardized treatment strategy and follow-up. On the basis of drug sensitivity data, reported resistance of T. whipplei to trimethoprim–sulfamethoxazole, and prior experience, a combination of doxycycline and hydroxychloroquine was used. A 12- to 18-month treatment strategy and analysis of the drug concentrations every 3 months seem reasonable. Patients must be forewarned about the risk for photosensitivity when taking doxycycline. All patients in our series have benefitted from heart valve removal; but in the future, to make the diagnosis before heart valve surgery is performed, we advise following the current recommendations for the surgical indications in infective endocarditis (21). Even if the approach lacks sensitivity, for patient follow-up, we suggest checking for the presence of T. whipplei in the saliva, fecal samples, and blood 2 months after the end of treatment. Subsequent analysis should be performed every 6 months for 2 years and every year for the life of the patient. Echocardiography should be performed yearly to detect relapses. We decided to treat T. whipplei recolonization, but we do not know if this measure is necessary.

T. whipplei endocarditis differs from classic Whipple disease. Classic Whipple disease involves most organs. Its diagnosis is based on the presence of T. whipplei–infected macrophages in intestinal tissues. T. whipplei endocarditis is an infection and not a potential cardiovalvular colonization with the bacterium because T. whipplei is the only infectious agent detected in heart valves, surrounded by an inflammatory process, and inside the macrophages. For white men >40 years of age with subacute endocarditis and arthralgia, T. whipplei infection should be suspected and the organism searched for in blood specimens by using specific repeat PCR and axenic culture, sampled in EDTA and heparin tubes, respectively.

**Acknowledgments**

We are grateful to all the patients who took part in this study and thank the staff members of the referring medical institutions for help in obtaining specimens.

This study was supported by the Crédit Ministériel “Programme Hospitalier de Recherche Clinique” 2009.

Dr Fenollar is a physician and research scientist at the Unité des Rickettsies, Aix-Marseille Université. Her main research interests include T. whipplei and Whipple disease.

**References**


Address for correspondence: Didier Raoult, Aix-Marseille Université, Unité des Rickettsies, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, CNRS-IRD-INSERM UMR 7278, Faculté de Médecine, 27 Blvd Jean Moulin, 13385 Marseille Cedex 05, France; email: didier.raoult@gmail.com
Atypical Scrapie Prions from Sheep and Lack of Disease in Transgenic Mice Overexpressing Human Prion Protein

Jonathan D.F. Wadsworth, Susan Joiner, Jacqueline M. Linehan, Anne Balkema-Buschmann, John Spiropoulos, Marion M. Simmons, Peter C. Griffiths, Martin H. Groschup, James Hope, Sebastian Brandner, Emmanuel A. Asante, and John Collinge

Public and animal health controls to limit human exposure to animal prions are focused on bovine spongiform encephalopathy (BSE), but other prion strains in ruminants may also have zoonotic potential. One example is atypical/Nor98 scrapie, which evaded statutory diagnostic methods worldwide until the early 2000s. To investigate whether sheep infected with scrapie prions could be another source of infection, we inoculated transgenic mice that overexpressed human prion protein with brain tissue from sheep with natural field cases of classical and atypical scrapie, sheep with experimental BSE, and cattle with BSE. We found that these mice were susceptible to BSE prions, but disease did not develop after prolonged post-inoculation periods when mice were inoculated with classical or atypical scrapie prions. These data are consistent with the conclusion that prion disease is less likely to develop in humans after exposure to naturally occurring prions of sheep than after exposure to epizootic BSE prions of ruminants.

Bovine spongiform encephalopathy (BSE) is the transmissible spongiform encephalopathy (TSE) or prion disease of domestic cattle. The BSE prion is an epizootic agent and causes variant Creutzfeldt-Jakob disease (vCJD) in humans after dietary exposure (1–4). Because the time lag between exposure and development of vCJD may be decades, uncertainty about the extent of the pathogenicity of BSE for humans continues (5), and subclinical forms of infection may exist (6,7). A recent immunohistochemical study that estimated prevalence of prion infection in the UK population by screening samples from surgically removed appendixes found 1 in 2,000 persons were positive for the disease-associated form of the prion protein (PrP) (8). Similar uncertainty exists in our understanding of scrapie, the TSE of small ruminants, which has been heightened in recent years by finding BSE in goats (9,10), the possibility of BSE in sheep (11), and the discovery of atypical scrapie (12,13), a form of small-ruminant TSE, which had evaded statutory diagnosis until the early 2000s.

Recent analysis of surveillance data of TSEs in small ruminants in Great Britain, collected over the past 10 years, has demonstrated a dramatic decrease (up to 90%) in number of confirmed cases of classical scrapie in the national flock. However, atypical scrapie continues to affect sheep bred for their relative resistance to the classical form of this prion disease, and the proportion of sheep with resistant genotypes in the national flock is likely to have increased over the past decade because of the National Scrapie Plan for Great Britain. This increase has rekindled speculation that atypical scrapie in small ruminants might be a source of human prion disease (11). Although atypical scrapie has been discovered retrospectively in 2 UK sheep culled in 1987 and 1989 (14,15), the level and duration of human exposure to atypical scrapie prions are unknown, and this lack of knowledge confounds a cause-and-effect investigation of epidemiologic links between this animal disease and some form of CJD (11).

Over the past 2 decades, surrogate methods have been developed to assess the relative pathogenicity of
animal prions for humans. One approach involves the experimental transmission of disease by inoculating homogenized brain tissue from affected animals into transgenic mice that are overexpressing 1 of the 2 common polymorphic forms of the human PrP (either methionine or valine at residue 129) on a mouse PrP null background (16). Such transgenic mice are fully susceptible to infection with human prions (16) and, to a lesser extent, cattle and ovine BSE prions (2,4,17), but appear resistant to chronic wasting disease prions from cervids (18–20). In this study, we inoculated transgenic mice that overexpressed human PrP with brain tissue from field sheep with natural cases of classical and atypical scrapie, sheep with serially-passaged experimental BSE, and cattle with BSE to assess the pathogenicity of natural scrapie prions relative to that of the known epizootic TSE agent, the cattle BSE prion strain.

Materials and Methods

Ovine and Bovine Prion Sources

From Great Britain’s Animal Health and Veterinary Laboratories Agency (AHVLA), we obtained 10% (w/v) brain homogenates prepared in sterile saline from sheep with neuropathologically confirmed prion disease and demonstrated ability to transmit prion disease to transgenic mice expressing ovine PrP or to wild-type mice (Table 1). We obtained scrapie-infected sheep brain from the Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health, Greifswald-Insel Reims, Germany) under a license granted by the Department for Environment, Food and Rural Affairs, according to the terms of the Importation of Animal Pathogens Order 1980. Brain samples from sheep with neuropathologically confirmed cases of classical and atypical scrapie were prepared as 10% (w/v) homogenates in sterile Dulbecco phosphate-buffered saline lacking Ca²⁺ and Mg²⁺ ions (D-PBS) by extrusion through syringe needles of decreasing diameter. Brains from cattle with neuropathologically confirmed cases of BSE (collected specifically for transmission studies in the early 1990s) were provided by the UK Central Veterinary Laboratory (now AHVLA). We used 10% (w/v) homogenates prepared from the brainstems of 5 cattle with natural BSE to generate pooled inocula, designated I038, which was previously shown to transmit prion disease to wild-type FVB/N and C57Bl/6 mice, and to transgenic mice overexpressing human PrP (2,4,23,24). All experimental procedures involving ovine or bovine prions were carried out in a microbiological containment level 3 facility with strict adherence to safety protocols.

Transgenic Mice

Transgenic mice homozygous for a human PrP 129V transgene array and murine PrP null alleles (Prnp⁰⁰), designated Tg(HuPrP129V⁰⁰ Prnp⁰⁰)-152 mice (129VV Tg152 mice), or homozygous for a human PrP 129M transgene array and murine PrP null alleles (Prnp⁰⁰), designated Tg(HuPrP129M⁰⁰ Prnp⁰⁰)-35 mice (129MM Tg35 mice), have been described (1,2,4,24–26). Both lines of mice were used to generate FVB/N-HuPrP¹⁰⁰ Prnp⁰⁰ congenic lines by backcrossing to FVB/N mice for 10 generations, followed by genetic testing (Charles River UK, Ltd., Margate, UK) by using 84 FVB-specific PCR microsatellite markers covering 19 chromosomes.

Table 1. Details of prion sources for ovine inocula*  

<table>
<thead>
<tr>
<th>Source code</th>
<th>Brain region</th>
<th>Prion agent</th>
<th>Ovine PrP genotype†</th>
<th>Attack rate (incubation period) Reference</th>
<th>Transmission data†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHVLA/SE1919/0077</td>
<td>Cerebral cortex</td>
<td>Classical scrapie</td>
<td>VRQ/VRQ</td>
<td>16/16 (64 ± 2 d)</td>
<td>(27); code SE1848/0005</td>
</tr>
<tr>
<td>AHVLA/SE1919/0080</td>
<td>Cerebral cortex</td>
<td>Classical scrapie</td>
<td>ARQ/ARQ</td>
<td>12/13 (155 ± 4 d)</td>
<td>(21); code SE1848/0008</td>
</tr>
<tr>
<td>FLI 1/06</td>
<td>Caudal medulla</td>
<td>Classical scrapie</td>
<td>ARQ/ARQ</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>FLI 83/04</td>
<td>Caudal medulla</td>
<td>Classical scrapie</td>
<td>ARQ/ARQ</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>FLI 107/04</td>
<td>Caudal medulla</td>
<td>Classical scrapie</td>
<td>ARQ/ARQ</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>AHVLA/SE1850/0001</td>
<td>Caudal medulla</td>
<td>Atypical scrapie</td>
<td>AHQ/AHQ</td>
<td>19/20 (210 ± 3 d)</td>
<td>(22); code 1</td>
</tr>
<tr>
<td>AHVLA/SE1850/0009</td>
<td>Caudal medulla</td>
<td>Atypical scrapie</td>
<td>ARR/ARR</td>
<td>19/19 (231 ± 6 d)</td>
<td>(22); code 9</td>
</tr>
<tr>
<td>FLI S7/06</td>
<td>Caudal medulla</td>
<td>Atypical scrapie</td>
<td>AHQ/ARQ</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>FLI 14/06</td>
<td>Caudal medulla</td>
<td>Atypical scrapie</td>
<td>ARR/ARR</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>FLI 26/06</td>
<td>Caudal medulla</td>
<td>Atypical scrapie</td>
<td>AHQ/ARQ</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>AHVLA/SE1929/0877</td>
<td>Caudal medulla</td>
<td>Ovine BSE</td>
<td>ARQ/ARQ</td>
<td>RII mice; 16/19 (422 ± 19 d)†</td>
<td>Unpub. data</td>
</tr>
<tr>
<td>AHVLA/SE11945/0032</td>
<td>Rostral medulla</td>
<td>2nd passage ovine BSE</td>
<td>ARQ/ARQ</td>
<td>RII mice; 18/20 (356 ± 9 d)‡</td>
<td>Unpub. data</td>
</tr>
</tbody>
</table>

*PrP, prion protein; AHVLA, Animal Health and Veterinary Laboratories Agency; ND, not done; NA, not applicable; FLI, Friedrich-Loeffler-Institut; BSE, bovine spongiform encephalopathy.
†Reports attack rate (no. infected mice as a proportion of no. inoculated mice) and mean incubation period in days ± SEM or SD and the reference in which the data were first published with original inocula code.
‡Ovine PrP codon 136,154,171 genotype.
§Transgenic for the ovine Prp VRQ allele on a mouse Prnp⁰⁰ background. Overexpression 8- to 10-fold of normal ovine brain.
¶Mean incubation period in days ± SEM.
at ≈20-cM intervals, to select breeding pairs positive for 100% of the FVB-specific markers. Selected congenic pairs were interbred to remove the endogenous murine PrP gene and to establish homozygosity of the human PrP transgene array. The resulting congenic lines, designated 129MM Tg35c and 129VV Tg152c, overexpress human PrP in brain at levels of 2× and 6× that of pooled human brain, respectively.

Transmission Studies

Work with animals was performed under a license granted by the UK Home Office and conformed to institutional guidelines of the University College London and ARRIVE (Animal Research: Reporting In Vivo Experiments guidelines of The National Centre for the Replacement, Refinement and Reduction of Animals in Research). Brain homogenates (10% w/v) were diluted to 1% (w/v) in sterile D-PBS and passed through a 25-gauge needle. Each mouse was inoculated with 30-μL of 1% (w/v) brain homogenate because this avoids excessive animal losses within the first 48 hours postinoculation (4). Brain homogenates from prion-infected sheep were inoculated intracerebrally into groups of 20 transgenic mice that overexpressed human PrP. Thereafter, mice were examined daily and killed if they were exhibiting signs of distress or once a diagnosis of clinical prion disease was established (4,24,25). Clinical diagnosis can be confounded by non-specific conditions that develop in mice as they age, and the mean lifespans of different lines of transgenic mice and the onset of aging artifacts vary greatly. On the basis of experience, we have limited these confounding effects by electively culling mice after postinoculation periods of >600 days. Notably, this also helps reduce the number of mice that die of old age, in which brain tissue can undergo autolytic deterioration that impairs immunohistochemical (IHC) analyses. At post-mortem, brains from inoculated mice were removed and divided sagittally, with half of the samples frozen and half fixed in formol-saline, and analyzed for abnormal PrP accumulation by IHC and immunoblotting.

Neuropathologic and Immunohistochemical Analyses

Brain fixed in 10% buffered formol-saline was immersed in 98% formic acid for 1 hour and embedded in paraffin wax. Serial sections (4-μm thick) were pretreated by boiling for 10 min in a low ionic strength buffer (2.1 mmol/L Tris, 1.3 mmol/L EDTA, 1.1 mmol/L sodium citrate, pH 7.8) before exposure to 98% formic acid for 5 min. Abnormal PrP accumulation was examined by using monoclonal antibody ICSM 35 against PrP (D-Gen Ltd., London, UK) on an automated IHC staining machine (Ventana Medical Systems, Inc.) before development with 3’ 3-diaminobenzidine tetrachloride as the chromogen (27). Conventional methods were used for Harris hematoxylin and eosin staining. Appropriate positive and negative controls were used throughout. Photographs were taken on an ImageView digital camera and composed with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Immunoblotting

Proteinase K (PK) digestion (50 or 100 μg/mL final protease concentration, 1 hour, 37°C), electrophoresis, and immunoblotting of 10% (w/v) transgenic mouse brain homogenates or 10% (w/v) brain homogenates from sheep with classical scrapie (prepared in D-PBS) were performed as described (27,28). Human PrP or ovine PrP was detected by using monoclonal antibodies 3F4 (29) or ICSM 35 against PrP (D-Gen Ltd.), respectively. Mouse brain homogenates found negative for disease-related PrP (PrPSc) after analysis of 10 μL 10% (w/v) brain homogenate were reanalyzed by sodium phosphotungstic acid (NaPTA) precipitation of PrPSc (30) from 250 μL of 10% (w/v) brain homogenate as described (28).

Atypical scrapie sheep brain was analyzed by using the procedure of Gretzschel et al. (31,32) with modifications. In brief, 200 μL of 10% (w/v) brain homogenate in D-PBS was centrifuged at 500 × g for 5 min, after which the supernatant was discarded, and the pellet was resuspended to 100 μL final volume with D-PBS, followed by the addition of 100 μL 4% (w/v) sodium lauroylsarcosine (sarkosyl) in D-PBS. After incubation at 37°C for 30 min with constant agitation and centrifugation at 500 × g for 5 min, 150 μL of the supernatant was transferred to a new tube. The supernatant fraction was treated with 2 μL of Benzonase (Benzonuclease purity 1; 25 U/μL; Merck, Nottingham, UK) for 30 min at 37°C with agitation and adjusted to a final concentration of 50 μg/mL PK (by adding 8 μL of a 1 mg/mL PK stock solution) and incubated at 37°C for 60 min with agitation. Samples were treated with 4 μL 100 mmol/L 4-(2-aminoethyl)-benzene sulfonyl fluoride, heated at 100°C for 5 min, adjusted with an equal volume of 2% (w/v) sarkosyl in D-PBS and 3 μL of Benzonase; they were then incubated for 30 min at 37°C with agitation before addition of 4% (w/v) NaPTA containing 170 mmol/L MgCl2, pH 7.4, to give a final concentration in the sample of 0.3% (w/v) NaPTA. After incubation for 60 min at 37°C, with constant agitation, samples were centrifuged at 16,100 × g for 30 min, and the supernatant fraction was discarded. The pellet fraction was re-suspended to a final volume of 10 μL in D-PBS containing 0.1% (w/v) sarkosyl and analyzed by electrophoresis, immunoblotting, and high sensitivity chemiluminescence (27,28), using monoclonal antibody ICSM 35 against PrP to detect ovine PrP.
Results

Scrapie Prions from Sheep and Lack of Disease in Transgenic Mice

We examined classical and atypical scrapie sheep brain homogenates from UK field cases (AHVLA) that contain PK-resistant ovine PrPSc and efficiently transmitted clinical prion disease to transgenic mice expressing ovine PrP (21,22) (Table 1), together with a series of PK-resistant PrP-positive brain homogenates from sheep in Germany with field cases of classical and atypical scrapie (Figure 1). All natural brain isolates examined produced no clinical prion disease or biochemical or histopathologic evidence for subclinical prion infection in transgenic mice that overexpressed human PrP after postinoculation intervals of >600 days (Table 2).

Consistent with the inability of IHC or high sensitivity immunoblotting to detect pathologic PrP in the brains of inoculated mice, neuropathologic examination of the brain showed no difference in spongiform change or gliosis from that observed in the brains of age-matched control mice (data not shown). From these findings, we conclude that both methionine and valine residue 129 variants of human PrP are refractory to pathologic conversion by these ovine prion strains in transgenic mice.

Transmission of Cattle BSE Prions to Transgenic Mice

Brain isolates from sheep with classical and atypical scrapie (including those with demonstrated prion infectivity in transgenic mice expressing ovine PrP) did not transmit prion disease to transgenic mice that were overexpressing human PrP. This fact contrasts markedly with the known susceptibility of these mice to transmission of multiple cattle BSE isolates (2,4,24,25) as well as to transmission of a wide range of human-acquired prion diseases (including kuru and vCJD) and sporadic prion disease isolates (2,4,24–26).

Concomitant with the current study, and as part of a separate experiment, we inoculated 129MM Tg35c mice intracerebrally with cattle BSE isolate I038. This BSE isolate has previously been shown to be transmissible to the parent 129MM Tg35 transgenic line, producing an attack rate of 8/20 inoculated mice (4) (Table 3). Affected 129MM Tg35 mice in these transmissions were culled (because of intercurrent illness or clinical prion disease) within 600 days of inoculation (Table 3) and demonstrated the presence of abnormal PrP in brain by IHC and immunoblotting (4). In 129MM Tg35c mice, cattle BSE isolate I038 produced an attack rate of 5/12 in intracerebrally inoculated mice (Table 3). Infection was characterized by the detection of abnormal PrP by IHC (Figure 2, panels A, B), which included large amorphous PrP deposits (Figure 2, panels C, E) and florid PrP plaques (Figure 2, panels D, F), and the detection of type 4 PrPSc in brain homogenate by immunoblotting (Figure 2, panel B inset). Intercurrent illness before 600 days postinoculation was seen in only one 129MM Tg35c mouse, with the remaining mice in the group (11/12) culled 611–853 days postinoculation (Table 3). Although most mice survived >600 days after inoculation, the attack rate of cattle BSE isolate I038 in 129MM Tg35c mice remained the same as observed in the parental 129MM Tg35 mouse line with ≈40% of inoculated mice becoming infected (Table 3). In addition, we found that 129MM Tg35 and 129MM Tg35c mice showed equivalent susceptibilities (100% attack rates) to vCJD or classical CJD prions (Table 3).
Experimental Ovine BSE in Transgenic Mice Expressing Human PrP 129 Methionine

Recently, 2 studies have concluded that experimental sheep BSE prions may propagate more efficiently than cattle BSE prions in transgenic mice that express human PrP 129 methionine (17,34). One of these studies convincingly established that sheep and goat BSE prions transmitted a molecular and neuropathologic phenotype congruent with transmission of vCJD prions in the same mice (17). These data strongly suggest that small ruminant BSE prions could act as causal agents of vCJD (17). In this study, we also examined the transmission properties of 2 experimental sheep BSE brain isolates derived from the primary transmission and secondary passage of cattle BSE in sheep. These AHVLA isolates were provided as brain homogenates that contained PK-resistant ovine PrP (Figure 3, panel A) and had known ability to transmit clinical prion disease to wild-type RIII mice (Table 1).

In the transgenic mice expressing human PrP, clinical prion disease was not produced by either of the 2 experimental sheep BSE isolates after postinoculation intervals >600 days (Table 2). Examination of brain from these inoculated mice by IHC and immunoblotting, after NaPTA precipitation for detection and appeared similar (but not identical) to type 4 PrP\textsuperscript{sc} seen in vCJD brain (Figure 3, panel B). Florid PrP plaques were not observed, and abundant PrP deposits were restricted to the corpus callosum (Figure 3, panel C), accompanied by occasional punctate PrP deposits in the thalamus and hypothalamus (data not shown). Secondary passages of this isolate in additional human PrP–expressing transgenic mice and wild-type FVB/N mice have been initiated to comprehensively define prion strain type.

**Table 2. Survival times of transgenic human PrP mice after inoculation of ovine prions**

<table>
<thead>
<tr>
<th>Source code</th>
<th>Prion agent</th>
<th>Transmission data</th>
<th>Prion agent</th>
<th>Transmission data</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHVLA/SE1919/0077</td>
<td>Classical scrapie</td>
<td>129MM Tg35c mice</td>
<td>Attack rate</td>
<td>Survival, d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Attack rate†</td>
<td>Survival, d‡</td>
<td>Attack rate</td>
</tr>
<tr>
<td>AHVLA/SE1919/0080</td>
<td>Classical scrapie</td>
<td>0/20</td>
<td>551, 551, 583, 615–666 (17)</td>
<td>0/16</td>
</tr>
<tr>
<td>FLI 1/06</td>
<td>Classical scrapie</td>
<td>0/19</td>
<td>580, 586, 586, 620–666 (16)</td>
<td>0/14</td>
</tr>
<tr>
<td>FLI 83/04</td>
<td>Classical scrapie</td>
<td>0/15</td>
<td>426, 475, 628–728 (13)</td>
<td>0/17</td>
</tr>
<tr>
<td>FLI 107/04</td>
<td>Classical scrapie</td>
<td>0/17</td>
<td>382, 382, 459, 573, 574, 578, 606–636 (11)</td>
<td>0/13</td>
</tr>
<tr>
<td>AHVLA/SE1850/0001</td>
<td>Atypical scrapie</td>
<td>0/18</td>
<td>213, 332, 437, 537, 537, 621–656 (13)</td>
<td>0/18</td>
</tr>
<tr>
<td>AHVLA/SE1850/0009</td>
<td>Atypical scrapie</td>
<td>0/18</td>
<td>440, 606–635 (17)</td>
<td>0/15</td>
</tr>
<tr>
<td>FLI S7/06</td>
<td>Atypical scrapie</td>
<td>0/16</td>
<td>498, 610–659 (15)</td>
<td>0/14</td>
</tr>
<tr>
<td>FLI 14/06</td>
<td>Atypical scrapie</td>
<td>0/18</td>
<td>538, 540, 545, 572, 601–728 (14)</td>
<td>0/15</td>
</tr>
<tr>
<td>FLI 26/06</td>
<td>Atypical scrapie</td>
<td>0/14</td>
<td>547, 553, 643–659 (12)</td>
<td>0/14</td>
</tr>
<tr>
<td>AHVLA/SE1945/0032</td>
<td>2nd passage ovine BSE</td>
<td>1/19</td>
<td>211, 337, 337, 434, 572, 517, 524, 616–661 (13)</td>
<td>0/17</td>
</tr>
</tbody>
</table>

\*PrP, prion protein; AHVLA, Animal Health and Veterinary Laboratories Agency; FLI, Friedrich-Loeffler-Institut; BSE, bovine spongiform encephalopathy.
†All mice were inoculated with 30 µL of 1% (w/v) brain homogenate. Attack rate is defined as the total number of clinically affected and subclinically infected mice as a proportion of the number of inoculated mice. Subclinical prion infection was assessed by immunohistochemical examination of brain for abnormal PrP deposition and for recipients of AHVLA inocula by sodium phosphotungstic acid precipitation of 250 µL 10% brain homogenate and analysis for PrP\textsuperscript{sc} by proteinase K digestion and immunoblotting.
‡The interval between inoculation and culling because of intercurrent illness, senescence, or termination of the experiment in days. Death dates of individual mice are shown together with the range for mice surviving beyond 600 d with the number of mice in this range shown in parentheses. Mice culled with postinoculation periods of ≤200 d due to intercurrent illness (all confirmed negative for prion infection) were not included in calculating attack rates.
Why the efficiency of transmission of experimental sheep BSE prions to 129MM Tg35c mice is low compared with that reported in different lines of human PrP 129 methionine–expressing mice (17,34) is unclear. One possible reason may simply relate to the prion titers in the inocula. Plinston et al. reported that 2 different inocula prepared from the same experimental sheep BSE brain had markedly different transmission efficiencies to gene-targeted mice expressing human PrP 129 methionine at endogenous levels (34). However, all AHVLA ovine prion isolates used in this study were chosen because they produced short survival periods and high attack rates in either ovine PrP transgenic mice or wild type mice (Table 1). Therefore, other possibilities must also be considered. In particular, studies involving different laboratories use different lines of genetically modified mice. Variation in genetic background and differences in PrP expression levels are known to influence host susceptibility to prion infection (16).

Discussion

In this study, we have shown that disease does not develop in transgenic mice overexpressing human PrP when mice are inoculated with ovine prions from sheep with natural cases of classical scrapie and atypical scrapie from Great Britain and Germany. These transgenic mice are susceptible to infection, and clinical disease develops when mice are challenged with brain tissue from cattle affected by classical BSE (2,4,24,25) or brain tissue from humans affected by classical (sporadic and iatrogenic) CJD, kuru, or vCJD (2,4,24–26). Therefore, this suggests that the transmission barrier associated with the interaction of human PrP and the prion strain causing epizootic BSE in cattle is lower than that associated with the prion strain causing atypical scrapie in sheep. Serial, blind passage of brain homogenates from “negative” challenged mice from this experiment into other lines of transgenic mice expressing either human PrP or ovine PrP will now be required to determine whether this transmission barrier is absolute.

Our findings complement those of other recent studies that have investigated the zoonotic potential of ruminant prion strains using other lines of human PrP–expressing mice. Gene-targeted human PrP–expressing mice have been shown to be resistant to infection with classical and atypical scrapie prions from sheep (34,35) and BSE prions from cattle (36) but are susceptible to infection with BSE prions from sheep (34). Transgenic mice with 6-fold over-expression of human PrP 129 methionine are susceptible to infection with cattle BSE prions but show greater susceptibility to ovine and caprine BSE prions (17).

Although we found evidence for transmission of experimental ovine BSE to transgenic mice expressing human PrP 129 methionine, the relative attack rate was lower than observed in the other lines of mice (17,34). The reasons underlying this are not clear but may relate to differences in the prion isolates themselves or differences in the various lines of mice. To definitively investigate interlaboratory differences in the apparent behavior of ovine BSE prions and reach a consensus, a panel of ovine prion inocula would need to formally undergo endpoint titration across the different lines of humanized mice and also in ovine PrP–expressing transgenic mice.

No strain variation has been found so far in the transmission, biochemical, or histopathologic characteristics of atypical scrapie prions (22,37), and so inferences from the present study are not confounded by sampling or strain considerations. This is not so for cases of classical scrapie
and, although our findings on atypical scrapie prions indicate that the zoonotic potential of this ovine prion strain is lower than for ruminant BSE prions, further transmission studies using a wider variety of field cases of classical scrapie are required to provide further reassurance of the low or negligible zoonotic potential of all sheep prions. Examining extraneural tissues (in particular, the spleen) in ovine prion-challenged mice will be critical because recent findings have shown that cross-species prion transmission efficacy can exhibit a dramatic tissue-dependence in the same host (38).

Acknowledgments

We thank all patients and their families for generously consenting to the use of human tissues in this research. We also thank our biological services team for animal care and R. Young for preparing the figures.

Some of the work was undertaken at University College London Hospital National Health Service Foundation Trust, which received a proportion of funding from the Department of Health’s National Institute for Health Research Biomedical Research Centre. This research was funded by the Medical Research Council (UK) and the European Union.

J.C. is a director and J.C. and J.D.F.W. are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination, and therapeutics. D-Gen markets the ICSM35 antibody used in this study.
Dr Wadsworth is a program leader within the UK Medical Research Council Prion Unit in London. His primary research interest is the molecular basis of mammalian prion strains.

References


Address for correspondence: Jonathan D.F. Wadsworth, Institute of Neurology, University College London, Medical Research Council Prion Unit, Queen Square, London WC1N 3BG, United Kingdom; email: j.d.wadsworth@prion.ucl.ac.uk

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.
Imported dengue cases pose the public health risk for local circulation in European areas, especially southeast France, where the *Aedes* mosquito is established. Using a capture–recapture method with Chao's estimator, we estimated the annual incidence of dengue fever and the completeness of existing mandatory notification and laboratory network surveillance systems. During 2007–2010, >8,300 cases with laboratory evidence of recent dengue infection were diagnosed. Of these cases, 4,500 occurred in 2010, coinciding with intense epidemics in the French West Indies. Over this 4-year period, 327 cases occurred in southeast France during the vector activity period. Of these, 234 cases occurred in 2010, most of them potentially viremic. Completeness of the mandatory notification and laboratory network systems were ≈10% and 40%, respectively, but higher in southeast areas during May–November (32% and 69%, respectively). Dengue surveillance systems in France provide complementary information that is essential to the implementation of control measures.

Dengue fever, caused by 4 virus serotypes, is the most common mosquito-borne viral disease in the world: an estimated 50 million cases occur annually (1). During the past 50 years, incidence has increased 30-fold with increasing geographic expansion (1). In Europe, imported cases among travelers returning from endemic or epidemic countries have been reported frequently during recent years. Considering the risk for a local cycle of transmission and subsequent epidemic, imported dengue cases pose a potential public health problem in European areas where a competent vector is established. Since 2004, the *Aedes albopictus* mosquito has been established in southeast France (2,3).

During 2010, the first 2 known cases of autochthonous dengue fever were diagnosed in persons in metropolitan France (4), which comprises continental France and the island of Corsica, located southeast of mainland France (Figure 1). Two cases were also reported in Croatia during 2010 (5), demonstrating that local transmission in continental Europe is a reality. Accordingly, in the context of implementing appropriate public health measures, dengue surveillance systems should be able to estimate the incidence of imported symptomatic cases, describe their geographic distribution in areas already or potentially colonized by the competent vector, and identify the countries where infection occurred. Using a capture–recapture method, we estimate the annual incidence of imported dengue cases and the completeness of the existing surveillance systems in metropolitan France during 2007–2010.

**Methods**

**Dengue Surveillance Systems**

As of 2010, the *Ae. albopictus* mosquito was designated as permanently established in 6 southeast departments (administrative districts) in metropolitan France as follows: Alpes-Maritimes (2004), the 2 departments that comprise Corsica, Haute-Corse (2006) and Corse-du-Sud (2007), Var (2007), Bouches-du-Rhône (2009), and
Alpes-de-Haute-Provence (2010). Entomological surveillance, based on data from the monitoring of ovitraps (3), enabled information on distribution of this mosquito to be updated within a few weeks. In addition to entomologic surveillance, since 2006, health authorities in France have implemented 3 complementary epidemiologic surveillance systems to identify new dengue and chikungunya infections: a notifiable diseases system that relies on mandatory notification, a laboratory-based surveillance system that operates at the national level, and an enhanced surveillance system, activated each year during May–November in the departments where the *Ae. albopictus* mosquito is established (3).

The notifiable diseases system requires mandatory notification by practitioners and biologists to collect clinical and biological information about recent symptomatic dengue cases. Notifiable cases are defined by recent fever (within 7 days of medical examination) associated with pain (headache, arthralgia, myalgia, low back pain, or retro-orbital pain) and positive test results for 1 of the following biologic results indicative of dengue infection: reverse transcription PCR (RT-PCR), nonstructural protein 1 [NS1] antigenic test, or IgM serologic analysis. Notification is centralized by the French Institute for Public Health Surveillance for the purpose of epidemiologic analysis. It has been shown that mandatory notification systems lack completeness (of unknown magnitude) and representativeness, and over-represent hospitalized case-patients (6), leading to unequal probability of being included in a sample (catchability) (7) of dengue cases.

The laboratory-based national surveillance system is a voluntary network that comprises 6 specialized laboratories that monitor the trends of dengue diagnosis (8). Dengue cases are defined by positive RT-PCR, NS1 or IgM serologic test results, regardless of clinical signs. These biologic tests are only to be prescribed when a patient has suggestive symptoms. They are reported weekly to the French Institute for Public Health Surveillance. A survey during 2006 showed that this laboratory network aggregated ≈85% of the biologic diagnoses of dengue performed in metropolitan France (9).

The enhanced surveillance system is implemented in the departments where the vector is established, during its period of activity from May 1–November 30 each year. Unlike mandatory notification, the basis of enhanced surveillance is the immediate reporting of all clinically suspected cases of dengue fever by practitioners to the regional health authorities. This facilitates accelerated biologic confirmation by the national reference laboratory for arboviruses and, when appropriate, the rapid implementation of local control measures such as perifocal vector control activities and active case finding (3,4).

The 3 surveillance systems are obviously interconnect- ed. For example, during the period of vector activity, dengue cases obtained from mandatory notification or from the laboratory network are immediately reported by the French
Institute for Public Health Surveillance to the regional officers supervising the enhanced surveillance system.

A person with an imported case was defined as having traveled in an area where the dengue virus circulates within the previous 15 days before the onset of symptoms. As no substantial local transmission cycle occurred during the study period, all cases without patient information on travel history were considered imported cases.

**Strategy for Statistical Analysis**

We used the capture-recapture method to estimate the incidence of imported dengue cases in metropolitan France during 2007–2010. By identifying common cases from several systems, this method provides an estimate of the number of cases not captured by any data sources. Consequently, the total number of cases and the capture probabilities of cases within each source can be estimated.

To identify common cases, we checked the 3 data sources to find the patient’s date of birth, sex, and postal code of residence or of the laboratory where blood samples were collected, and the date of blood sampling. We faced 2 main obstacles to using the capture-recapture method. First, 1 of the 3 data sources, the enhanced surveillance system, operates in a limited area during 7 months each year. This obstacle restricted the possibility of comparing the 3 sources for the analysis. Second, the functional interrelationships between the 3 dengue surveillance systems appear to be limitations to the use of the standard 2-source capture-recapture methods and need to be quantified. We therefore conducted the analysis in 2 steps.

First, dependencies between sources were statistically evaluated following suggestions of Wittes et al. ([10,11]). The odds ratio implemented with the capture-recapture technique, developed by Wittes et al., is an estimate of the increased probability of a dengue case being reported in a first source when it is also reported in a second source. To investigate the relationship between these sources, the analysis is restricted to cases identified by a third source. The dependence analysis of the sources was restricted to the year 2010 because of an insufficient number of dengue cases before this date. Figure 2 shows the distribution among the 3 surveillance systems of the 199 biologically confirmed dengue cases detected during May 1–November 30, 2010, in the 6 departments of southeast France where the mosquito was established. Table 1 details the calculation of statistical interdependence of the 3 sources. The enhanced surveillance system is highly dependent on both the mandatory notification and the laboratory network. In contrast, mandatory notification and the laboratory network are systems that do not seem to be substantially interdependent; accordingly, we retained only these 2 reporting mechanisms to estimate the annual dengue incidence.

Second, the capture-recapture method was applied to these 2 national-level sources by using 2 estimators of population size: the Chapman-Seber (CS) and the Chao estimates. The CS estimator ([12,13]) is a commonly used formula and is considered unbiased. This formula uses the hypotheses of independence between sources and equal catchability by each source. Instead, it has been shown recently that Chao’s estimator, as formulated by Brittain, is less biased than the CS estimator when there is dependence between sources or unequal catchability of cases ([14]). Therefore, in this study, we gave priority to the results obtained by using Chao’s estimator.

For both estimators, the 95% CIs associated with population size estimates were calculated with the log-transformation suggested by Burnham and used by Chao ([15,16]). The corresponding completeness values and their 95% CIs, obtained by using Monte-Carlo simulations, were calculated for mandatory notification, for the laboratory network, and for the combined surveillance systems.

Stratification was made for geographic areas where the *Ae. albopictus* mosquito was established versus other areas and by period of the year (vector activity period versus the rest of the year) to take into account and reduce the potential inequality in catchability. The total variance was calculated by adding the variance of each stratum. To acquire an understanding of the general shape of the curve of monthly number of cases and to compare it with that obtained for the French West Indies, we used the CS estimator with stratification according to geographic area, as many zero values precluded using the Chao estimator.

**Results**

During 2007–2010 in metropolitan France, 773 cases of dengue were reported by mandatory notification, 3,192 by the laboratory network, and 180 by the enhanced surveillance system. A total of 3,432 distinct cases were
For 1,204 cases (35%), anamnestic and biologic information on infection was made by IgM serology (83%) and by anamnestic and biologic information on infection was made by IgM serology (83%). Of those, 943 patients were hospitalized. Of those, 943 patients were hospitalized. Positive biologic diagnosis of dengue virus was determined by the viremic stage (viremia delay had exceeded 7 days) and 3.7% had data which were not compatible with the viremic stage (viremia delay had exceeded 7 days). Viremia usually occurs between the day before the onset of symptoms and the seventh day after.

The following additional clinical and biological information was available for 718 of the 773 cases of dengue reported by the mandatory notification system. Of the 718 patients, hemorrhagic symptoms were reported for 134 (19%) during the 4-year period. Eight (1%) of these had severe hemorrhagic symptoms (defined at the time of the study as tourniquet test, mucocutaneous bleeding, bleeding from puncture sites, or visceral bleeding). Of the patients for whom specific information was available, 51% (330/652) had thrombocytopenia (platelets ≤100,000/mL) and 47% (323/683) were hospitalized.

### Annual Incidence of Dengue Cases and Completeness of Surveillance Systems

Table 2 shows the total number of imported dengue cases in France during the study period: the annual number of cases estimated from mandatory notification and the laboratory network systems after stratification by area where the *Ae. albopictus* mosquito was established, and by the vector activity period. Using the Chao estimator, we calculated the global number of dengue cases to be 8,374 (95% CI [8,047–8,701]), which is consistent with the number of cases reported by the French departments (Figure 1). Of these cases, 3,423 were reported by 1 of the 2 national level sources: mandatory notification or the laboratory network. The male:female sex ratio of the patients was 0.99:1, and the median age of the patients was 41 years. Positive biologic diagnosis of dengue infection was made by IgM serology (83%) and by an antigenic method (17%, RT-PCR: 13% and NS1 test: 4%). For 1,204 cases (35%), anamnestic and biologic information was sufficient to determine the viremic status of the patients in metropolitan France. Among them, 48.5% were potentially viremic (positive RT-PCR or NS1 test), 47.8% were potentially viremic (the delay between the onset of symptoms and their subsequent return journey to France was <8 days) and 3.7% had data which were not compatible with the viremic stage (viremia delay had exceeded 7 days). Viremia usually occurs between the day before the onset of symptoms and the seventh day after.

The following additional clinical and biological information was available for 718 of the 773 cases of dengue reported by the mandatory notification system. Of the 718 patients, hemorrhagic symptoms were reported for 134 (19%) during the 4-year period. Eight (1%) of these had severe hemorrhagic symptoms (defined at the time of the study as tourniquet test, mucocutaneous bleeding, bleeding from puncture sites, or visceral bleeding). Of the patients for whom specific information was available, 51% (330/652) had thrombocytopenia (platelets ≤100,000/mL) and 47% (323/683) were hospitalized.

### Table 2. Annual number of dengue cases estimated from mandatory notification and laboratory network surveillance systems by using the Chao estimator, stratified according to geographic area and period of the year, metropolitan France, 2007–2010

| Year, region, period | No. observed cases | Chao estimator | | | | |
|----------------------|--------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                      | System             | Common cases   | Estimated no. cases (95% CI) | Completeness of MN, % (95% CI) | Completeness of LN, % (95% CI) | |
|                      | MN | LN | | | | |
| 2007                 | | | | | | |
| Southeast, May-Nov   | 4 | 21 | 4 | 39 (26–87) | 10.2 (2.4–17.2) | 53.8 (12.6–90.4) | | | |
| Other areas, remainder of year | 52 | 365 | 21 | 2,070 (1,444–3,071) | 2.5 (1.6–3.9) | 17.6 (10.9–27.1) | | | |
| Total                | 56 | 386 | 25 | 2,109 (1,481–3,109) | 2.7 (1.6–4.0) | 18.3 (11.3–27.5) | | | |
| 2008                 | | | | | | |
| Southeast, May-Nov   | 6 | 11 | 4 | 18 (14–37) | 33.2 (7.6–44.8) | 60.9 (13.9–82.2) | | | |
| Other areas, remainder of year | 58 | 293 | 42 | 733 (595–938) | 7.9 (5.9–10.0) | 40.0 (30.0–50.6) | | | |
| Total                | 64 | 304 | 46 | 751 (613–956) | 8.5 (6.3–10.6) | 40.5 (29.8–50.5) | | | |
| 2009                 | | | | | | |
| Southeast, May-Nov   | 5 | 19 | 4 | 36 (24–79) | 13.9 (3.1–22.9) | 52.8 (11.6–87.2) | | | |
| Other areas, remainder of year | 63 | 331 | 38 | 1,021 (806–1,339) | 6.2 (4.5–8.1) | 32.4 (23.7–42.4) | | | |
| Total                | 68 | 350 | 42 | 1,057 (841–1,375) | 6.4 (4.6–8.2) | 33.1 (23.6–42.4) | | | |
| 2010                 | | | | | | |
| Southeast, May-Nov   | 91 | 174 | 75 | 234 (216–264) | 38.9 (33.2–42.7) | 74.3 (63.6–81.6) | | | |
| Other areas, remainder of year | 494 | 1,951 | 354 | 4,222 (3,932–4,558) | 11.7 (10.8–12.6) | 46.2 (42.7–49.7) | | | |
| Total                | 585 | 2,125 | 429 | 4,456 (4,164–4,792) | 13.1 (12.2–14.1) | 47.7 (44.2–51.1) | | | |
| 2007–2010            | | | | | | |
| Southeast, May-Nov   | 106 | 225 | 87 | 327 (294–382) | 32.4 (17.2–35.7) | 68.8 (36.5–75.8) | | | |
| Other areas, remainder of year | 667 | 2,940 | 455 | 8,047 (7,217–9,045) | 8.3 (7.1–9.2) | 36.5 (31.1–40.7) | | | |
| Total                | 773 | 3,165 | 542 | 8,374 (7,543–9,371) | 9.2 (8.2–10.7) | 37.8 (33.5–43.8) | | | |

*Information on geographic area or period of the year was not available for 27 cases. MN, mandatory notification; LN, laboratory network.*
Finally, among the 199 patients in whom dengue was detected in southeast France during May–November 2010, 93 (47%) had viremic dengue infections, 64 (32%) were potentially viremic, and 13 (7%) were not viremic. There was not sufficient information for the remaining 29 patients (15%) to enable classification. Among the biologically and potentially viremic patients, the mean estimated duration of viremia while they were in metropolitan France was 6 days.

The completeness of the 2 surveillance systems differed greatly; completeness was much higher for the laboratory network (Table 2). Using the Chao estimator, we estimated the completeness at 3% in 2007 and 13% in 2010 for the mandatory notification surveillance system (9.2% for the 4-year period). We estimated completeness to be an average of ≈4 times higher for the laboratory network: 18% in 2007 and 48% in 2010 (37.8% for the 4-year period). Furthermore, for both surveillance systems, completeness was much higher in areas where the competent vector was established (20.3% for mandatory notification and 57.3% for the laboratory network over the 4-year period) than in other areas (8.6% and 37.1%, respectively), and also much higher during the vector activity period (12.5% and 44.2%, respectively) than during the rest of the year (3.4% and 25.3%, respectively) (Table 4). For the 4-year period, these figures were 32% and 69%, respectively, in Aedes spp.-infested areas during May–November (Table 2). The combination of the 2 surveillance systems increased the completeness compared with the use of the laboratory network alone, but this increase was limited: ≈2% to 4%

Table 3. Annual number of dengue cases estimated from MN and LN surveillance systems by using Chapman-Seber estimator, stratified according to geographic area and period of the year, metropolitan France, 2007–2010*

<table>
<thead>
<tr>
<th>Year, region, period</th>
<th>No. observed cases</th>
<th>Estimated no. cases (95% CI)</th>
<th>Completeness of MN, % (95% CI)</th>
<th>Completeness of LN, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>System</td>
<td>Common cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MN</td>
<td>LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast, May–Nov</td>
<td>4</td>
<td>21†</td>
<td>19.0†</td>
<td>100†</td>
</tr>
<tr>
<td>Other areas, remainder of year</td>
<td>52</td>
<td>365</td>
<td>21</td>
<td>881 (678–1,228)</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>386</td>
<td>902 (699–1,249)</td>
<td>6.2 (4.5–8.0)</td>
</tr>
<tr>
<td>2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast, May–Nov</td>
<td>6</td>
<td>11</td>
<td>16 (14–27)</td>
<td>38.0 (22.0–44.4)</td>
</tr>
<tr>
<td>Other areas, remainder of year</td>
<td>58</td>
<td>293</td>
<td>42</td>
<td>402 (360–479)</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>304</td>
<td>418 (376–495)</td>
<td>15.3 (12.9–17.0)</td>
</tr>
<tr>
<td>2009</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast, May–Nov</td>
<td>5</td>
<td>19</td>
<td>23 (20–38)</td>
<td>21.7 (13.1–24.4)</td>
</tr>
<tr>
<td>Other areas, remainder of year</td>
<td>63</td>
<td>331</td>
<td>38</td>
<td>544 (468–671)</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>350</td>
<td>567 (490–694)</td>
<td>12.0 (9.8–13.8)</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast, May–Nov</td>
<td>91</td>
<td>174</td>
<td>211 (200–232)</td>
<td>43.2 (39.3–45.4)</td>
</tr>
<tr>
<td>Other areas, remainder of year</td>
<td>494</td>
<td>1,951</td>
<td>354</td>
<td>2,721 (2,599–2,872)</td>
</tr>
<tr>
<td>Total</td>
<td>585</td>
<td>2,125</td>
<td>2,932 (2,809–3,083)</td>
<td>20.0 (19.0–20.8)</td>
</tr>
<tr>
<td>2007–2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast, May–Nov</td>
<td>106</td>
<td>225</td>
<td>271 (258–294)</td>
<td>39.2 (35.4–40.8)</td>
</tr>
<tr>
<td>Other areas, remainder of year</td>
<td>667</td>
<td>2,940</td>
<td>455</td>
<td>4,548 (4,262–4,907)</td>
</tr>
<tr>
<td>Total</td>
<td>773</td>
<td>3,165</td>
<td>4,818 (4,532–5,178)</td>
<td>16.0 (14.8–16.9)</td>
</tr>
</tbody>
</table>

*Information on geographic area or period of the year was not available for 27 cases; MN, mandatory notification; LN, laboratory network.
†95% CI not presented because of null variances.
Table 4. Number of dengue cases estimated from mandatory notification and laboratory network surveillance systems with Chao estimator after stratification according to geographic area and period of the year, metropolitan France, in 2010 and for 2007–2010*  

<table>
<thead>
<tr>
<th>Stratification type, Y</th>
<th>No. observed cases</th>
<th>Estimated total no. cases (95% CI)</th>
<th>Completeness of MN, % (95% CI)</th>
<th>Completeness of LN, % (95% CI)</th>
<th>Combined completeness, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Geographic stratification 2010</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southeast</td>
<td>2007–2010</td>
<td>97</td>
<td>229</td>
<td>80</td>
<td>332 (302–378)</td>
</tr>
<tr>
<td>Other areas</td>
<td>585</td>
<td>2,125</td>
<td>429</td>
<td>4,403 (4,120–4,730)</td>
<td>13.3 (12.3–14.2)</td>
</tr>
<tr>
<td><strong>Other areas 2007</strong></td>
<td>773</td>
<td>3,167</td>
<td>542</td>
<td>8,242 (7,446–9,194)</td>
<td>9.4 (8.4–10.4)</td>
</tr>
<tr>
<td><strong>Period stratification 2010</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>May–Nov†</td>
<td>524</td>
<td>1,694</td>
<td>386</td>
<td>3,186 (2,994–3,410)</td>
<td>16.4 (15.3–17.5)</td>
</tr>
<tr>
<td>Remainder of year</td>
<td>61</td>
<td>43</td>
<td>43</td>
<td>1,407 (1,118–1,821)</td>
<td>4.3 (3.2–5.6)</td>
</tr>
<tr>
<td>Total</td>
<td>585</td>
<td>2,125</td>
<td>429</td>
<td>4,594 (4,223–5,034)</td>
<td>12.7 (11.4–13.9)</td>
</tr>
<tr>
<td>May–Nov</td>
<td>656</td>
<td>2,325</td>
<td>468</td>
<td>5,263 (4,764–5,873)</td>
<td>12.5 (11.4–13.9)</td>
</tr>
<tr>
<td>Remainder of year</td>
<td>117</td>
<td>865</td>
<td>74</td>
<td>3,416 (2,761–4,303)</td>
<td>3.4 (2.7–4.2)</td>
</tr>
<tr>
<td>Total</td>
<td>773</td>
<td>3,190</td>
<td>542</td>
<td>8,679 (7,817–9,709)</td>
<td>8.9 (8.0–9.9)</td>
</tr>
</tbody>
</table>

*MN, mandatory notification; LN, laboratory network; information on geographic area or period of the year was not available for 27 cases. Southeast includes the departments (administrative regions) where the competent vector, the Aedes albopictus mosquito, was established.
†May–Nov is the period of activity of Aedes albopictus mosquitoes in metropolitan France.

(Table 4). Globally, the combined completeness was ≈40% for the 4-year period.

Geographic Area of Acquisition of Dengue Infection and Influence of Epidemics in French West Indies

Information on the county of acquisition of dengue infection was available for 1,335 patients (this information was available for mandatory notification and for 3 of the 6 laboratories). Dengue was acquired mainly from 2 geographic areas: the Caribbean and Southeast Asia, which represented 61% and 17% of cases, respectively, over the 4-year period (Table 5). In the Caribbean, the most frequent areas of acquisition (59% of all reported cases) were the French West Indies including Martinique, Guadeloupe, Saint-Barthelemy, and Saint-Martin. In Southeast Asia, dengue fever was primarily acquired in Thailand (7% of all cases) and Indonesia (5%).

In 2007 and 2010, respectively, 34% (37/109) and 71% (682/956) of all cases imported to France were acquired in Guadeloupe (19% in 2007, 41% in 2010) and in Martinique (14% in 2007, 30% in 2010); on each of the 2 islands, dengue epidemics affected nearly 20,000 persons in 2007 and 40,000 in 2010 (17,18). Figure 3 shows the monthly number of dengue cases estimated during 2007–2010 in metropolitan France (estimation from the 2 national sources by using capture–recapture with CS estimator) and in Guadeloupe and Martinique (estimation by regional health authorities from clinically suspected cases within the sentinel network of physicians). The curves roughly overlap, especially during epidemics in the French West Indies. More precisely, the peaks of imported cases in metropolitan France coincide with those of epidemics which occurred in the French West Indies, particularly for the year 2010.

Discussion

In this study, we estimated that >8,300 cases with laboratory evidence of recent dengue infection were imported into metropolitan France during 2007–2010. Approximately 4,500 of them occurred in 2010; this high number was mainly attributed to epidemics of unusually intense and long duration in the French West Indies (19). A correlation between a substantial number of imported cases of disease in metropolitan France and an intense epidemic in French overseas territories was observed with the dengue epidemic in the French West Indies in 2001 (20) and with the chikungunya epidemic on Reunion Island in 2006 (21). A similar contemporary association was observed in Germany, where an increase in imported dengue cases during 2002 was directly linked to an epidemic in Brazil (22).

An estimated 230 cases occurred during May–November 2010 in the 6 southeast departments of France where the Aedes albopictus mosquito was established, and >90% of the infected persons may have been viremic. The increase in the number of imported cases in southeast France and the high vector density in some urban areas were major factors in the emergence of a local transmission cycle. Two cases of autochthonous dengue fever were reported in the Alpes-Maritimes Department in September 2010 (4).

The capture–recapture method which we applied to estimate the incidence of imported dengue cases is widely used...
in epidemiologic surveillance studies when several sources of data are available (23). Several assumptions must be checked when using this method (23,24) to avoid biases. Among these, independence between sources and equal catchability of cases by each source, which are interdependent concepts (7), are of great importance. When log-linear modeling is used, the availability of at least 3 sources ensures that the dependence between sources and the unequal catchability when estimating the true number of cases (24,25) can be taken into account. When only 2 sources of data are available, as is the case for dengue surveillance in metropolitan France at the national level, alternative estimators seem worthwhile. In our study, the alternative used was the Chao estimator which relaxes the assumption that sources are independent, and provides more reliable estimates when the differences between the identifying probabilities of the 2 sources are large (26,27). In contrast, with increasing dependencies between sources, the commonly used CS estimator underestimates the true number of cases (14). This underestimation may explain the differences we found between the 2 estimates.

Furthermore, we reduced unequal catchability by stratifying the results by period of year and geographic area. Other factors associated with unequal catchability should be taken into consideration, but it was not possible to do so comprehensively in our study. In particular, patients with severe disease may have had a higher probability of being captured by surveillance systems, which would lead to an underestimation of dengue infections (28). Conversely, the risk for false positives when using IgM detection for dengue diagnosis may have led to an overestimation of this number.

As in other countries, the dengue surveillance systems in France aim to identify symptomatic patients. The proportion of asymptomatic or mildly symptomatic dengue infections fluctuates within endemo-epidemic countries (29) and equals ≈75% (29,30) of all dengue infections. Our estimate, based on symptomatic cases, must therefore be multiplied by 4 to provide a total number of imported dengue cases in France: ≈33,000 cases for the 2007–2010 period, including 1,300 cases in the area where the competent vector was established and during its period of activity. However, the role of asymptomatic dengue cases in the transmission of the virus to the competent vector is still not well known. In other words, viremia could be lower and shorter in duration in asymptomatic persons than in their symptomatic counterparts (31) and it is not certain that viremia of asymptomatic or mildly symptomatic persons is sufficient to be infective. From a public health point of view, the routine detection of asymptomatic infections returning from abroad is inconceivable.

Conclusions

Completeness of the 2 national-level surveillance systems differed greatly: ≈10% for mandatory notification and ≈40% for the laboratory network. For both surveillance systems, completeness was much higher in the area where the competent vector was established, and during the vector’s period of activity; these factors represent the main target of the surveillance system. Although this finding is comforting in terms of ensuring the implementation of measures aimed at limiting the risk for a local cycle transmission, additional efforts should be made to further increase completeness. The low completeness level of mandatory notification brings up the question of its real usefulness for the early detection of cases and the implementation of control measures, especially because it only marginally improves the completeness of the laboratory network. However, the mandatory notification system in France does monitor the trends of imported cases, including those from countries where no dengue surveillance systems are in place, as is the case in most countries in western Africa (32). Furthermore, the mandatory notification system collects additional clinical information (symptoms, severity) which can be analyzed according to

<table>
<thead>
<tr>
<th>Region†</th>
<th>2007 (n = 109)</th>
<th>2008 (n = 119)</th>
<th>2009 (n = 151)</th>
<th>2010 (n = 956)</th>
<th>2007–2010 (n = 1,335)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>2.8</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>East</td>
<td>6.4</td>
<td>1.7</td>
<td>0.7</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>West</td>
<td>4.6</td>
<td>21.8</td>
<td>11.9</td>
<td>1.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Americas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caribbean</td>
<td>42.2</td>
<td>22.7</td>
<td>19.9</td>
<td>75.0</td>
<td>61.4</td>
</tr>
<tr>
<td>Central America</td>
<td>3.7</td>
<td>3.4</td>
<td>4.0</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>South America</td>
<td>8.3</td>
<td>5.9</td>
<td>13.9</td>
<td>2.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>6.4</td>
<td>7.6</td>
<td>8.6</td>
<td>3.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Southeast</td>
<td>20.2</td>
<td>28.6</td>
<td>32.5</td>
<td>12.8</td>
<td>17.0</td>
</tr>
<tr>
<td>South Pacific</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polynesia</td>
<td>5.5</td>
<td>7.6</td>
<td>6.0</td>
<td>0.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Other areas in the South Pacific</td>
<td>0.0</td>
<td>0.0</td>
<td>2.0</td>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>


*Information was available for mandatory notification and for 3 of the 6 laboratories involved in the surveillance network.
where faster detection of the great majority of cases in the areas lance systems. The enhanced surveillance system leads to additional cases that were not detected by the other 2 surveil- 
- the framework. It contributes to the detection of a few ad-

increase of travelers. May increase because of the expansion of dengue and the

19 -

dengue continues to become hyperendemic in these ter-

intense dengue epidemics were among those who intro-

-ory diagnosis. Travelers returning from Antilles during

- plained by the global epidemiology of dengue, traveler

of imported cases can probably be simultaneously ex-

- ulation and the assessment of the risk for im-

- of spatial and temporal trends of dengue fever

mobilization of professionals during the vector activity

over, this local enhanced surveillance supports the annual

mediate implementation of local control measures. More

the serotype. This system is especially useful for surveil-

- The laboratory network system is used for the moni-

- The laboratory network system is used for the moni-

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.

Acknowledgments

We thank Henriette de Valk for reviewing the manuscript. We thank the reporting physicians for providing mandatory notifi-

- Dr La Ruche is an epidemiologist at the Department of Infectious Diseases, French Institute for Public Health Surveil-

- References

1. World Health Organization. Dengue guidelines for diagnosis,

treatment, prevention and control. Geneva: The Organization and

- The third system, enhanced surveillance, completes

- The third system, enhanced surveillance, completes

period, including health stakeholders in the areas where the vector is expanding.


Address for correspondence: Guy La Ruhe, 12 rue du Val d’Ose, 94415 Saint-Maurice Cedex, France; email: g.laruhe@invs.sante.fr
The widely used pseudorabies virus (PRV) Bartha-K61 vaccine has played a key role in the eradication of PRV. Since late 2011, however, a disease characterized by neurologic symptoms and a high number of deaths among newborn piglets has occurred among Bartha-K61–vaccinated pigs on many farms in China. Clinical samples from pigs on 15 farms in 6 provinces were examined. The PRV gE gene was detectable by PCR in all samples, and sequence analysis of the gE gene showed that all isolates belonged to a relatively independent cluster and contained 2 amino acid insertions. A PRV (named HeN1) was isolated and caused transitional fever in pigs. In protection assays, Bartha-K61 vaccine provided 100% protection against lethal challenge with SC (a classical PRV) but only 50% protection against 4 challenges with strain HeN1. The findings suggest that Bartha-K61 vaccine does not provide effective protection against PRV HeN1 infection.

Pseudorabies virus (PRV; family Herpesviridae, subfamily Alphaherpesvirinae, genus Varicellovirus) contains a double-stranded DNA genome with strong genetic stability. The virus has a broad host range and can infect most mammals and some avian species (1). Pigs are the natural reservoir for PRV; infection in adult pigs is called Aujeszky disease. Swine farmers with PRV-infected pigs can incur substantial economic costs from reproductive losses in sows and from weight loss in infected adults (2). PRV is especially prominent in regions of South America, Asia, and Europe with dense swine populations. There have been no reports of PRV in Norway, Finland, or Malta, and the disease has been eradicated from domestic pig populations in Germany, Austria, Sweden, Denmark, the United Kingdom, Canada, New Zealand, and the United States (3).

The swine industry worldwide has effectively used vaccines to control pseudorabies for >30 years; cases of the disease are now rarely reported from pig farms (4–7). Among the vaccines, Bartha-K61 is widely used and has played a key role in the eradication of pseudorabies. This vaccine is an attenuated strain of PRV produced by extensive in vitro passage and contains a well-characterized deletion of several viral proteins (i.e., complete gE and US9, partial gl and US2) that attenuates virulence (8–10). Thus, the gE ELISA is used for the differential diagnosis of infection with field PRV strains or vaccine strain in pigs.

In China, the first report of a PRV outbreak occurred in the 1950s, and the Bartha-K61 vaccine was imported from Hungary to China in the 1970s (11). From the 1990s until late 2011, >80% of pigs in China were vaccinated with the Bartha-K61 vaccine, and pseudorabies was well controlled (12). However, beginning in late 2011, pseudorabies has occurred on many large pig farms in animals that have been vaccinated with Bartha-K61 vaccine; during the first month of these outbreaks, 50% of samples were positive for pseudorabies gE antibody. The disease is characterized by stillbirth or the birth of weak piglets with neurologic symptoms that ultimately lead to death. The onset of clinical signs in 2- to 3-day-old piglets is sudden, spanning 5 hours from onset to death. The disease in piglets is characterized by shivering and opisthotonos, and 10%–50% of infected piglets die. Since the initial outbreak in late 2011, the disease has occurred in 6 provinces in China with extensive pig-raising industries and caused many piglet deaths and great economic loss (13).

The PRV Bartha-K61 vaccine is widely used to protect pigs against pseudorabies; there has been no reported...
resistance to the vaccine. In 2012, to determine the cause of recent PRV outbreaks among Bartha-K61–vaccinated pigs, we obtained clinical samples from piglets with suspected pseudorabies. A breakthrough PRV was isolated from the samples, and we identified the pathogenicity and immunologic protection of the novel isolate.

Materials and Methods

In 2012, we collected brain tissue from 154 dead piglets with suspected pseudorabies. The piglets were from 15 farms located in 6 provinces of China: Henan, Heilongjiang, Jilin, Liaoning, Inner Mongolia, and Jiangsu Provinces. All of the farms used PRV Bartha-K61 vaccine to protect their pigs against pseudorabies.

Cells, Vaccine, and Challenge Virus

Vero cells were used for virus propagation and titration in Dulbecco Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 µg/mL streptomycin, and 100 IU/mL penicillin. PRV Bartha-K61 vaccine with a virus titer of 10^0.5 50% tissue culture infectious doses (TCID_{50})/dose was purchased from Harbin Weike Biotechnology Development Co. (Harbin, China). According to the quality standards for this vaccine (14), the virus titer of qualified product is ≥5,000 TCID_{50}/dose. PRV SC strain, which is highly pathogenic to sheep and pigs (4), was isolated in 1980 and has been maintained in our laboratory. This strain has been used as a challenge virus to test the effectiveness of Bartha-K61 vaccine in China from the time the vaccine was first licensed in this country (14).

Viral Genome Extraction and PCR

DNA extraction was performed as described (15). The sense primer used for PCR was 5'-ATGCCG-GCCCTTTCTG-3', and the reverse primer was 5'-CGGTTCCTCCGGTATTTAAGC-3'. The thermal profile used for PCR was 95°C for 4 min; followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 10 min. This generated the complete sequence of the PRV gE gene (previously US8) and the flanking regions of gI (previously US7), and US9. The PCR products were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide for visualization.

Virus Isolation

PRV PCR–positive brain tissue homogenates were centrifuged at 10,000 g for 10 min. The supernatant was passed through a 0.45-µm filter and transferred to Vero cell monolayers. The cells were incubated at 37°C and examined daily for cytopathic effect (CPE). After the appearance of CPE, cells were collected and stored at −20°C, and a novel PRV was chosen for further investigation; we named the isolate HeN1. After 3 freeze-thaw cycles, PRV was cultured in Vero cells. The fifth passage of PRV was negatively stained with 2% phosphotungstic acid, and we examined the virus particle morphology by using a transmission electron microscope (H-7650; Hitachi High-Technologies Ltd., Tokyo, Japan).

Phylogenetic Analyses

Sixteen of the positive PCR products selected from different farms or collection times were cloned into the pMD18-T vector, and the insert was sequenced in both directions. We analyzed sequence data as described (16) and compared the complete sequences of the gE gene with all PRV gE sequences available in the GenBank database (Table 1). We used Lasergene sequence analysis software (DNASTAR, Madison, WI, USA) to perform multiple sequence alignments and phylogenetic analyses.

Experimental PRV Inoculation of Pigs

Six 3-month-old specific pathogen–free Bama miniature pigs were obtained from the Experimental Animal Center at the Veterinary Research Institute (Harbin, China). All pigs were confirmed to be free of PRV infection by using a gE ELISA kit (HerdChek PRV; IDEXX Laboratories, Westbrook, ME, USA) for PRV antibody detection and by using PCR. The animals were also determined to be free of porcine circovirus type 2, classical swine fever virus, porcine reproductive and respiratory syndrome virus, and porcine parvovirus by PCR.
syndrome virus, and swine influenza virus infections by using serologic methods or reverse transcription PCR or PCR as described (17,18).

The pigs were randomly assigned to 2 rooms and kept under Biosafety Level 2 conditions throughout the experiment. Five of the 6 pigs were in the test group (pigs 011–015); these pigs were injected intramuscularly with a 1-mL inoculum containing $1 \times 10^{7.0}$ TCID$_{50}$ of PRV strain HeN1. The sixth pig was used as a control and injected intramuscularly with 1 mL of Vero cell culture supernatant. Clinical symptoms were checked daily throughout the study, and rectal temperatures were recorded daily before feeding. We used the HerdChek PRV gE ELISA kit according to the manufacturer’s instructions to analyze PRV-specific antibodies in serum samples collected 0, 2, 5, 7, 14, 21, 28, and 35 days after inoculation. All animals were euthanized on postinoculation day 35. Tissue samples were obtained from the brains, lungs, hearts, testicles, and lymphoid nodes (mandibular, mesenteric, and superficial inguinal) for virus detection by PCR or virus isolation and for histopathologic examination.

**Virus Neutralization Assay**

We intramuscularly inoculated 5 PRV-free piglets with $10^{5.5}$ TCID$_{50}$ Bartha-K61 vaccine. Blood samples were collected weekly from each animal, and antisera were individually prepared and stored at -80°C until used. The virus neutralization assay was performed as described (4). All sera were heat-inactivated for 30 min at 56°C before testing. The assays were performed by mixing PRV SC and HeN1 strains, respectively, with 50 mL of serially diluted antiserum and 100 TCID$_{50}$ Bartha-K61 vaccine. Antiserum titers were expressed as the highest dilution that reduced the viral CPE by 50% relative to non-neutralized controls. All samples were analyzed in duplicate, and the results shown are the average of the duplicate assays.

**Experimental PRV Inoculation of Sheep**

Sheep are commonly used to examine the efficacy of Bartha-K61 vaccine in China. The experimental design, immune dose, and viral challenge level used were in accordance with China’s quality standards for veterinary biological products (14). Fourteen 18-month-old sheep were obtained from a farm determined, by PCR and gE ELISA (HerdChek PRV), to be free from PRV infection. Sheep were randomly assigned to 4 isolation rooms. Each sheep in Groups 1 and 3 was injected intramuscularly with $10^{5.0}$ TCID$_{50}$ Bartha-K61 vaccine. Sheep in Groups 2 and 4 were not vaccinated. After continuous observation for 14 days, the sheep in Groups 1 and 2 were each challenged with 1,000 50% lethal doses (LD$_{50}$) of PRV SC strain, and sheep in Groups 3 and 4 were challenged with 1,000 LD$_{50}$ of HeN1 strain. The LD$_{50}$ was titrated according to quality standards (14). According to the quality standards of Bartha-K61 vaccine, at least 2 sheep in the control group would become ill with pseudorabies and die, and all of the vaccinated sheep would be protected against infection.

**Results**

**PCR Survey of Clinical Samples**

PRV was detected by PCR in 88 (57.1%) of the 154 clinical samples tested. At least 1 sample from each of the 15 farms examined was positive (Table 2), suggesting that wild-type PRV infection is prevalent in China.

**PRV Isolation**

Positive brain tissue homogenate was filtered to remove bacteria and then inoculated onto Vero cells. CPE was characterized by the appearance of reticulated cells at 48 h (Figure 1). RNA and DNA extracted from the cell

![Figure 1. Cytopathic effect and morphology of pseudorabies virus strain HeN1. A) Uninfected control Vero cells. B) Pseudorabies virus–infected Vero cells. A) and B) Original magnification 200. The cytopathic effect, which was characterized by reticulated cells, was observed 48 h after inoculation. Spherical virus particles without (C) or with (D) viral envelope were observed by electron microscopy. Scale bars indicate 500 nm.](image-url)
cultures were tested by reverse transcription PCR or PCR and were PRV positive but negative for classical swine fever virus and porcine reproductive and respiratory syndrome. The HeN1 isolate was examined by electron microscopy, and spherical viral particles with or without viral envelope were observed (Figure 1).

## Phylogenetic Analysis
The complete gE genes of 16 isolates collected in 2012 from pig clinical samples were sequenced; each was 1,740 bp. Phylogenetic analysis revealed that the sequences of all 16 isolates clustered to a relatively independent region of the tree; this region was relatively distant from previously isolated strains of PRV (Figure 2, panel A). The PRV isolates shared 98.6%–99.8% nt and 95.0%–99.6% aa identity with previously isolated PRVs. Compared with Kaplan and Becker strains, these 16 isolates contained 2 aa insertions. Aspartate amino acid residues were inserted at positions 48 and 492–495, where the 2012 isolates contained 5 continuous residues and earlier isolates contained 4 continuous residues (Figure 2, panel B). Although amino acid insertion was also observed in a few early Chinese PRV isolates, the insertion in the new 2012 isolates was highly conserved.

## PRV HeN1–Inoculated, Bartha-K61–Vaccinated Pigs
Fever (rectal temperature \(\leq 41.0^\circ\text{C}\), reference temperature 39.0°C–39.5°C) developed in HeN1 PRV-inoculated pigs 2–6 days after challenge; temperatures returned to normal 7 days after inoculation and remained normal until the end of the experiment (Figure 3). Four to 6 days after challenge, loss of appetite was observed in the pigs; appetites subsequently returned to normal 6–7 days after inoculation without any other clinical symptoms. PRV gE antibodies were detected in serum samples for all pigs 5–7 days after inoculation (Figure 3). Pathologic
examination showed brain hemorrhage in all infected pigs (Figure 4); noteworthy damage did not occur in other organs. In the brain, the histopathologic changes were characterized by local bleeding of the meninges, chronic meningitis, and lymphocyte infiltration around the small blood vessels of the brain cortex. PRV HeN1 was not detectable in serum samples by PCR or virus isolation, but it was detectable in all brains and most testicles by PCR (Table 3).

**Virus Neutralization Assay**

At different times, we collected antiserum samples from PRV Bartha-K61–vaccinated pigs and measured the virus neutralization capacity against Bartha-K61, SC, and HeN1 PRV strains. The virus neutralization titer of antisera to Bartha-K61 was typically 20- to 40-fold. The capacity of neutralizing heterologous PRVs was lower, and the virus neutralization titer of antisera was 10- to 15-fold against SC strain and 10-fold against the novel HeN1 strain (Figure 5).

**Immunologic Protection in Sheep**

To determine the protective effect of Bartha-K61 vaccine strain against PRV HeN1 challenge, we vaccinated sheep, challenged them with PRV strain HeN1 or SC, and then continuously observed them for 14 days. The control (unvaccinated) sheep challenged with HeN1 or SC strain

### Table 3. Virus detection in clinical samples from 5 Bartha-K61–vaccinated pigs inoculated with pseudorabies virus HeN1, China, 2012*

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Serum sample on postinoculation day no.</th>
<th>Brain</th>
<th>Lung</th>
<th>Mandibular</th>
<th>Mesenteric</th>
<th>Superficial inguinal</th>
<th>Testicle</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>011</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>012</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>013</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>014</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>015</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Virus was detected in serum samples by PCR and virus isolation; virus was detected in organs by PCR only.−, negative; +, positive.
The neutralization titer to Bartha-K61 was 20- to 40-fold; the neutralization titers to pseudorabies virus SC and HeN1 strains were 10- to 15-fold and 10-fold, respectively. The virus neutralization assay was performed with antiserum from 5 individual piglets; error bars represent the SD of the 5 experiments.

Discussion

Pigs are the reservoir host for PRV. Nonpregnant adult pigs do not show obvious clinical signs and symptoms of infection, except for weight loss; however, among pregnant sows, the disease causes stillbirths and the birth of weak piglets with neurologic symptoms that lead to death. Newborn piglets infected with virulent PRV occasionally show diarrhea, neurologic signs, and a higher rate in brain tissue than in blood; thus, brain tissue is likely to be the most reliable clinical sample for diagnosing PRV infection.

PRV antibody can be detected in sheep after vaccination. However, protection against PRV challenge is not closely related to the level of antibody because the virus is nonviremic and spreads predominantly by mucosal infection and neuronal innervation (19). Results of a microneutralization assay suggested that serum generated by pigs vaccinated with Bartha-K61 vaccine had neutralizing ability; however, this neutralizing ability was substantially decreased for currently circulating virulent PRV strains. Moreover, there was no correlation between the neutralizing antibody titer and in China, sheep are typically used for determining the efficacy of Bartha-K61 vaccine in quality standards for veterinary biologic products (14). In addition, lethal challenge with 1,000 LD₅₀ PRV SC strain has also been documented in these quality standards.

The findings in this study showed that sheep vaccinated with Bartha-K61 vaccine were protected from a lethal challenge with PRV SC strain, proving that the vaccine was effective. However, only half of the sheep vaccinated with Bartha-K61 vaccine survived challenge with the novel HeN1 strain, suggesting that the vaccine does not provide full protection against this PRV strain. In China, PRV Bartha-K61 vaccine has been widely applied in the field for ≈30 years, and is recognized as an excellent vaccine strain. Nevertheless, since late 2011, cases of pseudorabies have occurred on many farms, and the disease has gradually become widespread in China. Animals on the affected farms had been vaccinated according to normal procedures with PRV Bartha-K61 vaccine. Brain tissue was collected from dead piglets with suspected pseudorabies on 15 pig farms in 6 China provinces and tested for the presence of PRV by PCR and virus isolation. Wild PRV was present in all 15 samples, and gE gene sequencing showed the isolates to be phylogenetically distant from previously characterized PRV isolates.

Table 4. Protective effect of Bartha-K61 vaccine in sheep challenged with PRV strains HeN1 and SC, China, 2012

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Vaccinated with Bartha-K61</th>
<th>No. PRV antibody-positive before challenge/no. total</th>
<th>Challenge virus</th>
<th>No. sick after challenge/no. total</th>
<th>No. deaths after challenge/no. total</th>
<th>No. protected sheep/no. total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>4/4</td>
<td>HeN1</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>0/3</td>
<td>HeN1</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>3/4</td>
<td>SC</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>4</td>
<td>No</td>
<td>0/3</td>
<td>SC</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*Sheep from a farm determined to be free from PRV infection were randomly assigned to 4 isolation rooms (Groups 1–4). After vaccination, sheep were challenged with PRV strain HeN1 or SC and continuously observed for 14 days. PRV, pseudorabies virus.
protection rate, so evaluation of vaccine efficacy should not be judged purely by the levels of neutralizing antibodies. Thus, the protective immune response afforded by existing vaccine strains against the currently circulating, virulent PRV HeN1 isolate remains to be elucidated.

The control and prevention of pseudorabies requires depopulation of infected animals, zoning for restricted movement of commercial animals, and improved strategies for detecting PRV infection and vaccinating against the disease (3). Vaccination and DIVA (differentiating infected from vaccinated animals) form the basis of control and prevention (20). In some European Union countries, the disease has been well controlled or eradicated by using a gE-deleted vaccine along with gE ELISA for the differential diagnosis of infection with field PRV or the vaccine strain in pigs (21,22). In China, the epidemiologic surveillance of PRV has been strengthened, PRV-positive pigs are being separated from noninfected pigs, and PRV-free pig farms have been advised to vaccinate their animals. In addition, research on the pathogenicity of PRV is ongoing in China, and a new effective vaccine is also in development in China.

This study was supported by the National Natural Science Foundation of China (grant no. 31270045) and the National High Technology Research and Development Program (863 plan) (grant no. 2011AA10A208).

Dr An is an associate professor at the State Key Laboratory of Veterinary Biotechnology, Veterinary Research Institute, in Harbin, China. His research interests focus on the epidemiology and molecular biology of porcine reproductive and respiratory syndrome virus and pseudorabies virus.

References


Address for correspondence: Guang-Zhi Tong, Shanghai Veterinary Research Institute, CAAS No. 518, Ziyue Rd, Minhang District, Shanghai 200241, China; email: gztong@shvri.ac.cn
Understanding global influenza migration and persistence is crucial for vaccine strain selection. Using 240 new human influenza A virus whole genomes collected in Vietnam during 2001–2008, we looked for persistence patterns and migratory connections between Vietnam and other countries. We found that viruses in Vietnam migrate to and from China, Hong Kong, Taiwan, Cambodia, Japan, South Korea, and the United States. We attempted to reduce geographic bias by generating phylogenies subsampled at the year and country levels. However, migration events in these phylogenies were still driven by the presence or absence of sequence data, indicating that an epidemiologic study design that controls for prevalence is required for robust migration analysis. With whole-genome data, most migration events are not detectable from the phylogeny of the hemagglutinin segment alone, although general migratory relationships between Vietnam and other countries are visible in the hemagglutinin phylogeny. It is possible that virus lineages in Vietnam persisted for >1 year.

Understanding influenza dynamics in tropical regions is crucial for understanding global influenza epidemiology because dynamics between temperate and tropical regions are closely linked. Phylogenetic studies have supported eastern Asia, Southeast Asia, and the tropics as potential ecological sources of global influenza circulation (1,2), but others have suggested a variety of geographic regions as potential sources (3–5). Consequently, the role played by the tropics in the global epidemiology of influenza is still uncertain. Viral gene sequence data from tropical countries are crucial for understanding virus migratory routes within the tropics and between tropical and temperate countries.

Vietnam is an example of a tropical country that may play a major role in global influenza dynamics but for which relatively little is known about influenza epidemiology and genetic population structure of the viruses. Sentinel surveillance suggests that in Vietnam, influenza peaks 1–2 times per year, but neither the influenza-like illness (ILI) data nor the virologic confirmation data show a simple seasonal pattern; the trends for confirmed influenza cases fluctuate more than trends for ILI (6,7). Serologic studies indicate that annual influenza incidence in Vietnam is between 17% and 26% (8). The population of Vietnam is relatively young; according to contact patterns, most cases should occur among younger persons (9,10). Given Vietnam’s high population density and strong travel connections to eastern Asia, Southeast Asia, and Australia/New Zealand, Vietnam is as likely as any other country in eastern or Southeast Asia to support continuous, year-round circulation of a single influenza lineage (persistence) and potentially act as a global source of influenza viruses.

Previous global phylogenetic studies of influenza have demonstrated virus mixing globally (3,4,11), a lack of interseasonal persistence in temperate regions (1,2,11,12), and some evidence of persistence in subtropical regions (5,13). Time-series studies of confirmed influenza suggest (with exceptions [14]) that influenza does not exhibit the same strong and regular seasonality in
tropical countries as it does in temperate zones (15–19) and that it could be constantly circulating throughout the year (20,21); however, in the latter 2 studies, phylogenetic analyses were not performed. We analyzed 240 newly sequenced influenza virus whole genomes from Vietnam, sampled through the Vietnam National Sentinel Surveillance System during 2001–2008 (6). We determined the relative strength of influenza migratory connections between Vietnam and the rest of the world, and we interpreted these results in the context of a sampling bias that seems to affect all sequence-based studies aiming for phylogeographic interpretations. On the basis of frequent sampling in 2007 and 2008, we assessed whether influenza viral lineages persisted in Vietnam during this period. However, we could not definitively conclude whether Vietnam represents a sink or a source population for influenza transmission.

Understanding global influenza migration and persistence patterns is crucial for maintaining a coordinated and efficient biannual strain selection process for influenza vaccine. Choices for future vaccine components will depend on recent availability of samples, and understanding each region’s contribution to global influenza circulation will help inform decisions based on viruses coming from highly connected or weakly connected regions.

Methods

Samples

During 2001–2008, as part of the Vietnam National Influenza Surveillance System, nasopharyngeal or throat swab samples were collected from patients seeking care for ILI at hospitals (6). Specimens were tested for influenza A and B viruses and were further subtyped for H1, H3, and H5 by reverse transcription PCR by using primers, probes, and reagents recommended by the Centers for Disease Control and Prevention and the World Health Organization (WHO). Samples that were positive for influenza A by PCR were selected for virus isolation, and isolates reaching a titer of 1:8 in hemagglutination assays were selected for sequencing analysis. All isolates were subtyped by using hemagglutination assays with reference antigens and antiserum from the WHO reagent kit. A total of 242 samples were shipped to the National Institutes of Health Influenza Genome Sequencing Project (USA) (22) for whole-genome sequencing at the J. Craig Venter Institute. Of the 242 samples, 2 were excluded from this analysis (1 that could not be sequenced and 1 from a patient with a mixed infection). The final dataset of the 240 whole-genome sequences comprised 145 influenza subtypes H3N2 and 95 H1N1 (GenBank accession nos. CY103972–CY105893). Table 1 shows the numbers and locations of the viruses.

Datasets

For phylogeographic analysis, we compiled influenza virus sequences of subtypes H1N1 and H3N2 into 2 datasets: a regional dataset of whole-genome sequences from Asia and Australia/New Zealand and a global dataset of geographically subsampled sequences (50 replicates) of the hemagglutinin (HA) segment (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/11/13-0349-Techapp1.pdf). For subsampling, we randomly sampled 12 sequences per geographic region per year.

Phylogenetic Inference

Sequences were aligned by using the MUSCLE program, version 3.8 (23). Maximum-likelihood trees were inferred by using RAxML version 7.3.0 with 2,000 bootstrap replicates (24,25). For the regional HA datasets, phylogenetic trees with sampling date information were inferred by using BEAST version 1.6.2 (26) and a relaxed molecular clock (uncorrelated lognormal). The nucleotide substitution model was SRD06 + HKY85 + Γ, and the demographic models used were constant population size and Bayesian skyride (online Technical Appendix).

Analysis of Regional Migration

Migration analysis was conducted by using a straightforward parsimony method in the PAUP* program (27,28). The 2,000 bootstrap trees and the best maximum-likelihood tree inferred by RAxML were read into PAUP*, and nucleotide sequences were replaced by single-letter location codes assigned to a set of predefined global regions (online Technical Appendix Figures 5, 6). Changes in location code were mapped onto the branches of the trees.
by using an ACCTRAN parsimony criterion (28). Analysis was performed on all segments to determine whether migration histories differed among them, which could have been caused by reassortment among influenza virus RNA segments. A strong reassortment signal was verified when standard mosaic and phylogenetic criteria were used (29) (online Technical Appendix Figure 2).

Analysis of Global Migration
For comparison, we performed the same migration analysis on the global dataset of 50 subsampled replicates for influenza virus subtypes H3N2 and H1N1. Migration matrices were built describing numbers of connections between 27 subtype H3N2 or 29 subtype H1N1 predefined geographic regions. We used Gephi software (30) to visualize the connections in the matrix. For regions with sufficient samples, we computed minimum distances to the trunk of the rooted phylogeny for all 50 subsampled trees (subtype H3N2 only) to determine whether viruses from different regions could be described as ancestral (close to the trunk) or derived (far from the trunk).

Results
Regional Migration of Influenza Virus (H3N2) HA
The relationship between the subtype H3N2 HA sequences from Vietnam and other viruses sampled in the region is shown in Figure 1. Representative samples from Vietnam are available for 2003–2008 but not for 2006, when subtype H1N1 predominated. Viruses isolated in Vietnam show close relationships to viruses isolated in Hong Kong, Taiwan, Singapore, and Australia/New Zealand. Inferred from this tree were 20 parsimony-unambiguous migration events, 9 showing Vietnam–Hong Kong migration, 7 showing Vietnam–Australia/New Zealand migration, 2 showing Vietnam–Taiwan migration, 1 showing Vietnam–South Korea migration, and 1 showing Vietnam–Qatar migration. Clearly, because Hong Kong (68 sequences) and Australia/New Zealand (>500 sequences) were overrepresented in the regional dataset, most Vietnam migrations were associated with these 2 locations. Because we were initially uncertain how well a geographically regional phylogenetic tree would reflect the true migration patterns of influenza virus, we performed the same migration analysis on the global dataset of 50 subsampled replicates for influenza virus subtypes H3N2 and H1N1.

Figure 1. Maximum-likelihood phylogenetic tree (hemagglutinin segment) of the 787 sequences that comprise the regional influenza (H3N2) dataset. Tree is rooted on A/Canterbury/179/1999, and bootstrap values are shown on key nodes. Branches are colored by location: red, Vietnam; purple, Australia or New Zealand; green, Hong Kong; blue, Taiwan; orange, Singapore. Labels are shown directly to the left or right of the cladesthey are describing, with 2 exceptions: the label "VN Jun 2003–Jan 2004" refers to the viruses directly above it, and the label "ANZ 2000/2001" refers to the 2 major clades below it and above it. KG, Kyrgyzstan; VN, Vietnam; JP, Japan; KR, South Korea; QA, Qatar; KW, Kuwait; TW, Taiwan; HK, Hong Kong; ANZ, Australia and New Zealand. Scale bar indicates nucleotide substitutions per site.
viruses in Vietnam, we performed a validation exercise to determine what proportion of global migration of influenza subtype H3N2 virus from Vietnam could be detected in a regional phylogenetic tree (online Technical Appendix). Approximately 70% of Vietnam influenza migrations from a global analysis were also observed in the regional tree.

**Regional Migration of Influenza Virus (H1N1) HA**

The relationship between influenza subtype H1N1 HA sequences from Vietnam and other regional viruses is shown in Figure 2. Representative samples from Vietnam are available for 2001–2008 but not for 2004 and 2007. Inferred from this tree are 10 parsimony-unambiguous migration events, 6 showing Vietnam–Taiwan migration, 2 showing Vietnam–Japan migration, and 2 showing Vietnam–Australia/New Zealand migration. As in the analysis for subtype H3N2, these migration links correspond with the viruses that were sequenced from the region during 2001–2008.

**Whole-Genome Migration Patterns**

Because the regional trees included only sequences for which whole genomes were available, migration patterns were compared systematically across all 8 influenza segments. Because influenza viruses reassort, different event histories should be visible in phylogenies inferred separately for the 8 virus segments. Indeed, for the subtype H3N2 dataset, we observed a median of 14 parsimony-unambiguous migration events for the neuraminidase segment and a median of 41 for the matrix protein segment; the other segments fell somewhere in between (Table 2). Again, most migrations were with Australia/New Zealand and Hong Kong, indicating that the pattern of migration is similar across segments, although different numbers of migrations and different individual migration events are visible when different segments are analyzed. For the matrix protein and nonstructural protein segments of subtype H3N2 viruses, the large number of migration events may result from the larger number of topologically uncertain and polytomic nodes in these trees that had to be randomly resolved to compute the number of migration events; that is, sequences from one country could be mistakenly mixed with sequences from other countries, thus generating some artificial migration events in the parsimony analysis. The low confidence in the

---

**Figure 2. Maximum-likelihood phylogenetic tree (hemagglutinin segment) of the 300 sequences that comprise the regional influenza (H1N1) dataset. Tree is rooted on A/New Caledonia/20/1999, and bootstrap values are shown on key nodes. Branches are colored by location: red, Vietnam; purple, Australia or New Zealand; yellow, Japan; blue, Taiwan. Labels are shown directly to the left or right of the clades they are describing. JP, Japan; VN, Vietnam; TW, Taiwan; ANZ, Australia and New Zealand. Scale bar indicates nucleotide substitutions per site.**
Vietnam–Singapore migration link for subtype H3N2 may result from the small number of whole-genome sequences available from Singapore, all of which were collected in 2003.

For subtype H1N1 viruses, we observed 6–16 migration events across the trees inferred for the 8 segments (Table 2). In the bootstrapped data, the Vietnam–Taiwan and Vietnam–Japan migratory connections seem to be approximately equal, despite the fact that the best maximum-likelihood tree showed 6 Vietnam–Taiwan connections and 2 Vietnam–Japan connections. The migratory connection between Vietnam and Australia/New Zealand seems to be somewhat weaker, possibly because of substantially less sampling of Australia/New Zealand viruses in the subtype H1N1 dataset. In the subtype H1N1 dataset, the number of migration events for the matrix protein and nonstructural protein segments did not increase.

Table 2. Observed migration of influenza virus between Vietnam and other countries*  

<table>
<thead>
<tr>
<th>Virus subtype, country</th>
<th>PB2</th>
<th>PB1</th>
<th>PA</th>
<th>HA</th>
<th>NP</th>
<th>NA</th>
<th>MP</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H3N2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aus/NZ</td>
<td>9 (5–13)</td>
<td>9 (5–13)</td>
<td>7 (4–10)</td>
<td>7 (3–10)</td>
<td>11 (6–16)</td>
<td>5 (2–8)</td>
<td>26 (19–33)</td>
<td>27 (18–36)</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>4 (1–7)</td>
<td>4 (1–8)</td>
<td>6 (2–10)</td>
<td>5 (2–8)</td>
<td>6 (2–10)</td>
<td>7 (3.5–12)</td>
<td>12 (6–18)</td>
<td>5 (1–10.5)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>2 (1–3)</td>
<td>3 (2–4)</td>
<td>3 (2–4)</td>
<td>3 (1–4)</td>
<td>2 (1–4)</td>
<td>1 (0–3)</td>
<td>3 (1–5)</td>
<td>2 (1–5)</td>
</tr>
<tr>
<td>Singapore</td>
<td>1 (0–3)</td>
<td>1 (0–3)</td>
<td>0 (0–2)</td>
<td>1 (0–3)</td>
<td>1 (0–6)</td>
<td>1 (0–3)</td>
<td>0 (0–2)</td>
<td>1 (0–3)</td>
</tr>
<tr>
<td><strong>H1N1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>3 (1–6)</td>
<td>3 (1–5)</td>
<td>4 (2–7)</td>
<td>5 (2–7)</td>
<td>5 (2–8)</td>
<td>4 (2–8)</td>
<td>5 (2–10)</td>
<td>5 (2–8)</td>
</tr>
<tr>
<td>Aus/NZ</td>
<td>2 (0–5)</td>
<td>1 (0–4)</td>
<td>2 (0–5)</td>
<td>2 (0–5)</td>
<td>4 (1–8)</td>
<td>2 (0–4)</td>
<td>5 (2–9)</td>
<td>2 (0–6)</td>
</tr>
<tr>
<td>Japan</td>
<td>2 (1–4)</td>
<td>2 (1–4)</td>
<td>2 (1–4)</td>
<td>3 (1–4)</td>
<td>4 (1–8)</td>
<td>1 (0–2)</td>
<td>6 (2–9)</td>
<td>4 (2–7)</td>
</tr>
</tbody>
</table>
*Data from 2,000 bootstrapped trees for all 8 segments of regional datasets for influenza subtypes H3N2 and H1N1. PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleocapsid protein; MP, matrix protein; NS, nonstructural protein; Aus/NZ, Australia/New Zealand.

Migration in Subsampled Global HA Trees

Because it is clear that the presence and number of samples from different regions influence migration analysis, migratory patterns were reanalyzed on the global subsampled dataset to reduce the geographic bias present from having higher numbers of samples available from some regions than others. Using global HA trees for subtype H3N2 and H1N1 sequences, we constructed full migration matrices including all parsimony-unambiguous migration events among our predefined regions (online Technical Appendix Figures 5, 6). These migration networks are shown in Figure 3, where the United States is a major hub of influenza migration and eastern Asia and Australia/New Zealand play major roles. The subtype H3N2 data show Vietnam connected with most other countries in eastern and Southeast Asia, with the United States, and weakly with southern Asia. The subtype H1N1 data show Vietnam connected with the United States and Europe but weakly with other Asian countries. For both subtypes, the total number of migration events associated with each node in the network is correlated with the number of samples available for that node (all p values were <10\(^{-5}\); Kendall and Spearman tests).

Note that sample numbers are not identical for each node because for some regions <12 sequences per year were available, and these regions did not need to be subsampled for those years. Hence, undersampling and oversampling can generate this correlation. Despite our attempt to reduce geographic bias in the global dataset, inference on migration events is still closely associated with regional availability of samples; this bias appears to affect all phylogeographic studies.

For H3N2 sequences, to determine whether any region has the characteristics of an ecological source, we computed the phylogenetic distance of sequences from 6 well-sampled regions (China, Hong Kong, Japan, Vietnam, Australia/New Zealand, and the United States) to the trunk of the global maximum-likelihood phylogenetic tree (Figure 4). In 2003, for example, across all 50 subsampled trees, sequences isolated in China were typically closest to the trunk of the phylogenetic tree, indicating that these sequences are ancestral to other viral sequences sampled in 2003; this finding is consistent with the global replacement of subtype H3N2 viruses by the A/Fujian/411/2002-lineage that occurred in 2003. In general, for the years 2003–2007, in no region were sequences consistently ancestral, indicating that it is unlikely that there is a single global source of human influenza viruses. The more likely global migration model involves periodic global strain replacements originating in different regions in different years (3, 4). There were not sufficient samples from all regions/years to perform this analysis on the subtype H1N1 dataset.

Lineage Persistence

Figure 5 shows a Bayesian subtype H3N2 phylogenetic tree inferred from the time-stamped regional sequence data. The insets of this figure detail the 2007–2008 part of the phylogeny (87 sequences) and the coalescent times for the tips of these branches. It is difficult to draw a complete persistence picture for these viruses because of undersampling during the second quarter of 2007 and the first half of 2008 despite PCR-confirmed evidence of influenza virus activity during these periods (6). The median coalescent time for viruses from Vietnam sampled during this period...
is 37 days (interquartile range 21–72 days), and the insets in Figure 5 suggest that one of the lineages persisted in Vietnam for the 13 months from January 2007 through January 2008. An absence of samples from February through May 2008 makes it impossible to determine conclusively if this lineage persisted in Vietnam for the entire 2-year period.

Figure 3. Global migration maps from fully subsampled global hemagglutinin tree for A) influenza (H3N2), based on 1,140 sequences, and B) influenza (H1N1), based on 554 sequences. The size and color of the nodes corresponds to the number of migration events associated with that location (median from 50 subsamples). The thickness of the lines corresponds to the number of migration events between 2 nodes. Red lines join Vietnam to other locations; blue lines join other locations. UK, United Kingdom; USA, United States.
Figure 4. Minimum phylogenetic distance to the trunk, computed for the 50 subsampled global influenza (H3N2) phylogenies. Minimum distances are shown by year and by region, for 6 regions with sufficient sampling during 2003–2007. ANZ, Australia/New Zealand; VN, Vietnam; HK, Hong Kong; CN, China; JP, Japan; US, United States. Red lines show medians across 50 subsamples. For Vietnam in 2006 and Hong Kong in 2007, there were insufficient virus sequences.

To determine whether the lack of samples from other countries created an artifactual picture of lineage persistence in Vietnam during 2007–2008, we assembled a sequence set of all 672 viruses from Asia and Australia/New Zealand from 2006 through 2008. The maximum clade credibility tree of these sequences (online Technical Appendix Figure 1) indicates that the Vietnam lineages separate into >10 distinct lineages when viewed in the context of all Asian/Australia/New Zealand influenza viruses. One of these lineages persisted in Vietnam for 15 months (online Technical Appendix Figure 1 panel A), and another persisted for 10–12 months (online Technical Appendix Figure 1, panel B), suggesting that lineage persistence of >1 year may have occurred in Vietnam during 2007–2008. However, this type of analysis is very sensitive to phylogenetic uncertainty because the individual lineages (or subclades) contain few sequences and may not be robust to small changes in tree topology.

Discussion

According to our analysis, the major migratory routes of influenza virus pass through the United States, eastern Asia, and Australia/New Zealand. Europe—despite its population density and consistency of wintertime influenza epidemics—was slightly less connected to other parts of the world when compared with the United States. These results are consistent with those of previous studies that showed eastern Asia (2) and tropical Asia (1) as key influenza source populations and the United States as a major contributing region (3). The new sequence data in this analysis support strong migratory connections between Vietnam and neighboring countries, the United States, and Europe. Our regional phylogenetic analysis supports a strong connection between Vietnam and Australia/New Zealand, but the global analysis reveals that Australia/New Zealand sequences are more closely related to sequences from Asian countries other than Vietnam. In addition, the inferred phylogenies provide evidence of virus persistence in Vietnam for >1 year. This is a major finding because strong migratory links and persistence are the 2 key features for a proposed source region for influenza transmission; long-term persistence in tropical regions may be associated with more antigenic evolution and immune escape if it can be shown that longer persistence gives the virus population more time to accumulate and fix antigenic changes (2,31,32).

In general, persistence analyses are difficult even with regular sequence sampling and weekly virologic confirmations. When attempting to assess the likelihood of influenza persistence in a focal region (e.g., Vietnam), we must sample outside the focal region to determine whether local viruses have been reintroduced from elsewhere. However, the more sampling in the nonfocal region, the more likely it becomes that we sample nonfocal viruses similar to focal viruses and that more diversity is detected in the nonfocal region, making it seem basal (closer to the root) to the focal region. There are no clear criteria for whether we have undersampled or oversampled the focal or the nonfocal region; thus, it is extremely difficult to state with certainty that an apparently local lineage has persisted in the same location. For the 2007–2008 Vietnam influenza sequences, viruses were sampled for most of this period and coalescence times were generally short, indicating that most of these viruses have a relatively recent ancestor in Vietnam. These data are consistent with and provide evidence for lineage persistence in Vietnam during this time. However, we know of no unbiased test that can reject the possibility of virus introduction. The perfect dataset for demonstrating lineage persistence would seem to be 52 viruses sampled in 52 weeks, with consecutive viruses differing at 0 or 1 nt positions.

A major limitation of all migration analyses performed with sequence data is geographic sampling bias: undersampling and oversampling. The more sequences that are available for a given location, the more likely it is that 1 of these sequences will be a recent immigrant, identifiable by the presence of similar sequences from other locations. To overcome this bias, subsampling is typically conducted (3,5) to ensure that the same numbers of sequences are used...
from each region. In the situation when too few sequences are available from a particular location, a smaller number of migratory links will be able to be inferred for that location. This second bias cannot be corrected with a subsampling strategy.

Our analysis of the global subsampled dataset showed that sample counts and strength of migratory connections were highly correlated. It has so far been impossible to determine the causal direction in this correlation. A migration signal can be weak because of a dearth of samples. Conversely, the small number of samples can be the result of low influenza activity and a corresponding weak migratory connection with other regions. The directionality of causation cannot be determined from sequence data alone. A sequence sampling strategy must be devised in the context of an influenza surveillance system, and the epidemiologic data and sequence data must be analyzed jointly. Disease prevalence and sequence data should be directly linked to provide a denominator to help determine whether undersampling or oversampling are truly occurring, which would allow for correction of sampling numbers across regions.

Despite this seemingly obvious point about oversampling, the counterpoint is that oversampling in influenza sequence data occurs with a high degree of pseudoreplication. Influenza sequence sampling in most scientific studies and public health contexts is conducted in such a way that each additional sequence sample is not an independent observation but, rather, is an observation with a high degree of correlation to recently collected samples (33). These pseudoreplicated samples should not, in principle, generate additional artificial migration events into the analysis because the dependency structure of the samples is entirely accounted for in the phylogeny. Nevertheless, a correlation between sample number and migration strength persists in the data, partially, at least, because a larger number of samples increases the probability that a distant recently introduced lineage is sampled.
New approaches are needed in order to fully account for all spatial, evolutionary, and epidemiologic dependencies in phylogeographic analyses. For recent phylogeographic studies, Bayesian approaches have been the method of choice (1,3,4,34–37), primarily because of their ability to account for uncertainty in evolutionary, demographic, and migratory parameters, but especially because of their ability to incorporate topological uncertainty into phylogenetic analyses. If these methods can be further developed to incorporate representativeness uncertainty—essentially, a prior distribution on the size of the sampling pool to account for the fact that some parts of the phylogeny will be oversampled while others will be undersampled—then this type of Bayesian analysis could serve as a powerful auxiliary tool in phylogeography, enabling us to determine whether sampling bias has a larger effect in some regions than others. Another role for Bayesian analysis of influenza sequences will be the application of Bayesian phylogeographic methods on whole-genome sequence data (1). For highly reassortant datasets, the presence of independent migration signals in 8 phylogenies (for the influenza virus 8 RNA segments) should act to reduce uncertainty for the inferred migration parameters.

We intended to elucidate the migratory pathways of influenza into and out of Vietnam and the likelihood of virus persistence in Vietnam. For each of these objectives, we recommend that future studies link phylogenetic analyses with prevalence data, allowing for correction of known biases and providing crucial complementary epidemiologic evidence for migration and persistence. If the source–sink framework is an oversimplification of global influenza circulation (3–5), Vietnam probably plays both roles on different occasions, given its close connections to other countries in Asia, Europe, and the United States.

Acknowledgments

We thank WHO Vietnam, the US Centers for Disease Control and Prevention, H. Suzuki, and R. Saito for helping establish influenza sentinel surveillance in Vietnam. Thanks to E.C. Holmes for critical review of this manuscript.

H.M.L., P.H., M.F.B. are supported by the Wellcome Trust (089276/B/09/7, 098511/Z/12/Z, WT/093724). T.T.L. is supported by a Royal Society Newton International Fellowship (UK). Computing and hardware costs were funded by the Li Ka Shing, University of Oxford Global Health Programme (LG05). This project has been funded in part with federal funds from the Department of Health and Human Services, National Institutes of Health, National Institute of Allergy and Infectious Diseases, under contract no. HHSN2722009000007C.

Dr Le is head of the Virology Department at the Vietnam National Institute for Hygiene and Epidemiology, Hanoi, Vietnam. Her research interests are virology, public health, and human and avian influenza viruses.

References


Address for correspondence: Maciej F. Boni, Oxford University Clinical Research Unit, Hospital for Tropical Diseases, 764 Vo Van Kiet St, Q5, Ho Chi Minh City, Vietnam; email: mboni@oucru.org

---

**Get the content you want delivered to your inbox.**

Sign up to receive emailed announcements when new podcasts or articles on topics you select are posted on our website.

[www.cdc.gov/ncidod/eid/subscribe.htm](http://www.cdc.gov/ncidod/eid/subscribe.htm)
Data on influenza epidemiology in HIV-infected persons are limited, particularly for sub-Saharan Africa, where HIV infection is widespread. We tested respiratory and blood samples from patients with acute lower respiratory tract infections hospitalized in South Africa during 2009–2011 for viral and pneumococcal infections. Influenza was identified in 9% (1,056/11,925) of patients enrolled; among influenza case-patients, 358 (44%) of the 819 who were tested were infected with HIV. Influenza-associated acute lower respiratory tract infection incidence was 4–8 times greater for HIV-infected (186–228/100,000) than for HIV-uninfected persons (26–54/100,000). Furthermore, multivariable analysis showed HIV-infected patients were more likely to have pneumococcal co-infection; to be infected with influenza type B compared with type A; to be hospitalized for 2–7 days or >7 days; and to die from their illness. These findings indicate that HIV-infected persons are at greater risk for severe illnesses related to influenza and thus should be prioritized for influenza vaccination.

Knowledge is limited about influenza virus–associated illness and death in persons infected with HIV type 1, particularly in sub-Saharan Africa (1,2). In 2009, South Africa had ≈5 million HIV-infected persons, and HIV prevalence among pregnant women was 29% (3,4). Influenza virus circulates seasonally in South Africa, during the Southern Hemisphere winter (5).

Studies from the United States suggest that, in the absence of highly active antiretroviral therapy (HAART), HIV-infected adults have an increased risk of seasonal influenza hospitalization (1), death (6), and prolonged illness compared with the general population. This risk decreased following the widespread introduction of HAART (6,7). In 2011, ≈52% of eligible HIV-infected adults in South Africa were receiving HAART (8), and HAART-naive HIV-infected children had an 8-fold greater risk for influenza-associated pneumonia hospitalization and a trend toward a higher case-fatality rate (CFR) (8% vs. 2% in HIV-uninfected children) (2,9). Adults in South Africa with AIDS had similar influenza-associated death rates to those for adults in the United States with AIDS in the pre-HAART era (6). In Kenya, HIV-infected adults were at increased risk for influenza-associated pneumonia hospitalization compared with HIV-uninfected adults (10,11).

Data from low HIV prevalence countries where most persons evaluated had access to HAART and influenza antivirals suggested that HIV-infected persons were more likely to be hospitalized for influenza A(H1N1)pdm09 compared with the general population, but rates of intensive care and death did not differ (1,12). Nevertheless, high HIV prevalence (53%) was observed among patients who died with confirmed influenza A(H1N1)pdm09 in South Africa (13). We investigated the incidence of hospitalization for
influenza-associated acute lower respiratory tract infection (LRTI) and the clinical course of illness in persons with and without HIV infection in South Africa.

**Methods**

**Surveillance Program**

Beginning in February 2009, active, prospective, hospital-based surveillance (the Severe Acute Respiratory Illness program) was implemented in 3 of the 9 provinces of South Africa: Chris Hani-Baragwanath Hospital (CHBH) in an urban area of Gauteng Province; Edendale Hospital in a peri-urban area of KwaZulu-Natal Province; and Matikwana and Mapulaneng Hospitals in a rural area of Mpumalanga Province. In June 2010, an additional surveillance site was introduced at Klerksdorp and Tshepong Hospitals in a peri-urban area of the Northwest Province (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/11/13-0546-Techapp1.pdf).

**Case Definition**

A case of acute LRTI was defined as a hospitalized person who had illness onset within 7 days of admission and who met age-specific clinical inclusion criteria. We included children ages 2 days through <3 months who had physician-diagnosed sepsis or acute LRTI, children ages 3 months through <5 years with physician-diagnosed LRTI (e.g., bronchitis, bronchiolitis, pneumonia, pleural effusion), and persons ≥5 years of age who met the World Health Organization (WHO) case definition for severe acute respiratory infection (14): sudden onset of fever (≥38°C) or reported fever, cough or sore throat, and shortness of breath or difficulty breathing.

**Study Procedures**

All patients admitted during Monday through Friday were eligible, except for adult patients at CHBH, where enrollment was limited to 2 of every 5 working days (selected days varied systematically) per week because of large patient numbers and limited resources. The overall numbers of persons admissions, cases meeting study definitions, and persons enrolled were recorded. Study staff completed case report forms until discharge and collected nasopharyngeal and throat swabs from patients ≥5 years of age or nasopharyngeal aspirates from patients <5 years of age and blood specimens from consenting patients. Hospital and intensive care unit admission and collection of specimens for bacterial culture, tuberculosis testing, and CD4+ T-cell counts were performed according to attending physician discretion.

**Laboratory Methods**

Respiratory specimens were transported in viral transport medium at 4–8°C to the National Institute for Communicable Diseases within 72 hours of collection. Respiratory specimens were tested by multiplex real-time reverse transcription PCR for 10 respiratory viruses as described and included influenza A and B viruses (15). Influenza-positive specimens were subtyped by using real-time reverse transcription PCR (16). *Streptococcus pneumoniae* was identified by quantitative real-time PCR detecting the *lytA* gene from whole-blood specimens (17).

**Definitions**

Underlying medical conditions were defined as asthma, other chronic lung disease, chronic heart disease, liver disease, renal disease, diabetes mellitus, immunocompromising conditions excluding HIV infection, neurologic disease, or pregnancy. These conditions were considered absent if indicated as such in medical records or if there was no direct reference to the condition. Invasive isolates were defined as bacterial pathogens, excluding likely contaminants, isolated from blood, cerebrospinal fluid, or another sterile site from a specimen taken within 48 hours of hospitalization. Current tuberculosis was defined patients who had laboratory-confirmed diagnosis of tuberculosis or who were receiving or initiated on anti-tuberculosis treatment during the current admission.

**Evaluation of HIV Serostatus**

HIV infection status was determined from results of testing undertaken as part of standard-of-care or through anonymized linked dried blood spot specimen testing, by HIV PCR for children <18 months of age and by ELISA for persons ≥18 months of age (18). CD4+ T-cell counts were determined by flow cytometry (19). Patients were categorized into 2 immunosuppression categories (1): mild immunosuppression (CD4+ T-lymphocytes ≥200/mm³ or equivalent age-appropriate CD4+ percentage for children <5 years of age), or (2) severe immunosuppression (CD4+ T-lymphocytes <200/mm³ or equivalent age-appropriate CD4+ percentage for children <5 years of age) (20).

**Calculation of Incidence**

Calculation of incidence was conducted at CHBH, the only site for which population denominator data were available. This hospital is the only public hospital serving a community of ≈1.3 million black African persons in 2011, of whom ≈10% have private medical insurance (21). Most (>80%) uninsured persons and ≈10% of insured persons seek care at public hospitals; consequently, most persons requiring hospitalization from this community are admitted to CHBH. We estimated the incidence of influenza hospitalizations per 100,000 persons by using the number of acute LRTI hospitalizations for which the patient tested positive for influenza virus, adjusting for nonenrollment (i.e., refusal to participate, nonenrollment
during weekends, nonenrollment in 3 of 5 adult wards) by age groups and HIV status divided by the midyear total population estimates (22) for each year, multiplied by 100,000. HIV prevalence in the study population was estimated from the projections of the Actuarial Society of South Africa AIDS and Demographic model (3). We assumed that the HIV prevalence by age group and influenza subtype among patients not tested for HIV was the same as that among those tested. For 14 patients for whom influenza A virus subtyping was not performed, we imputed the influenza subtype on the basis of date of specimen collection and circulating influenza subtypes.

CI s for incidence estimates were calculated by using Poisson distribution. Age-specific and overall age-adjusted risk of influenza hospitalization in HIV-infected and -uninfected persons was determined by using log-binomial regression. To explore the possible effect of missing data on estimates of HIV-specific incidence, a sensitivity analysis was conducted in which all cases not tested for HIV were assumed to be HIV uninfected.

Analysis of Risk Factors for HIV-Positive Serostatus

Univariate and multivariable analyses were performed in Stata version 9 (StataCorp LP, College Station, TX, USA). Multivariable logistic regression models were evaluated starting with all variables that were significant at p<0.1 on univariate analysis and dropping nonsignificant factors with stepwise backward selection. All 2-way interactions were evaluated. Two-sided p values <0.05 were considered significant. For each univariate analysis, we used all available case information. For the multivariable model, patients with missing data for included variables were dropped. Age group, duration of hospitalization, and year were defined as categorical variables in multiple levels. All other variables were defined as the presence or absence of the attribute, excluding missing data. To explore possible bias, patients tested for HIV were compared with those not tested.

Results

Demographics, Clinical Characteristics, and Seasonality of Influenza-associated Acute LRTI

During February 2009–December 2011, a total of 14,725 persons who fulfilled the LRTI case definition were approached for study enrollment; 2,562 (17%) were not enrolled. The most common reasons for nonenrollment were study refusal (n = 779, 30%), unavailable legal guardian (n = 758, 30%), and patients being confused or too ill to consent (n = 242, 9%). Of 12,163 patients enrolled, 11,925 (98%) were tested for influenza; 1,056 (9%) had positive results (online Technical Appendix Figure 2). The influenza detection rate varied by age group: 7% (266/4,046) for those <1 year of age, 11% (252/2,292) for those 1–4 years of age, 12% (111/934) for those 5–24 years of age, 9% (270/2,930) for those 25–44 years of age, 9% (119/1,395) for those 45–64 years of age, and 12% (38/328) for those ≥65 years of age (p<0.001). The overall influenza detection rate was similar among HIV-infected (358/4,208 [9%]) and HIV-uninfected (461/4,473 [10%]) persons (p = 0.163). Most patients (8,961/12,163 [74%]) were enrolled at CHBH.

In 2009, influenza circulation in South Africa was biphasic, with a peak of influenza A(H3N2) infections (190/386 [49%] of annual cases), followed by a second peak of influenza A(H1N1)pdm2009 infections (158/386 [41%] of annual cases). In 2010, influenza B was the predominant subtype (172/289 [60%] of annual cases). In 2011, there were again 2 influenza peaks; influenza A(H1N1)pdm09 predominated (152/381 [40%] of annual cases) initially, followed by influenza B and A(H3N2) (129/381 [34%] and 100/381 [26%] of annual cases, respectively) (Figure 1).

Of the 1,056 patients who had positive test results for influenza, 819 (78%) had an available HIV infection status result (597 [73%] by anonymized HIV testing; 83 [10%] tested by ward clinicians; 139 [17%] by anonymous and clinician testing) (online Technical Appendix Figure 2). Age-specific HIV prevalence findings were not substantially different when only patients tested through anonymized unlinked testing were included (data not shown). The proportion of influenza-positive patients with available HIV results increased during the study period, from 62% (239/386) in 2009 to 89% (339/381) in 2011 (p<0.001), and increased with increasing age, from 65% (335/518) among children <5 years of age to 90% (484/538) among persons ≥5 years of age (p<0.001). When we compared patients tested for HIV to those not tested for HIV, controlling for year of test and age group, no differences in patient epidemiologic characteristics or CFRs were seen (data not shown). The proportion of patients tested for HIV and the HIV prevalence among tested patients did not differ between surveillance sites (data not shown). The overall HIV prevalence among influenza-positive case-patients was 44% (358/819) and varied by age group: 10% (16/164) for those <1 year of age, 17% (29/171) for those 1–4 years of age, 46% (38/82) for those 5–24 years of age, 84% (212/251) for those 25–44 years of age, 54% (61/113) for those 45–64 years of age, and 5% (2/38) for those ≥65 years of age (p<0.001).

Among patients who had positive influenza test results, 10% (106/1,056) had tuberculosis co-infection, 7% (63/889) had pneumococcal co-infection, and 7% (78/1,056) had another underlying medical condition. Among 106 patients classified as having tuberculosis, only 31 (29%) were laboratory confirmed. Three pregnant women identified in this surveillance tested influenza positive; all were HIV infected. No influenza-positive patient
Influenza and HIV, South Africa

reported receiving influenza vaccine or oseltamivir treatment. Forty-eight HIV-infected and 116 HIV-uninfected patients with influenza had sterile site specimens submitted for bacterial culture; test results were positive for 3 HIV-infected patients (2 *S. pneumoniae* and 1 *Haemophilus influenzae*) and 2 HIV-uninfected patients (1 *Neisseria meningitidis* and 1 *S. pneumoniae*).

**Incidence of Influenza Hospitalization in HIV-Infected and -Uninfected Patients**

The incidence of hospitalization for influenza-associated acute LRTI among patients at CHBH was highest for patients ages 0–4 years in all study years and for all influenza subtypes, with the highest incidence for those <1 year of age (Table 1; Figure 2). Smaller peaks in incidence were observed in the adult (25–54 years) and elderly (≥65 years) age groups each year (Figure 2). HIV-infected persons experienced a 4–8 times greater incidence of influenza-associated acute LRTI (age-adjusted relative risk [aRR] 4.2 [95% CI 3.6–4.8] in 2009, aRR 7.5 [95% CI 6.4–8.8] in 2010, and aRR 5.5 [4.7–6.3] in 2011) (Table 1). The incidence of hospitalization among HIV-infected persons compared with HIV-uninfected persons was 3–5 times greater for influenza A(H3N2) (aRR 3.3 [95% CI 2.7–4.0] in 2009 and aRR 4.9 [3.5–6.5] in 2011), 4–6 times greater for influenza A(H1N1)pdm09 (aRR 4.4 [95% CI 3.6–5.4] in 2009 and 5.6 [95% CI 4.4–7.1] in 2011), and 9 times greater for influenza B [aRR 8.7 (13.2–38.5) in 2010 and 8.7 [4.4–7.2] in 2011] (online Technical Appendix Table 1). The relative risk for hospitalization for influenza-associated acute LRTI among HIV-infected persons was elevated in all age groups (generally highest in age group
Table 1. Incidence of laboratory-confirmed influenza-associated lower respiratory tract infection hospitalizations per 100,000 population by year and HIV status at Chris Hani-Baragwanath Hospital, South Africa*

<table>
<thead>
<tr>
<th>Year and patient age range, y</th>
<th>No. HIV-positive/no. tested (%)</th>
<th>% HIV prevalence</th>
<th>Incidence rate (95% CI)</th>
<th>Relative risk (95% CI)</th>
<th>HIV infected vs. uninfected</th>
<th>Sensitivity analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All patients</td>
<td>HIV infected patients</td>
<td>HIV uninfected patients</td>
<td>HIV infected vs. uninfected</td>
<td>HIV infected vs. uninfected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIV infected vs. uninfected</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIV infected vs. uninfected</td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td>103/188 (55)</td>
<td>11</td>
<td>336 (304–370)</td>
<td>766 (553–1,021)</td>
<td>314 (284–349)</td>
<td>2.4 (1.7–3.3)</td>
</tr>
<tr>
<td>5–24</td>
<td>18/29 (62)</td>
<td>39</td>
<td>27 (23–33)</td>
<td>194 (142–261)</td>
<td>17 (14–22)</td>
<td>11.0 (7.4–16.1)</td>
</tr>
<tr>
<td>25–44</td>
<td>41/44 (93)</td>
<td>88</td>
<td>59 (52–67)</td>
<td>198 (173–227)</td>
<td>9 (7–14)</td>
<td>20.3 (13.8–31.3)</td>
</tr>
<tr>
<td>≥45</td>
<td>27/27 (100)</td>
<td>41</td>
<td>67 (57–78)</td>
<td>260 (201–331)</td>
<td>44 (36–54)</td>
<td>5.9 (4.2–8.2)</td>
</tr>
<tr>
<td>Total</td>
<td>189/288 (66)</td>
<td>34</td>
<td>78 (73–83)</td>
<td>228 (206–254)</td>
<td>54 (50–60)</td>
<td>4.2 (3.6–4.8)</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIV infected vs. uninfected</td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td>54/84 (64)</td>
<td>9</td>
<td>153 (131–177)</td>
<td>317 (187–514)</td>
<td>145 (124–170)</td>
<td>2.2 (1.3–3.6)</td>
</tr>
<tr>
<td>5–24</td>
<td>15/22 (68)</td>
<td>33</td>
<td>14 (11–18)</td>
<td>89 (57–135)</td>
<td>10 (7–13)</td>
<td>8.8 (5.2–15.2)</td>
</tr>
<tr>
<td>25–44</td>
<td>73/78 (94)</td>
<td>89</td>
<td>60 (53–68)</td>
<td>203 (178–231)</td>
<td>9 (6–13)</td>
<td>22.9 (15.4–34.7)</td>
</tr>
<tr>
<td>≥45</td>
<td>38–39 (97)</td>
<td>55</td>
<td>47 (40–56)</td>
<td>243 (191–307)</td>
<td>24 (18–31)</td>
<td>10.3 (7.2–14.8)</td>
</tr>
<tr>
<td>Total</td>
<td>180/223 (81)</td>
<td>53</td>
<td>49 (45–53)</td>
<td>197 (176–219)</td>
<td>26 (23–29)</td>
<td>7.5 (6.4–8.8)‡</td>
</tr>
<tr>
<td>2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIV infected vs. uninfected</td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td>81/96 (84)</td>
<td>6</td>
<td>186 (162–212)</td>
<td>273 (151–463)</td>
<td>182 (159–209)</td>
<td>1.5 (0.8–2.6)</td>
</tr>
<tr>
<td>5–24</td>
<td>13/13 (100)</td>
<td>46</td>
<td>8 (6–11)</td>
<td>71 (42–111)</td>
<td>5 (3–7)</td>
<td>15.4 (7.7–30.3)</td>
</tr>
<tr>
<td>25–44</td>
<td>88/89 (99)</td>
<td>80</td>
<td>68 (61–76)</td>
<td>206 (180–234)</td>
<td>19 (15–24)</td>
<td>10.9 (8.2–14.7)</td>
</tr>
<tr>
<td>≥45</td>
<td>42/43 (98)</td>
<td>38</td>
<td>54 (48–65)</td>
<td>192 (146–247)</td>
<td>39 (32–48)</td>
<td>4.9 (3.5–6.9)</td>
</tr>
<tr>
<td>Total</td>
<td>224/241 (93)</td>
<td>43</td>
<td>56 (50–58)</td>
<td>186 (167–207)</td>
<td>34 (31–37)</td>
<td>5.5 (4.7–6.3)‡</td>
</tr>
</tbody>
</table>

*Boldface indicates significance.
†Assuming that all patients not tested for HIV are HIV negative.
‡Age-adjusted.

Among influenza virus–positive case-patients, the CFR was 4 times greater for HIV-infected (19/356, 5%) than for HIV-uninfected (6/461, 1%) persons (p = 0.002). In each age group except for the elderly, CFRs were significantly higher for HIV-infected compared with HIV-uninfected persons: 7% (36/509) vs. 1% (34/3,630) for ages 0–4 years (relative risk [RR] 7.6, 95% CI 4.7–12.1); 6% (28/433) vs. 1% (3/298) for ages 5–24 years (RR 6.4, 95% CI 2.0–21.1); 7% (164/2,381) vs. 3% (8/308) for ages 25–44 years (RR 2.7, 95% CI 1.3–5.4); 12% (100/833) vs. 7% (34/456) for ages 45–64 years (RR 1.6, 95% CI 1.1–2.4); and 4% (2/50) vs. 9% (23/246) for age ≥65 years (RR 0.4, 95% CI 0.1–1.8).

Results from multivariable analysis indicate that, among patients with influenza-associated hospitalization, those with HIV infection (compared with those without HIV infection) were more likely to be age group 5–24 years (odds ratio [OR] 4.4, 95% CI 2.4–8.2), 25–44 years (OR 24.2, 95% CI 14.1–41.7), or 45–64 years (OR 6.2, 95% CI 3.4–11.3); female sex (OR 1.9, 95% CI 1.2–2.8); black African race (OR 4.0, 95% CI 1.1–14.6); co-infected with pneumococcus (OR 2.3, 95% CI 1.0–5.0); infected with influenza type B (vs. type A) (OR 1.6 95% CI 1.0–2.4); hospitalized for 2–7 days (OR 2.8 95% CI 1.5–5.5) or >7 days (OR 4.5, 95% CI 2.1–9.5); and more likely to die (OR 3.9, 95% CI 1.1–14.1) (Table 2). In contrast, those with HIV infection were less likely than those without HIV infection to have underlying medical conditions other than HIV (OR 0.4, 95% CI 0.2–0.8).

A total of 118 (33%) HIV-infected patients had available CD4+ T-cell count data; 7 were <5 years of age. Most (60%, 70/118) had severe immunosuppression (CD4+ T cell counts <200/mm³ or age-specific equivalent). CFRs were not significantly different between patients with (13%, 9/70) and without (6%, 3/47; p = 0.258) severe immunosuppression, although numbers were small. The duration of hospitalization was longer for those with severe immunosuppression (median 7 days, interquartile range 2–11 days) than for those without (median 5 days, interquartile range 1–7 days; p = 0.02). Of those with available data, 51% (111/218) reported currently receiving HAART and 25% (60/241) reported receiving prophylaxis with trimethoprim/sulfamethoxazole. CFRs were similar for patients receiving (7/113, 6%) and not receiving (8/107, 7%) HAART (p = 0.706).

Discussion

We have shown that HIV-infected persons experienced elevated incidence of hospitalization, prolonged hospitalization, and increased risk of in-hospital death resulting from influenza. In contrast to most other countries...
Table 2. Comparison of the clinical and epidemiologic characteristics of HIV-infected and uninfected patients hospitalized with influenza-associated acute LRTI at 4 sentinel surveillance sites, South Africa 2009–2011*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-infected patients†</th>
<th>HIV-uninfected patients†</th>
<th>Univariate analysis OR (95% CI)</th>
<th>p value</th>
<th>Multivariable analysis OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age group, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>45/358 (13)</td>
<td>290/461 (63)</td>
<td>Referent</td>
<td>0.001</td>
<td>Referent</td>
<td>0.001</td>
</tr>
<tr>
<td>5–24</td>
<td>38/358 (11)</td>
<td>44/461 (10)</td>
<td>5.6 (3.3–9.5)</td>
<td>4.4 (2.4–8.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25–44</td>
<td>312/358 (59)</td>
<td>39/461 (8)</td>
<td>35.0 (22.0–55.7)</td>
<td>24.2 (14.1–41.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45–64</td>
<td>61/358 (17)</td>
<td>52/461 (11)</td>
<td>7.6 (4.7–12.3)</td>
<td>6.2 (3.4–11.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;65</td>
<td>2/358 (1)</td>
<td>36/461 (8)</td>
<td>0.4 (0.1–1.5)</td>
<td>0.2 (0.04–0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female sex</td>
<td>253/358 (71)</td>
<td>224/461 (49)</td>
<td>2.5 (1.9–3.4)</td>
<td>&lt;0.001</td>
<td>1.9 (1.2–2.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>Black African race</td>
<td>353/358 (99)</td>
<td>445/461 (97)</td>
<td>2.4 (0.9–6.6)</td>
<td>0.096</td>
<td>4.0 (1.1–14.6)</td>
<td>0.036</td>
</tr>
<tr>
<td>Year of hospitalization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>88/358 (25)</td>
<td>151/461 (33)</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>127/358 (35)</td>
<td>114/461 (25)</td>
<td>1.9 (1.3–2.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011</td>
<td>143/358 (40)</td>
<td>196/461 (43)</td>
<td>1.3 (0.9–1.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-infections and underlying medical conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underlying condition excluding tuberculosis and HIV‡</td>
<td>25/358 (7)</td>
<td>47/461 (10)</td>
<td>0.7 (0.4–1.1)</td>
<td>0.109</td>
<td>0.4 (0.2–0.8)</td>
<td>0.008</td>
</tr>
<tr>
<td>Smoking§</td>
<td>32/299 (11)</td>
<td>24/151 (16)</td>
<td>0.6 (0.4–1.1)</td>
<td>0.117</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consumed alcohol§</td>
<td>28/299 (9)</td>
<td>26/151 (17)</td>
<td>0.5 (0.3–0.9)</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Underlying tuberculosis</td>
<td>60/357 (17)</td>
<td>19/461 (4)</td>
<td>4.7 (2.7–8.0)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumococcal co-infection on PCR¶</td>
<td>37/345 (11)</td>
<td>17/389 (4)</td>
<td>2.7 (1.5–5.0)</td>
<td>&lt;0.001</td>
<td>2.3 (1.0–5.0)</td>
<td>0.043</td>
</tr>
<tr>
<td>Viral respiratory co-infection#</td>
<td>82/358 (23)</td>
<td>152/461 (33)</td>
<td>0.6 (0.4–0.8)</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza type B (vs. A)</td>
<td>148/358 (41)</td>
<td>133/461 (29)</td>
<td>1.7 (1.3–2.3)</td>
<td>&lt;0.001</td>
<td>1.6 (1.0–2.4)</td>
<td>0.035</td>
</tr>
<tr>
<td>Received ≥2 doses of pneumococcal conjugate vaccine**</td>
<td>3/39 (8)</td>
<td>53/242 (22)</td>
<td>0.3 (0.1–1.0)</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical findings and treatment course</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptoms ≥2 d before admission</td>
<td>296/358 (83)</td>
<td>295/461 (64)</td>
<td>2.7 (1.9–3.7)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admission to intensive care</td>
<td>0/357 (0)</td>
<td>6/461 (1)</td>
<td>Undefined</td>
<td>0.031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>2/357 (1)</td>
<td>4/461 (1)</td>
<td>0.6 (0.1–3.5)</td>
<td>0.612</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen required</td>
<td>142/357 (40)</td>
<td>141/461 (31)</td>
<td>1.5 (1.1–2.0)</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimicrobial drugs prescribed at admission</td>
<td>351/358 (98)</td>
<td>438/460 (95)</td>
<td>2.5 (1.1–6.0)</td>
<td>0.036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of hospitalization, d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>20/352 (6)</td>
<td>149/460 (32)</td>
<td>Referent</td>
<td>0.001</td>
<td>Referent</td>
<td>0.001</td>
</tr>
<tr>
<td>2–7</td>
<td>217/352 (62)</td>
<td>241/460 (52)</td>
<td>6.7 (4.1–11.1)</td>
<td>2.8 (1.5–5.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;7</td>
<td>115/352 (33)</td>
<td>70/460 (15)</td>
<td>12.2 (7.0–21.3)</td>
<td>4.5 (2.1–9.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median duration of hospitalization, d (range)</td>
<td>6 (4–8)</td>
<td>3 (1–6)</td>
<td>1.1 (1.05–1.13)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case-fatality rate</td>
<td>19/356 (5)</td>
<td>6/461 (1)</td>
<td>4.3 (1.7–10.8)</td>
<td>0.002</td>
<td>3.9 (1.1–14.1)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

*ORs and p values are shown for all variables included in the multivariable model. LRTI, lower respiratory tract infection; OR, odds ratio.
†Values are patients/total no. in category (%) except as indicated. Some data are missing or were not recorded.
‡Asthma, other chronic lung disease, chronic heart disease (valvular heart disease, coronary artery disease, or heart failure excluding hypertension), liver disease (cirrhosis or liver failure), renal disease (nephrotic syndrome, chronic renal failure), diabetes mellitus, immunocompromising conditions excluding HIV infection (organ transplant, immunosuppressive therapy, immunoglobulin deficiency, malignancy), neurologic disease (cerebrovascular accident, spinal cord injury), seizures, neuromuscular conditions) or pregnancy. Coexisting illnesses were considered absent in cases for which the medical records stated that the patient had no underlying medical condition or when there was no direct reference to that condition.
§Question asked of patients >12 y of age only.
¶Co-infection with influenza and ≥1 of the following: parainfluenza virus 1, 2, or 3; respiratory syncytial virus; enterovirus; human metapneumovirus; adenovirus; rhinovirus.
**Verified only for children <5 y of age.

(12), HIV infection (>40%) was the most common underlying risk factor for influenza-associated LRTI hospitalization in South Africa. This factor resulted in a W-shaped age-distribution of influenza hospitalizations, with peaks among young children and the elderly and an additional peak among young adults associated with HIV infection. These findings highlight the need to target HIV-infected persons for influenza vaccination.

Bacterial co-infections may have contributed to some of the influenza-associated LRTI hospitalizations and deaths in the HIV-infected group, among whom we observed an elevated risk of pneumococcal co-infection. An elevated risk of hospitalization for invasive pneumococcal disease has been documented in HIV-infected persons (23), and a synergistic relationship exists between influenza and pneumococcus (24,25). Whereas real-time PCR is more sensitive than blood culture for diagnosing pneumococcal pneumonia, additional cases of pneumococcal co-infection may still have been missed (23,26). Pneumococcal DNA in the blood may reflect occult bacteremia in some persons (27,28).

HIV-infected persons with influenza-associated acute LRTI were more likely to have underlying tuberculosis,
although not all tuberculosis cases were laboratory-confirmed. Tuberculosis was also common in a South African case-series of influenza A(H1N1)pdm09 deaths (13). An association between tuberculosis and influenza-associated death has been suggested (11,29) but warrants further corroboration.

The observed prevalence of underlying medical conditions was lower for HIV-infected (7%) than HIV-uninfected persons (10%) and lower than has been observed in the United States, where 68% of HIV-infected and 74% of hospitalized HIV-uninfected adults had influenza A(H1N1)pdm09 (12). This discrepancy could be because our documentation was incomplete or may reflect a true difference in the relative contribution of underlying risk conditions in our setting.

The increased risk for hospitalization for influenza-associated acute LRTI among HIV-infected persons appeared to be greater for influenza B (≈8-fold) than influenza A (≈3–4-fold). Reasons for this are unclear. Influenza B severity is intermediate, falling between those for influenza A(H3N2) and A(H1N1). Bacterial superinfection may contribute to death in patients (particularly adults) with influenza B, and severe and fatal disease due to influenza B has been described in previously healthy persons (30).

Influenza vaccination is safe and efficacious in HIV-infected adults in Africa (31,32), whereas the efficacy among HIV-infected persons is unclear (33). No patients reported receiving influenza vaccination or antiviral treatment, despite national recommendations for influenza vaccination of risk groups and for antiviral treatment for influenza infection in persons with severe illness or underlying risk conditions (34). Influenza vaccine (170,000–1,000,000 doses for a population of ≈50 million each year) and oseltamivir treatment are made available free of charge through the public health sector in South Africa, although challenges in procurement and distribution may limit access. The low uptake of oseltamivir may be because clinicians doubt its effectiveness when patients delay seeking health care; >80% of HIV-infected persons reported symptoms for >48 hours before admission. The effectiveness of antiviral treatment for influenza-associated LRTI hospitalization in settings similar to ours needs to be evaluated. An additional contributing factor to the low use of oseltamivir could be a low index of suspicion for influenza as an etiologic agent in HIV-infected persons with LRTI, because they are also at risk for respiratory disease from other pathogens, such as pneumococcus, Pneumocystis jirovecii, and tuberculosis (12). Maternal immunization against influenza has been suggested as a strategy to reduce the high rates of influenza infection among infants <6 months of age (35), but the effectiveness of this intervention in settings with a high prevalence of maternal HIV infection is unknown.

Our study has several limitations. The low rate of HIV testing among children may have introduced bias if their characteristics differed from those who were tested. Surveillance programs such as ours may underestimate the true number of deaths because severely ill patients may be less amenable to study inclusion or may die before or shortly after hospital admission. Our estimates of incidence also assumed that all persons in Soweto access care at CHBH hospital. Therefore, our estimates likely represent minimum rates. Nevertheless, the estimates of relative risk by HIV status should be robust, unless patients had differential access to care by HIV-infection status (12). Incidence data were derived from a temperate urban area and may not be representative of more subtropical rural areas, but incidence among HIV-uninfected persons was similar to that described for other developing countries (36,37). This analysis included the years after the introduction of influenza A(H1N1)pdm09, and thus we cannot comment on age-specific influenza incidence before this period. Several studies have suggested that pregnancy is a major risk factor for severe disease and death associated with influenza virus infection (38,39). Few pregnant women were enrolled in our study; these patients may have been missed because review of admissions to maternity wards was not always consistent. The case definition of physician-diagnosed acute LRTI in children ages 3 months–<5 years relied on subjective clinician assessment and did not include fever as a criterion because acute LRTI may be afebrile and fever reporting may be subjective in this age group. CD4+ cell count data were only available for one third of HIV-infected patients, and CD4+ cell counts among tested patients may have differed from those in untested patients.

In conclusion, we have demonstrated that, in a high HIV-prevalence setting, HIV infection is a major risk factor for influenza hospitalization and severe disease. Further studies are warranted on the effectiveness of influenza vaccine among HIV-infected children and HIV-infected adults with advanced immunosuppression or tuberculosis co-infection.

to Avian and Pandemic Influenza in South Africa funds from the US Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Cooperative Agreement No. U51/IP000155-04).

Dr C. Cohen is a medical epidemiologist and co-head of the Centre for Respiratory Diseases and Meningitis at the National Institute for Communicable Diseases in South Africa. Her research interests include the epidemiology of respiratory diseases and meningitis and vaccine-preventable diseases.

References


Address for correspondence: Cheryl Cohen, Centre for Respiratory Diseases and Meningitis, National Institute for Communicable Diseases, Private Bag X4, Sandringham, 2131, Gauteng, South Africa; email: cherylc@nicd.ac.za
This study aimed to compare the epidemiology of *Rickettsia felis* infection and malaria in France, North Africa, and sub-Saharan Africa and to identify a common vector. Blood specimens from 3,122 febrile patients and from 500 nonfebrile persons were analyzed for *R. felis* and *Plasmodium* spp. We observed a significant linear trend (p<0.0001) of increasing risk for *R. felis* infection. The risks were lowest in France, Tunisia, and Algeria (1%), and highest in rural Senegal (15%). Co-infections with *R. felis* and *Plasmodium* spp. and occurrences of *R. felis* relapses or reinfections were identified. This study demonstrates a correlation between malaria and *R. felis* infection regarding geographic distribution, seasonality, asymptomatic infections, and a potential vector. *R. felis* infection should be suspected in these geographical areas where malaria is endemic. Doxycycline chemoprophylaxis against malaria in travelers to sub-Saharan Africa also protects against rickettioses; thus, empirical treatment strategies for febrile illness for travelers and residents in sub-Saharan Africa may require reevaluation.

**Common Epidemiology of**

*Rickettsia felis* Infection and Malaria, Africa


Investigations examining the etiologic spectrum of fever of unknown origin in Africa rapidly progressed during 2008–2011 (1–3), providing increased knowledge about bacterial infections. Bacterial agents that have been most frequently identified in North and sub-Saharan Africa by culture are non-typhoidal *Salmonella*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium tuberculosis* (2). Several studies have assessed the effect of fastidious bacterial infections in systemic febrile illness, including *Rickettsia felis* (4–6), *Coxiella burnetii* (7), *Tropheryma whippelii* (3), and *Borrelia* spp. (1,8). Tourism, immigration, international business travel, international aid work, and the deployment of troops overseas were documented as contributors to a tremendous increase in international travel during 1996–2004 (9). International tourist arrivals reached 940 million worldwide during 2010, an increase of 6.6% over 2009, and the current total number of international migrants has increased to an estimated 214 million persons in 2012 (10). Consequently, physicians in the Western hemisphere increasingly encounter febrile patients returning from international travel who were exposed to tropical infections that the physicians are unfamiliar with (9,10). Among international travelers, malaria, dengue, and rickettsiosis are among the most identified etiologies of febrile illness, and exposure to mosquitoes is reported as the most common source of fever (11).

*Rickettsia felis*, an obligate intracellular Gram-negative bacterium belonging to the spotted fever group of *Rickettsia*, has been shown to be a common agent of bloodstream infections in among humans Senegal and Kenya, identified in 7% of the population evaluated (4–6). However, the epidemiology (including vectors and reservoirs)
and clinical picture of this emerging infection in the rest of Africa is largely unknown (12,13). During 2011, a possibly primary infection with *R. felis*, named “yaaf,” was hypothesized in the case of an 8-month-old girl in Senegal with polymorphous skin lesions (12).

The considerable frequency of *R. felis* infections observed in febrile patients in malaria-endemic regions and the many relapses previously reported (4,5) led us to investigate the possible correlation of *R. felis* and that of the parasite, *Plasmodium falciparum*, a known vector of malaria. The reservoirs for malaria and many rickettsial species are mammals, including humans; humans have long been known to be a reservoir for malaria, and were documented as the reservoir for *R. prowazekii*, the agent of epidemic typhus (14). Vectors for both organisms are arthropods: for rickettsial diseases vectors are typically ticks, lice or mites, and infected humans are susceptible to relapse (such as epidemic and scrub typhus) (14).

The vectors for malaria are mosquitoes of the genus *Anopheles* that breed in warm and humid areas (15). Malaria is particularly common among young patients, because progressive immunity develops following multiple infections as the child grows older. Great apes in Cameroon were recently identified as targets or possibly the origin of malaria (16). *R. felis* has recently been detected in *Anopheles gambiae* mosquitoes in molecular form S, in *Aedes albopictus* mosquitoes, and in gorilla fecal samples (17–19). These elements suggest comparable features within the epidemiologic cycles of malaria and *R. felis* infection. In addition, co-infections by *R. felis* and *P. falciparum* have been reported in Kenya (5). To prove the hypothesis of the similar epidemiology of malaria and *R. felis* infection, target populations, clinical phenomena (relapses and bacteremia in apparently asymptomatic patients), and geographic and seasonal distribution should be compared. The objective of this work is to clarify the epidemiology of *R. felis* infection and to compare it with malarial epidemiology.

**Materials and Methods**

**Study Areas and Participants**

**Febrile Patients**

During June 2010–March 2012, a cohort of 2,075 patients (67% <15 years of age; sex ratio, 1:1) from 14 health centers distributed throughout rural Senegal (Senegal study sites S1–S6) were enrolled in this study. The study sites spanned various ecosystems, from dry regions in the north (Dielmo, Senegal study region 1–S1, Ndiop–S2, Keur Momar Sarr–S3, and Niakhar–S4) to humid regions in the south (Basse-Casamance–S5 and Kedougou–S6) that had a rainy season during June through October (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/19/11/13-0361-Techapp1.pdf). In addition, patients from various medical facilities were included: 100 from rural Mali dispensaries; Diankabou-Mali study site M1 and Kole-Mali study site M2; 50 from Franceville, in urban Gabon (pediatric consultation); 183 from Sfax, Tunisia (infectious diseases and pediatric departments); 266 from Oran, Algeria (department of infectious diseases); 48 from the Kenitra region, rural Morocco (dispensaries); and 400 from Marseille, France (hospital emergency units) (Figure 1). Questionnaires and informed consent forms were completed upon enrollment in the study. For each febrile patient (axillary temperature >37.5°C), an interview was conducted, a blood sample (200 µl blood containing EDTA) was collected, and a medical examination was performed. The national ethics committees of Senegal, Gabon, and France approved this project (No. 0–00.87MSP/DS/CNERS and No. 001380MSP/DS/CNERS).

**Control Group**

Samples were obtained from 400 afebrile persons (62% >15 years of age) from S1–2 who participated in a longitudinal study of malaria (20) and 100 persons from France who were under the medical care of 1 of the authors (D.R.) for conditions other than malaria.

**Arthropod Collection in Senegal**

Arthropod specimens collected in Senegal consisted of 949 adult mosquitoes from 3 locations (Table 1, 154 mosquito larvae from Mariste, Dakar, 370 ticks from 2 locations, 160 adult bed bugs from 6 locations, and 384 midges from 2 locations. The *Anopheles arabiensis* mosquito larvae were collected from breeding sites in Mariste, Dakar. The pooled larvae were maintained under laboratory conditions until they grew to the adult stage. In sites S1–2, 144 adult ticks (2 *Rhipicephalus* spp., 4 *Argas persicus*, and 138 *Ornithodoros sonrai*) from 55 burrows inside of 16 human dwellings were collected. A total of 226 *Ornithodoros capensis* ticks were manually collected from the nests of great cormorants (*Phalacrocorax carbo*) in Sarpan Island (îles de la Madeleine) near Dakar. Bed bugs were manually captured from the beds of ill persons. The collection of *Culicoides* spp. was performed in S1–2 by using overnight posed CDC light traps with 0.7-mm mesh size. The arthropods were identified at the species level by using morphological characteristics according to identification keys.

**Molecular Analysis**

DNA was extracted by using the 2-stage protocol for a QIAamp kit (QIAGEN, Hilden, Germany) for the S1–6 groups (3,4,7), and a Biorobot EZ1 Workstation (QIAGEN, Courtaboeuf, France) was used to extract DNA from...
samples from S₁₋₂, Algeria, Tunisia, Morocco, and France. In Gabon, the DNA Blood Omega Bio-tek-E.Z.N.A method (Omega Bio-tek, Norcross, GA, USA) was used according to the manufacturer’s protocol. For all locations, DNA was eluted in 100 µL of elution buffer, and 5 µL was used per reaction.

Quantitative real-time reverse transcription PCR (qRT-PCR) was performed by using a 7900HT-thermocycler (Applied Biosystems) with the QuantiTect-Probe PCR Kit (QIAGEN, Courtabeuf, France). Only samples positive for the β-actin gene product were considered reliable (3); thus, 51 and 9 samples from Senegal and Algeria, respectively, were excluded. All samples were screened by using a *Rickettsia* genus-specific qRT-PCR targeting the gltA gene and an *R. felis*-specific qRT-PCR targeting the bioB gene (4). The positive samples were tested by a second *R. felis*-specific qRT-PCR targeting the orfB gene (18). A sample was considered positive when the qRT-PCRs were positive for the 2 different specific genes. Positive samples from arthropods were further tested for plasmid pRFδ (21) and by a newly designed *R. felis*-specific qRT-PCR targeting the vapB1 gene with the primers VapB1.R (5′-AGGCGAAAGCTTTGAC-GTG-3′) and VapB1.F (5′-TGTCTTTTCATGAATT-GATCAGCA-3′) and the probe VapB1.P (6-FAM-5′-AAGGCTTTGTTTCTGCAGGC-3′TAMRA).

Blood smears stained with Giemsa were examined for the samples collected in Gabon. All other samples were tested by using a *Plasmodium*-genus specific qRT-PCR targeting the Cox-1 gene found in all *Plasmodium* species; the primers Psp_15.F (5′-AGGAATTCGACTGGCCTACA-3′) and Psp_16.R (5′-CCAGCGACGCGTATTATCT-3′) and the (6FAM-5′-CGAACGCTTTTAACGCTGACATGG-3′TAMRA) probe were used. The positive samples were subsequently tested by *Plasmodium*-genus specific qRT-PCR targeting 18S rRNA with the primers Plasmo_18S_2_MBF (5′-AGGCAACACAGGTCTGTGA-3′) and Plasmo_18S_2_MBR (5′-GCAATAATCTAATCCATCACG-3′) and the (6FAM-5′-GAACTAGGCTGCACGC-GTGCTACA-TAMRA-3′) probe.

**Statistical Analysis**

Statistical analyses were performed by using the Statcalc module of Epi Info 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA) to calculate the χ² values for the incidence rate trends calculated for each country. PASW Statistics software 17.0 (IBM, SPSS Inc., Armonk, NY, USA) was used to perform Pearson correlation analyses. The relative risk (RR) and the 95% CI of the risk were calculated by using either the Mantel-Haenszel χ² test or Fisher’s exact test. The statistical significance of the χ² values was evaluated at α = 0.05. The attack rates of *R. felis* infection and malaria were calculated for each country, site, sex, and age range. In contrast, the incidence rates of *R. felis* infection and malaria for S₁₋₂ were calculated monthly and yearly from June 13, 2010 through October 13, 2011. The data from a study performed in 2009 (4) were combined with those of this study to determine the frequency of relapses or re-infections of *R. felis* infections in S₁₋₂.
Table 1. Detection of *Rickettsia felis* DNA in mosquitoes, Senegal, March 2010–September 2012

<table>
<thead>
<tr>
<th>Geographic location in Senegal</th>
<th>Period of collection</th>
<th>Collection method</th>
<th>Mosquito species, morphological identification</th>
<th>DNA samples</th>
<th><em>Rickettsia felis</em> detection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferlo, 15°52'N, 15°15'W</td>
<td>Mar 2010</td>
<td>CDC type light trap collections*</td>
<td><em>Aedes luteocephalus</em></td>
<td>203</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Culex quinquefasciatus</em></td>
<td>186</td>
<td>0/186</td>
</tr>
<tr>
<td>Dakar, 14°41'N, 17°26'W</td>
<td>Dec 2011</td>
<td>Immature stages-lab conditions</td>
<td><em>Anopheles arabiensis</em></td>
<td>154 †</td>
<td>2 (&lt;1)‡</td>
</tr>
<tr>
<td>Diebelo, 13°34'N, 16°24'W</td>
<td>Jul 2012</td>
<td>Human landing catches</td>
<td><em>An. arabiensis</em></td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. welcomei</em></td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Mansonia uniformis</em></td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. quinquefasciatus</em></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sep 2012</td>
<td>Pyrethrum spray catches</td>
<td><em>An. ziemanni</em></td>
<td>7</td>
<td>1 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. pharoensis</em></td>
<td>10</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>M. uniformis</em></td>
<td>8</td>
<td>2 (25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. welcomei</em></td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. funestus</em></td>
<td>7</td>
<td>2 (29)</td>
</tr>
<tr>
<td>Elinkine, 12°30'N, 16°39’W</td>
<td>Sep 2012</td>
<td>CDC-type light trap collections</td>
<td><em>An. gambiae</em></td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Culex sp.</em></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrethrum spray catches</td>
<td><em>An. gambiae</em></td>
<td>290</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. squamosus</em></td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>An. ziemanni</em></td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Culex sp.</em></td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Aedes sp.</em></td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Mansonia sp.</em></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>1,103</td>
<td>9 (&lt;1)</td>
</tr>
</tbody>
</table>

*Manufactured by John W. Hock Company, Gainesville, FL, USA.
†Including 20 male mosquitoes.
‡*R. felis* DNA was detected in 1 male mosquito.

Results

*Rickettsia felis* Detection

**Senegal**

The attack rate of *R. felis* infections in febrile patients was 15% (312/2,024); those infections occurred primarily during the rainy season rather than the dry season (207/1,105 vs. 105/916, respectively; p<0.0001). The risk of developing *R. felis* infection was 1.6× higher during the rainy period (95% CI 1.3–2) than during the dry period. When calculated by site, substantial differences in the rates of *R. felis* infection were observed (Table 2). The highest attack rates were observed in S5–6, reaching 40% (92/231) from August–October 2011. The lowest attack rate was observed in S1–2 (7%–8%) and was significantly lower than that observed at the 4 other sites S3–4 (p<0.001) (Table 2).

Incidence rates were obtained from 2 health centers (Figure 2). In 2011, the incidence rate of *R. felis* in S was 6.7 (4.8–9.0) per 100 person-years or 0.55 (0.39–0.76) per 100 person-months; the incidence rate in S was 3.1 (1.8–4.9) per 100 person-years or 0.26 (0.15–0.41) per 100 person-months during the same period. In S5–6, a significant difference was found between the incidence of *R. felis* for patients <15 years of age, which was 0.23 (0.16–0.31) per 100 person-months, and the incidence in patients >15 years of age, which was 0.10 (0.06–0.15) per 100 person-months (relative risk [RR] 2.38, 95% CI 1.34–4.28, p = 0.003). When the incidence rates by age group were calculated according to sex, a significant difference was observed only in the male group, in which the incidence rate was significantly higher in the patients <15 years of age than in the patients >15 years of age (0.29 vs. 0.07 per 100 person-months, RR 5.97, 95% CI 2.28–17.15, p = 0.001).

Table 3 shows the age distribution of *R. felis* infection. The occurrence of *R. felis* infection was significantly lower in patients 1–3 years of age (10%) than in patients >4 years of age (p = 0.03 for patients 4–6 years of age (15%); p = 0.003 for patients 7–15 years of age (16%); p = 0.004 for patients 16 to 29 years of age (16%); p = 0.002 for those >30 years of age (17%). The sex ratio for *R. felis* was 145M/162F (1:1.1). No deaths associated with *R. felis* infection were registered.

Combining these data with our preliminary report of 8 infected patients during 2008–2009 in S1–2 (4), we identified 61 patients with *R. felis* infections among a total of 456 villagers tested in S1–2. A second *R. felis* infection was diagnosed in 5 patients after 44 to 911 days, and 1 patient was positive for *R. felis* infection a second and third time at days 378 and 441, respectively. The 6 patients (4 male, 2 female) who had relapses or re-infections were from S1, and 5 were <6 years of age.

**Other Countries**

Samples from 3 patients (3%, 3/100) in rural Mali (M1, 1/50; M2, 2/50), 5 patients (10%, 5/50) in urban
and Senegal) (p < 0.0001). The probability of R. felis infection was 1.4× higher during the rainy period than during the dry period (95% CI 1.2–1.7, p<0.0001). The highest rate was in southeastern S6, whereas the lowest rate, 11% (37/350), was in southwestern S5 (Table 2). During the same time period, the incidence rate of malaria was 17.6 per 100 person-years or 0.42 per 100 person-months for S5 and 5.1 per 100 person-years or 0.22 per 100 person-months, RR 2.51, 95% CI 1.73–3.65, p<0.0001).

When the incidence rate by age group was calculated according to sex, the highest incidence was found among girls <15 years of age: 0.47 (0.37–0.65) versus 0.25 (0.16–0.37) per 100 person-months, RR 1.82, 95% CI 1.18–2.82, p = 0.002. The probability of R. felis infection was 1.00 for Algeria (baseline), 2.8 for Morocco, 4 for Mali, 14.5 for Gabon, and 24 for Senegal.

Malaria

Senegal

The attack rate of Plasmodium spp. in febrile persons from Senegal was 21% (400/1868, 206 females); those infections occurred significantly more often during the rainy season compared with the dry season (256/1042 vs. 144/822, respectively; p = 0.0002). The risk for malaria was 1.4× higher during the rainy period than during the dry period (95% CI 1.2–1.7, p<0.0001). The highest rate was in southeastern S6, whereas the lowest rate, 11% (37/350), was in southwestern S5 (Table 2). During the same time period, the incidence rate of malaria was 17.6 per 100 person-years or 0.42 per 100 person-months for S5 and 5.1 per 100 person-years or 0.22 per 100 person-months for S5. The highest incidence of malaria was among patients <15 years of age in S1-2 (0.55 (0.44–0.67) versus 0.22 (0.16–0.30) per 100 person-months, RR 2.51, 95% CI 1.73–3.65, p<0.0001).

When the incidence rate by age group was calculated according to sex, the highest incidence was found among girls <15 years of age: 0.47 (0.37–0.65) versus 0.25 (0.16–0.37) per 100 person-months, RR 1.82, 95% CI 1.18–2.82, p = 0.002. The probability of R. felis infection was 1.00 for Algeria (baseline), 2.8 for Morocco, 4 for Mali, 14.5 for Gabon, and 24 for Senegal.

Table 2. Attack rate of Rickettsia felis infection and malaria by country and geographic site, Africa, 2010–2012

<table>
<thead>
<tr>
<th>Participant status, country, and study site (site abbreviation)</th>
<th>Collection period</th>
<th>No. samples*</th>
<th>R. felis</th>
<th>Plasmodium spp.</th>
<th>R. felis/Plasmodium spp. co-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Febrile patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senegal</td>
<td>Jun 2010–Mar 2012</td>
<td>2,024</td>
<td>312/2,024 (15)</td>
<td>400/1,867† (21)</td>
<td>66/265 (23)*</td>
</tr>
<tr>
<td>Dieblo (S1)</td>
<td>Jun 2010–Feb 2012</td>
<td>540</td>
<td>39/540 (7)</td>
<td>118/509 (23)</td>
<td>8/36 (22)</td>
</tr>
<tr>
<td>Ndiop (S2)</td>
<td>Jun 2010–Feb 2012</td>
<td>246</td>
<td>20/246 (8)</td>
<td>33/237 (14)</td>
<td>3/18 (17)</td>
</tr>
<tr>
<td>Keur-Momar Sarr (S3)</td>
<td>Mar–Nov 2011</td>
<td>223</td>
<td>36/223 (16)</td>
<td>44/196 (22)</td>
<td>9/33 (27)</td>
</tr>
<tr>
<td>Niakhar (S4)</td>
<td>Oct 2010–Mar 2012</td>
<td>316</td>
<td>76/316 (24)</td>
<td>74/303 (24)</td>
<td>18/74 (24)</td>
</tr>
<tr>
<td>Basse-Casamance (S5)</td>
<td>Jan 2011–Mar 2012</td>
<td>411</td>
<td>84/411 (20)</td>
<td>37/350 (11)</td>
<td>7/69 (10)</td>
</tr>
<tr>
<td>Kedougou (S6)</td>
<td>2011</td>
<td>288</td>
<td>57/288 (20)</td>
<td>94/272 (34)</td>
<td>21/55 (38)</td>
</tr>
<tr>
<td>Gabon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Franceville</td>
<td>2011</td>
<td>50</td>
<td>5/50 (10)</td>
<td>19/50 (38)†</td>
<td>2/5 (40)**</td>
</tr>
<tr>
<td>Mali</td>
<td>2011</td>
<td>100</td>
<td>3/100 (3)</td>
<td>90% (90/100)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Diakambou (M1)</td>
<td>Oct</td>
<td>50</td>
<td>1/50 (2)</td>
<td>82% (41/50)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Kole (M2)</td>
<td>Nov</td>
<td>50</td>
<td>2/50 (4)</td>
<td>98% (49/50)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Algeria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oran</td>
<td>Jul–Sep 2012</td>
<td>257</td>
<td>2/257 (1)</td>
<td>1/257 (0.4%)</td>
<td>0/1</td>
</tr>
<tr>
<td>Morocco</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casablanca</td>
<td>May–Jun 2006</td>
<td>48</td>
<td>1/48 (2)</td>
<td>0/38†</td>
<td>0</td>
</tr>
<tr>
<td>Tunisia</td>
<td>2012</td>
<td>183</td>
<td>0/183</td>
<td>0/183</td>
<td>0</td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marseille</td>
<td>2012</td>
<td>400</td>
<td>0/400</td>
<td>0/400</td>
<td>0</td>
</tr>
<tr>
<td>Afebrile persons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senegal (S1–S2)</td>
<td>Dec 2011–Apr 2012</td>
<td>391</td>
<td>17/391 (4)</td>
<td>5/391 (1)</td>
<td>0/5</td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marseille</td>
<td>2011–2012</td>
<td>100</td>
<td>0/100</td>
<td>0/100</td>
<td>0</td>
</tr>
</tbody>
</table>

†Reliable samples.
††There were insufficient DNA samples for the analysis of Plasmodium spp., as decided a posteriori.
+Positive by blood smear.
Other Countries

Plasmodium DNA was detected in 90% of the blood samples collected in Mali; 3 patients with malaria from Mali were co-infected with R. felis (Table 2). In Gabon, samples from 38% (19/50) of the patients tested positive for malaria by using blood smears; of those patients were co-infected with R. felis. We most likely misdiagnosed malaria among the patients in Gabon, as based on the lower sensitivity and high specificity of microscopy versus PCR as the standard (22). Plasmodium DNA was not detected in the samples from Tunisia, Morocco, or France. However, 1 Plasmodium spp.-positive sample was collected in Algeria from a 21-year-old woman who was hospitalized for high fever, chills, and sweats after having spent >2 months visiting her family in Niger without malaria chemoprophylaxis.

Correlation of R. felis with Malaria

Using the Pearson correlation test, we found a significant correlation between the number of patients infected with R. felis and those infected with Plasmodium spp. (p<0.002): a higher number of R. felis infections correlated with a higher number of malaria cases. A significant correlation was also found for seasonality for infection by both pathogens: most cases occurred during the rainy period (p<0.0001). In addition, children <3 years of age were infected with both organisms less often than persons >4 years of age, and the Pearson test showed a significant correlation between R. felis and malaria (p = 0.001) for this age group.

Control Group

R. felis DNA was detected in 4% of the afebrile persons (17/391) from Senegal, 12 of whom were children (<15 years of age); malaria was detected in 5 afebrile persons, 3 of whom were children. Both pathogens were detected significantly less often in afebrile patients than in febrile patients (p<0.001). DNA from R. felis and Plasmodium were not detected among persons in the control group in France.

Arthropod Study

Samples from 9 mosquitoes (≈1%, 9/1,103) and 1 bed bug (≈1%, 1/160) tested positive in 2 R. felis-specific qRT-PCRs (Tables 1,4). The pRFδ plasmid was detected in 8 mosquito samples (21). In Dakar, 1% (2/154) of the An. arabiensis mosquitoes collected were positive for R. felis, including 1 male, suggesting transovarian transmission. One Aedes luteocephalus from Ferlo 0.5% (1/203) was positive for R. felis. In S1, 15% (6/40) mosquitoes collected in September 2012 were positive for R. felis, including 1 An. ziemanni, 1 An. pharoensis, 2 Mansonia uniformis, and 2 An. funestus. None of the 24 mosquito samples collected from this region in July tested positive. In addition, 1 Cimex hemipterus bed bug (3%) (1/39), collected from a household in S1 in February 2012, tested positive. No R. felis DNA was detected in soft or hard ticks or in Culicoides species.

Discussion

This study shows that Rickettsia felis is an emerging pathogen commonly detected in sub-Saharan rural

Table 3. Age distribution of infections with Rickettsia felis and Plasmodium spp. among patients in Senegal, by age group, 2010–2012*

<table>
<thead>
<tr>
<th>Species</th>
<th>&lt;1 y</th>
<th>1–3 y</th>
<th>4–6 y</th>
<th>7–15 y</th>
<th>16–29 y</th>
<th>&gt;30 y</th>
<th>ND</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium spp.</td>
<td>20/169 (12)</td>
<td>60/327 (16)</td>
<td>57/251 (23)</td>
<td>134/401 (33)</td>
<td>56/271 (21)</td>
<td>58/310 (19)</td>
<td>15/78 (19)</td>
<td>400/1,867 (21)</td>
</tr>
<tr>
<td>R. felis</td>
<td>27/184 (15)</td>
<td>40/422 (10)</td>
<td>40/270 (15)</td>
<td>69/425 (16)</td>
<td>49/298 (16)</td>
<td>57/336 (17)</td>
<td>30/89 (34)</td>
<td>312/2,024 (15)</td>
</tr>
</tbody>
</table>

*ND, no age data available.
We are confident that our molecular results are reliable and that the negative results in samples from France illustrate a correlation between *R. felis* infection and malaria with regard to the geographic distribution and seasonality. A trend of higher risk for *R. felis* infection in southern countries than in northern countries was revealed; the highest risk for *R. felis* infection was in rural Senegal (24 times than in Algeria). In Senegal, DNA from *Plasmodium* spp. and *R. felis* were detected at high levels, mostly during the rainy season and among children <15 years of age (Figure 2), but no coincidental relationship was found. The incidence of co-infection of *R. felis* and malaria was lower in Senegal (23%) than in Kenya (79%) (5), but higher than the rate of simultaneous bacterial bloodstream infections and malaria parasitemia, which ranged from 6% in rural Mozambique (23) to 11% in Nairobi (24). Mixed infections for rickettsioses, including co-infections with malaria or with other bacteria (*Leptospira* spp., *Coxiella burnetii*, and *Burkholderia pseudomallei*) have been described (25).

*R. felis* was detected in afebrile persons, most of whom were children <15 years of age, confirming the previously reported results in Kenya (5). Although rickettsioses have not previously been reported in afebrile persons, low-grade *Plasmodium* parasitemia has been reported among persons without a fever (26). This result should be confirmed by culture, but *R. felis* has never been isolated, even from acutely ill patients. Nonetheless, the absence of positive tests in the control group located in France confirmed the specificity of our tests. The S1–2 population was screened serologically for *R. felis*, and low titers were identified in 1 of 479 serum samples tested (27), which is substantially lower than the seroprevalence of other spotted fever group rickettsiae. The mechanism of absence of a serologic response and the occurrence of multiple re-infections or relapses of *R. felis* should be investigated further.

In this work, we demonstrated a greater frequency of *R. felis* during the rainy season among children in the sub-tropical zones, a period coinciding with circulation of *P. falciparum*. There are other seasonal diseases, including influenza, which are most common during the rainy season in subtropical Africa, particularly in Senegal (28). Influenza is a disease found throughout the year, with seasonal peaks, in Africa; none of the tested patients had influenza symptoms. Furthermore, leptospirosis, for which rickettsial disease could be mistaken, has not been documented in Senegal. Last, the most common seasonal disease in the most northern part of the intertropical area is malaria; a disease, however, which is common in all seasons in equatorial wetlands. These data, for which confirmation is needed, show a seasonal correlation between *R. felis* and malaria; the correlation is related to the presence and activity of *Anopheles* mosquitoes. Although the cat flea, *Ctenocephalides felis*, is currently the only known vector of *R. felis*, a variety of other arthropods have been suspected, including different flea species, ticks, mites, and lice (13). In Senegal, the source of *R. felis* is yet to be determined. We did not detect *R. felis* in fleas that were screened during 1 year in S1 and S2 (13). In other studies, *R. felis* was not detected in soft or hard ticks (27,29), tsetse flies (30), or mites. These findings support the hypothesis of the role of *Anopheles* in the transmission of *R. felis*; this hypothesis should be confirmed or refuted by future studies.

The clinical findings for *R. felis* infection are often unclear and are typically misdiagnosed as other febrile illnesses (12,31). Recently, the primary infection was described in a patient with polymorphous skin lesions, including papules, vesicles, erosions, and ulcers (12), similar to patients from Mexico (32). In the current study, a high incidence of *R. felis* infection was identified in children <15 years of age, as described (4). Fortunately, such patients improve rapidly with doxycycline treatment (12). For travelers to sub-Saharan Africa.
Africa, the medications recommended for the chemoprophylaxis of malaria include doxycycline, which has the added advantage of being effective against rickettsioses (33).

This study showed the wide distribution and high incidence of *R. felis* infection; therefore, rickettsiosis should be considered one of the major causes of febrile diseases in sub-Saharan Africa. The demonstrated geographic distribution, seasonality, target population, incidence of relapses or re-infections, and asymptomatic infections of *R. felis* infection are similar to malaria. Further studies are needed to investigate the hypotheses that humans, as for epidemic typhus, another vector-borne relapsing rickettsiosis, or apes could be reservoirs and mosquitoes could be a vector for *R. felis* infection.

Acknowledgments

We thank the villagers who participated in this study. We also thank Masse Sambou, Aliou Diallo, Khadim Leye, Babacar Ndao, Malick Diop, Arsène Mabika, Marielle Bedotto, Denis Pyak and Annick Bernard for technical support.

This study was funded by the Agence National de Recherche grant 2010 (MALEMAF), Foundation Méditerranée Infecton, Fondation Mériel, and a collaborative grant to Josselin Thuilliez, University of Paris, Paris, France.

Dr Mediannikov is an infectious disease specialist and research scientist working at the Unit of Research on Emergent Infectious and Tropical Diseases in Marseille, France and Dakar, Senegal. His main research interests include vector-borne diseases and medical entomology.

Dr Socolovschi is a physician of infectious diseases diseases and tropical medicine at the Medical School of Marseille, France. Her research interests focus on vector-borne infectious tropical diseases and medical entomology.

Dr Mediannikov is an infectious disease specialist and research scientist working at the Unit of Research on Emergent Infectious and Tropical Diseases in Marseille, France and Dakar, Senegal. His main research interests include vector-borne diseases and medical entomology.

Dr Socolovschi is a physician of infectious diseases and tropical medicine at the Medical School of Marseille, France. Her research interests focus on vector-borne infectious tropical diseases and medical entomology.

References

22. Ndao M, Bandiyayera E, Kokosk E, Gyorkos TW, MacLean JD, Ward BJ. Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria


Address for correspondence: Didier Raoult, Université Aix-Marseille, URMITE, UMR CNRS 7278, IRD 198, INSEM 1095, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 5 France; email: didier.raoult@gmail.com
In mainland China, most avian influenza A(H7N9) cases in the spring of 2013 were reported through the pneumonia of unknown etiology (PUE) surveillance system. To understand the role of possible underreporting and surveillance bias in assessing the epidemiology of subtype H7N9 cases and the effect of live-poultry market closures, we examined all PUE cases reported from 2004 through May 3, 2013. Historically, the PUE system was underused, reporting was inconsistent, and PUE reporting was biased toward A(H7N9)-affected provinces, with sparse data from unaffected provinces; however, we found no evidence that the older ages of persons with A(H7N9) resulted from surveillance bias. The absolute number and the proportion of PUE cases confirmed to be A(H7N9) declined after live-poultry market closures (p<0.001), indicating that market closures might have positively affected outbreak control. In China, PUE surveillance needs to be improved.

Since 2004, the Chinese Center for Disease Control and Prevention (China CDC) has conducted surveillance for pneumonia of unknown etiology (PUE) to facilitate timely detection of novel respiratory pathogens, such as severe acute respiratory syndrome (SARS) and avian influenza. On March 31, 2013, health authorities in China reported the first human infection with avian influenza A(H7N9) virus to the World Health Organization (1). In response to the emergence of A(H7N9), China CDC and provincial and local CDCs introduced testing for A(H7N9) virus of all persons with reported PUE. As of May 3, 2013, a total of 127 laboratory-confirmed A(H7N9) cases, resulting in 24 deaths, had been reported from 10 provinces and municipalities in mainland China (hereafter referred to as affected areas). The median age of these case-patients was 62 years; most (71%) were males.

Most confirmed case-patients had severe disease (2–4), and an analysis of national influenza-like illness surveillance data has not found evidence of widespread A(H7N9)-associated mild illness (5). After preliminary epidemiologic and virologic information pointed to live-poultry markets (LPMs) as a possible source of infection (2,4), retail and wholesale LPMs were closed in several major cities in which A(H7N9) was confirmed, including Shanghai, Nanjing, and Hangzhou. The number of new cases declined in these cities after LPM closures (6).

However, these reports of A(H7N9) geographic occurrence, demographic patterns, and effectiveness of control measures depend not only on the number of confirmed A(H7N9) cases but also on surveillance and on reporting and testing patterns. Although the number of cases has been studied at length, reported cases are a function of surveillance, and A(H7N9) reporting and testing patterns have not been examined in detail. We describe the PUE surveillance system in China and analyze the proportion of tested persons who test positive in mainland China for A(H7N9) by province, age, and sex before and after LPM closures to assess the possible role of surveillance bias.

Methods

Surveillance for PUE before A(H7N9) Emergence

From 2004 through March 2013, health care facilities of all types in China were required to report any patient
Surveillance for PUE after A(H7N9) Emergence

In response to the emergence of A(H7N9), 3 key changes in this system were implemented. First, starting on March 31, 2013, all specimens from reported PUE cases were required to be tested not only for influenza A(H5N1) but also for seasonal influenza A, influenza B, and influenza A(H7N9) by real-time reverse transcription PCR (3). If a specimen was positive for influenza A but could not be subtyped, further testing would be performed. If test results for both influenza types A and B were negative, specimens would be tested for SARS-CoV and Middle East respiratory syndrome coronavirus. Second, local-level evaluation of cases was streamlined in early April 2013. After cases were reported, specimens were sent directly for testing to local and/or provincial CDCs, bypassing the expert consultation committees. Third, to avoid delay in A(H7N9) diagnosis, the fourth reporting criterion above (antimicrobial treatment failure) was replaced with a requirement that the pneumonia etiology could not be attributed to an alternative clinical or laboratory diagnoses. Clinicians were given flexibility to determine how to interpret this criterion, and specific tests were not specified.

Respiratory specimens collected from patients whose illnesses meet the modified PUE case definition are sent to the local and/or provincial influenza network laboratory for testing for A(H7N9). (The first A[H7N9] case in a province is confirmed by China CDC and subsequent cases by the provincial CDC.) In addition, as of April 5, clinicians could also specify whether a patient had a suspected or confirmed A(H7N9) case by using a separate specific case definition and laboratory evidence of possible A(H7N9) infection (7) and reported directly to CISDCP. In this analysis, we focused only on the historical and current performance of the PUE surveillance system.

To better understand testing patterns during the A(H7N9) outbreak, we looked at historical reporting in the PUE surveillance system from January 2004 through March 2013. We also examined all PUE cases reported to China CDC during March 30–May 3, 2013, and calculated the proportion positive for A(H7N9) by province and in different age and sex groups. To assess whether LPM closures helped control the epidemic and to account for any reduction in testing, we examined the number of confirmed A(H7N9) cases and the proportion of PUE case-patients who tested positive for A(H7N9) in the week before and the 2 weeks after LPM closures in Shanghai (population 30.5 million), Nanjing (population 8.2 million), and Hangzhou (population 8.8 million). The LPMs were first closed in these cities on April 6, April 8, and April 15, respectively. At the time the study was conducted, the estimated median incubation period of A(H7N9) infection was 6 days (interquartile range 4–7) (China CDC, unpub. data). We separated postclosure results into those in the first and second weeks after closure in each LPM (1–7 days and 8–14 days, respectively) and compared proportions before and after LPM closure using a \( \chi^2 \) test for trend. A Pearson \( \chi^2 \) test was used to compare the proportion of men and women who tested positive for A(H7N9), and significance was defined by \( \alpha<0.05 \). SPSS software version 19.0 (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

During January 2004–March 30, 2013, a total of 1,016 cases were reported to the PUE surveillance system, of which 976 (96%) had a final diagnosis available. Thirty-nine (4%) cases were identified as A(H5N1), accounting for 91% of the 43 avian influenza A(H5N1) confirmed in humans in mainland China during 2005–2013. No SARS cases were identified. 744 (76%) PUE cases had no clear
RESEARCH

final diagnosis. In most months <10 PUE cases were reported, and a mean of 10 cases were reported each month (range 0–168). The number of reported cases increased during identified outbreaks, such as the SARS outbreak in 2004, when the system was first established, and avian influenza A(H5N1) outbreaks in humans during the winter and spring of 2005–06 and early 2009 (Figure 1).

During March 30–May 3, 2013, a total of 1,118 PUE cases were reported from 24 provinces, with earliest onset on January 26. PUE cases peaked at 61 per day on April 8, 2013, and then dropped rapidly in the following 3 weeks (Figure 2). A total of 1,002 (90%) PUE cases reported were from affected areas, which constitute 43% of the Chinese population, and 116 (10%) were from from unaffected areas (57% of the population). Most PUE cases were reported from Shanghai (468 [42%] of 1,118) and Zhejiang (388 [36%]). Of the 1,002 PUE cases from affected areas, 94 (9%) were confirmed as A(H7N9), which represents 74% of all 127 confirmed A(H7N9) cases in mainland China as of May 3. The remaining 33 cases were reported either through the influenza-like illness surveillance system (6 cases) or directly to CISDCP (27 cases).

Among the affected areas, Jiangsu reported the highest percentage of PUE positive for A(H7N9) (74%). This was followed by Hunan (33%), Henan (27%), Fujian (18%), Zhejiang (14%), Jiangxi (10%), Shanghai (4%), Beijing (3%), and Anhui and Shandong (0 cases each) (Table 1).

Of all PUE cases from the affected areas, 288 (29%) occurred in persons <25 years of age; 399 (40%) were 25–59 years, and 315 (31%) were ≥60 years. The number of PUE cases among female patients was lower overall (449 [45%] of 1,002) and in each age group except the 15–24-year and 25–59-year groups. Among persons ≥60 years of age, many more men than women were reported through the PUE systems (198 men vs. 117 women) (Table 2).

Of PUE cases confirmed to be A(H7N9), 1 (1%) was in the 5–14-year age group, 42 (45%) were in patients 25–59 years of age, and 51 (54%) were in patients ≥60 years of age. The proportion of PUE cases positive for A(H7N9) was higher in adults (11% and 16% in persons 25–59 and ≥60 years of age, respectively) than in children, teenagers, and young adults (0%, 1%, and 0% in persons <1–4, 5–14, and 15–24 years of age, respectively). Overall, more positive A(H7N9) cases occurred in men than in women (62 vs. 32), and men and women differed significantly in the proportion positive for A(H7N9) (11% vs. 7%, p = 0.027). In persons ≥60 years of age, twice as many A(H7N9) cases occurred in men than in women (34 vs. 17), although the proportion of PUE cases that were positive for A(H7N9) was not significantly higher in men than in women (17% vs. 15%; p = 0.539) (Table 2).

The total number of PUE reported cases declined after LPM closures in Hangzhou and Nanjing but increased in Shanghai in the 1–6 days after closure, then dropped in the 7–14 days after closure. The number of confirmed A(H7N9) cases in Shanghai and Hangzhou after officials closed LPMs declined from 11 and 15 cases, respectively, in the week before closure to 4 and 4 cases during the 1–7 days after closure. In the 8–14 days after closure, 1 and 0 cases were confirmed in those cities, respectively. The proportion of PUE cases positive for A(H7N9) also declined from 14% and 25% before closure to 2% and 12% 1–7 days later and 1% and 0% 8–14 days later, respectively (χ² test for trend, p<0.001 in Shanghai; p = 0.056 in Hangzhou). In Nanjing, 5 positive A(H7N9) cases occurred in the week before LPM closure, with 1 in the 14 days after closure (p = 0.564). When data from the 3 areas are combined, the number of positive cases declined from 31 cases in the week before closure (21% of PUE cases positive for A(H7N9)) to 8 cases (4% positive) 1–7 days after closure; it decreased...
further to 2 cases (2% positive) in the 8–14 days after closure (p<0.001). In Shanghai, >1.5 times the number of PUE cases were tested for A(H7N9) in the 8–14 days after LPM closure than before closure, although testing decreased in Hangzhou and Nanjing after LPM closure. These data suggest that the decline in absolute numbers was not a surveillance artifact but a real effect (Table 3; Figure 3).

Discussion

Our study examined the Chinese national PUE surveillance system and its utility during the influenza A(H7N9) outbreak in the spring of 2013. Historically, the PUE system had been underused, and reporting had been inconsistent. The number of reported PUE cases increased above minimum levels only during known outbreaks of A(H5N1) and SARS, the only pathogens for which there had been testing. We describe several changes made to the PUE system during the A(H7N9) outbreak that increased its sensitivity and timeliness, resulting in increased reporting; yet, we demonstrated low frequency of PUE reporting from unaffected provinces. Moreover, some provinces were clearly prescreening possible A(H7N9) PUE cases before reporting, which resulted in wide variations in percent positivity. Nevertheless, data from the PUE system demonstrated that 1) A(H7N9) cases were indeed more common in elderly persons; 2) men are at higher risk than women for PUE and A(H7N9) virus infection; and 3) the decline in reported cases after LPM closure probably reflects a true decline in the number of cases, not merely a decline in testing.

Historical data from the PUE surveillance system demonstrated that the system has consistently been underused. Before the A(H7N9) outbreak, it was used to report most A(H5N1) cases in China. However, the PUE system was not (and still is not) used consistently. In 1 study, which examined all cases of community-acquired pneumonia in 6 hospitals over 1 year (April 1, 2008–March 31, 2009), 442 (29%) of the 1,506 community-acquired pneumonia cases met PUE criteria and should have been reported to the PUE system (8). In contrast, only 1,016 PUE cases in all of China were reported during a 9-year period. We showed that the number of cases surged when an outbreak occurred, either during the SARS outbreak or during publicized A(H5N1) outbreaks. This surge may reflect enhanced administrative requirements from health authorities (9) or enhanced clinician awareness of respiratory viruses.

Before April 2013, the administrative burden of reporting a case to the PUE system gave clinicians little incentive to participate. Reporting a PUE case triggered
requirements, such as cooperating with an epidemiologic investigation, collecting specimens, providing clinical information for expert committees, and moving patients to isolation wards. In return, clinicians received little information; 76% of reported PUE cases had no final specific diagnosis, and clinicians were told only whether the cases were SARS or A(H5N1). Streamlining the PUE reporting system and decreasing the requirements involving expert consultation committees probably contributed to the large increase in PUE reporting during the A(H7N9) outbreak; more PUE cases were reported during the study period than in the prior 9 years of PUE surveillance.

During the A(H7N9) epidemic, reporting increased substantially only in affected areas, leading to huge variation between provinces in PUE reporting. Of most concern is that during the A(H7N9) outbreak, areas with no human cases grossly underreported PUE cases. Most (92%) reported PUE cases were negative for A(H7N9) and were probably caused by other etiologies. Thus, we would expect to see a comparable number of PUE cases reported in affected and unaffected areas. However, 68% of all PUE cases were reported from Shanghai and Zhejiang province; together, these 2 provinces constitute only 6% of the total population of China. By contrast, only 10% of all PUE cases were reported in the 21 unaffected provinces; these constitute 57% of the population.

In addition to surveillance bias away from provinces unaffected by the A(H7N9) outbreak, variation probably occurs among provinces in the screening that precedes reporting a PUE case. Some provinces reported PUE cases before extensive testing; in other provinces, clinicians may send specimens directly to the local CDC for testing first, then report only those that had a positive result as PUE cases. This scenario was documented in a previous analysis of the PUE system during 2004–2007 (11). The discrepancy in the proportion of positive cases in different provinces (74% in Jiangsu vs. 4% in Shanghai) indicates that prescreening was most likely a factor in PUE reporting practices during the A(H7N9) outbreak. The sharp decline in PUE reporting noted after mid-April also might reflect increased availability of A(H7N9) testing at the local and provincial levels. The ability to test for A(H7N9) locally enables clinicians and local health officials to bypass PUE reporting and instead report a case to CIDSP as a suspected or confirmed A(H7N9) case; this raises the question of how much the PUE system will be used if future large outbreaks of A(H7N9) occur.

Despite the limitations of the PUE reporting system, it yielded important epidemiologic information. First, we found that the older age distribution of persons with A(H7N9) was probably true and not a result of surveillance bias because testing was extensive among young persons, and the percentage positive increased in persons ≥60 years of age. This contrasts sharply with A(H5N1) cases in China in which the median age of infection is 26 years (12). Second, more PUE cases were reported among men who were also more likely to test positive; the reason may be that men are at higher risk for any pneumonia, perhaps because of underlying respiratory comorbidities, but the increased percentage positive for A(H7N9) among men also suggests a specific risk for A(H7N9), especially among working-aged men. The reason may be that these men are more

Table 2. Number of reported PUE cases and number positive for influenza A(H7N9) virus in 10 affected areas, mainland China, March 30–May 3, 2013*

<table>
<thead>
<tr>
<th>Location</th>
<th>No. PUE</th>
<th>A(H7N9) positive, no. (%)</th>
<th>A(H7N9) positive, no. (%)</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanghai</td>
<td>81</td>
<td>11 (14)</td>
<td>188</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Nanjing</td>
<td>7</td>
<td>5 (71)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hangzhou</td>
<td>60</td>
<td>15 (25)</td>
<td>34</td>
<td>4 (12)</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>31 (21)</td>
<td>222</td>
<td>8 (4)</td>
</tr>
</tbody>
</table>

†p value comparing proportion positive among males with proportion positive among females. Pearson χ².

Table 3. Reported PUE cases that were positive for influenza A(H7N9) virus before and after closure of live-bird markets in 3 cities, mainland China, March 30–May 3, 2013*

<table>
<thead>
<tr>
<th>Location</th>
<th>0–6 d before closure†</th>
<th>1–7 d after closure†</th>
<th>8–14 d after closure†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanghai</td>
<td>81</td>
<td>11 (14)</td>
<td>188</td>
</tr>
<tr>
<td>Nanjing</td>
<td>7</td>
<td>5 (71)</td>
<td>0</td>
</tr>
<tr>
<td>Hangzhou</td>
<td>60</td>
<td>15 (25)</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>31 (21)</td>
<td>222</td>
</tr>
</tbody>
</table>

†p value comparing proportion positive among males with proportion positive among females. Pearson χ².
Pneumonia Surveillance and Influenza A(H7N9) exposed to poultry through occupation or behavior. Third, PUE surveillance analysis suggested that LPM closure did reduce A(H7N9) transmission to humans, whereas a previous report indicated that the number of new A(H7N9) cases declined after LPM closure (6), this decline could have reflected decreased testing and not an actual decline in A(H7N9) incidence. Our analysis shows that, although the number of persons reported with PUE and tested for A(H7N9) virus decreased after LPM closure, the proportion of PUE cases positive for A(H7N9) also decreased in the weeks after closure. Investigation of A(H7N9) cases in China has found that 77% of cases for which information was available have had poultry exposure, many through contact with LPMs (2). In the 1997 outbreak of A(H5N1) in Hong Kong, poultry were culled and LPMs closed (13). These measures controlled the outbreak, and A(H5N1) disease was not reported again in humans until 2003.

Our study has several limitations. First, the incidence of A(H7N9) in the 3 areas with LPM closure that we studied may have decreased regardless of LPM closure. It is possible that LPM closures were associated with—but not the cause of—the waning number of cases. This decreasing incidence could have been the case had there been a short wave of infected poultry passing through LPMs. Also possible is that, as with A(H5N1), A(H7N9) may be seasonal in birds and therefore in humans, with lower transmission during the spring and summer months. Second, although we demonstrate that the proportion of PUE cases positive for A(H7N9) decreased after LPM closure, the substantial decrease in reporting and testing immediately after market closure in Hangzhou may have resulted in missed cases and exaggerated the apparent effect of closure. In addition, how much increased local testing for A(H7N9) may have affected PUE reporting is unknown.

This study identified several major problems with the PUE surveillance system, including low and uneven levels of participation and inconsistency among provinces in how the system is used. Given its potential value in monitoring future A(H7N9) activity, the system’s overall objectives and reporting procedures should be further evaluated. The continued threat of additional viral adaptation to human hosts leading to increased transmissibility lends added urgency to the ongoing improvement of the PUE system to better understand the epidemiology of A(H7N9), detect outbreaks, and evaluate control measures.

Acknowledgments
We thank Yachun Dai, Jianfen Wang, and Yanfei Li for technical assistance on exporting the PUE database from CISDCP.

Figure 3. Reported PUE cases and confirmed influenza A(H7N9) cases reported before and after LPM closures, Shanghai (A), Nanjing (B), and Hangzhou (C), mainland China, March 30–May 3, 2013. PUE, pneumonia of unknown etiology; LPM, live-poultry market.
This work was supported by the China–US Collaborative Program on Emerging and Re-emerging Infectious Diseases and a grant from National Ministry of Science and Technology Emergency Research Project on human infection with avian influenza A(H7N9) virus (Epidemiology Research Project) (KJYJ-2013-01-02).

Dr Xiang is an epidemiologist at the Office for Emerging Infectious Disease, Public Health Emergency Center, Chinese CDC. Her research interests are the surveillance of emerging infectious diseases, prevention and control strategies for emerging infectious diseases, and pandemic influenza preparedness.

References


Address for correspondence: Zijian Feng, No.155 Changbai Rd, Changping District, Beijing 102206, China; email: fengzj@chinacdc.cn
Inflammatory bowel disease (IBD) is a risk factor for *Clostridium difficile* infections (CDIs). Because of similar disruptions to the intestinal microbiome found in IBD and in obesity, we conducted a retrospective study to clarify the role of obesity in CDI. We reviewed records of patients with laboratory-confirmed CDIs in a tertiary care medical center over a 6-month period. Of 132 patients, 43% had community onset, 30% had health care facility onset, and 23% had community onset infections after exposure to a health care facility. Patients with community onset infections had higher body mass indices than the general population and those with community onset after exposure to a health care facility had higher rates of IBD, and lower prior antibacterial drug exposure than patients who had CDI onset in a health care facility. Obesity may be associated with CDI, independent of antibacterial drug or health care exposures.
costs range from $496 million to >$1 billion (1,2). C. difficile is a leading cause of infectious diarrhea in hospitalized patients: the annual number of diagnoses of CDI on discharge has more than doubled, from ≈139,000 to 336,600 during this decade (3). The epidemiology of CDI has also shifted. A greater number of community onset cases have been recorded in traditionally low-risk populations (4,5), raising the concern for whether there are unidentified risk factors increasing the probability of CDI among this subset of persons. Association of CDI with novel risk factors can contribute to improved clinical surveillance of persons at highest risk for infection in the hospital setting or the community.

Inflammatory bowel disease (IBD) has been identified as an independent risk factor for C. difficile colonization and disease; patients with IBD have increased severity of illness and death rates from CDI (6,7). This relationship appears to be modulated by a dysbiosis of intestinal microbiota (7,8). Similar to changes noted with use of antibacterial drugs and IBD, studies have shown that obesity may be associated with decreased diversity and changes in composition of the intestinal microbiome (9–11). Given the similarities in derangements of the intestinal microbiota seen secondary to antibacterial drug use, IBD, and obesity, obesity may also predispose persons to CDI.

Before 2010, the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America guidelines (SHEA-IDSA guidelines) defined CDIs as having community onset (CO) or inpatient health care facility onset (HO). Reflecting the changing epidemiology of CDI, the definition was expanded by the 2010 update of clinical practice guidelines to include an additional category of disease: community-onset health care facility–associated (CO-HCFA) (12). This category, CO-HCFA, is defined as onset of disease in CDI patients in the community who had exposure to health care facilities during the previous 4 weeks. We believe the introduction of this category has removed cases from the CO cohort who had recent exposure to health care facilities and may help detect associations between CDI and novel risk factors in patients with few other traditional exposures.

This study aims to identify possible demographic and risk factor differences between patients who develop community onset CDI compared with their HO and CO-HCFA counterparts. In particular, we examine whether obesity is overrepresented in patients with community onset infections who did not have exposure to health care facilities, antibacterial drugs, or the diagnosis of IBD. Furthermore, we examine the health care delivery sites represented among patients with CO-HCFA infections. The identification of these sites will facilitate targeted training and education of staff and improved allocation of infection control resources to decrease future incidence of disease.

Methods

This study was a retrospective analysis of the infection control databases, microbiology results, and medical records of all patients who had laboratory proven CDI at Boston Medical Center (BMC) that serves as a regional safety net hospital. At the time of the study, the 508-bed academic medical center had a network of 15 community health centers. The study was approved by the BMC Institutional Review Board.

Our institution adopted the 2010 SHEA-IDSA guideline classifications for CDI in November 2011. All CDI cases in adults during November 2011–April 2012 were reviewed. Case-patients were defined as persons who had fecal samples positive for C. difficile by using the C. Diff Quik Chek Complete enzyme immunoassay (TechLab, Blacksburg VA, USA) or GeneXpert PCR (Cepheid, Sunnyvale, CA, USA) during the study period. At BMC, only non-formed stools are accepted for microbiological analysis for CDI. Samples are tested by enzyme immunoassay for toxins A and B; if the result is inconclusive or clinical suspicion of disease is high, PCR is used.

By using the former classification, the case-patients with laboratory proven CDI were first categorized as having either community or nosocomial onset disease. Patients were then recategorized by using the new SHEA-IDSA guidelines as having CO, CO-HCFA, or HO disease. The CO category included patients who had symptoms and a positive fecal sample test and no exposure to health care facilities or associated sites for >30 days before the clinic visit or hospital admission. CO-HCFA case-patients were exposed to health care facilities within the previous 30 days. Case-patients with HO disease had onset of symptoms >48 hours after admission and had positive results for CDI laboratory tests. Patients who had new symptoms and a positive assay and a previous positive test for C. difficile >30 days but <8 weeks prior to examination were classified as having recurrent disease. Because of the small sample size of this group, recurrent case-patients were excluded from analysis to facilitate statistical comparison.

An exposure to hemodialysis centers, day surgery, chemotherapy suites, or long-term care facilities was considered an encounter with the health care system. Demographic data extracted from the patient chart included age, sex, race and ethnicity, height, and weight. Factors that have been identified as risk factors for CDI were also documented and included the presence of certain coexisting medical conditions, use of anti-ulcer medications, admission to a hospital intensive care unit, duration of hospital stay, and antibacterial use during the preceding month (13). IBD was cataloged separately from other immunocompromising conditions. Obesity was defined as body mass index (BMI) >30, calculated as weight (kg)/height (m)^2.
Statistical analyses were performed by using SPSS software version 20 (SPSS, Inc., Chicago, IL, USA). Descriptive statistics, including student t-test, 1-way analysis of variance, and χ² statistics, were acquired for the data where appropriate. The proportion of CO case-patients diagnosed with obesity was compared with data gathered during 2011 in Massachusetts: population statistics provided by the US Census Bureau (14) and weight classification by BMI data provided by the Centers for Disease Control and Prevention Behavioral Risk Factor Surveillance System (15). Age was used as a continuous variable to calculate the means in univariate analysis but categorized as either <65 or ≥65 years for multivariable regression. All reported p values were 2-sided; results with a p value <0.05 were considered significant.

Three binary regression analyses were performed with either CO and CO-HCFA, CO and HO, or CO-HCFA and HO as outcomes. CO-HCFA was used as the reference category for the first model, while HO served as the reference for the second and third models. A stepwise backward elimination method and likelihood ratios were used to find the best fitted model that also contained clinically relevant variables. All 2-way interaction terms were examined; none were found to have a substantial impact. A p value of 0.10 was used for exclusion from the regression model of nonclinically relevant covariates.

Results

A total of 137 cases of CDI were identified in patients at BMC during the study period. Five patients had recurrent disease and were excluded, and the remaining 132 cases were analyzed. According to the former definitions of location of onset of CDI, 91 cases were CO and 41 were HO. By using the definitions described in 2010, 35.2% (32/91) of the CO cases were found to be HCFA-CO (Figure). Of these, 62.5% (20/32) had a prior hospital admission as a risk factor, while 28.1% (9/32) were from a long-term care facility. Other risk factors (accounting for those with >1 risk factor) included recent surgery (12.5%), hemodialysis (9.4%), or outpatient chemotherapy (3.4%). Results for patient demographics among the 3 CDI categories are shown in Table 1. Among hospitalized patients from each category (109/132), patients with nosocomial infections had a longer length of stay (p<0.001) and were more likely to have been admitted to an intensive care unit (ICU) (p = 0.002) (Table 1).

In univariate analysis testing for differences across the 3 groups, there were lower percentages of patients with IBD in the HO and HCFA categories compared with the CO group (p = 0.018). A higher percentage of patients in the CO category were noted to be obese, and this finding approached statistical significance (p = 0.08). The percentage of patients in the CO group who were obese (34%) was statistically higher than the state average (23%) (odds ratio 1.7, 95% CI 1.02–2.99). HO cases were more likely to have had prior exposure to antibacterial drugs compared with the CO and HCFA groups (p <0.001). The use of antiulcer medication and coexisting conditions such as immunosuppressive conditions, diabetes, and end stage renal disease were identified with statistically similar frequency in the 3 groups. CDI in HO group was associated with longer hospital stays and higher likelihood of an ICU stay than that in the CO or HCFA groups (p<0.01 for both).

In binomial logistic regression, the CO cohort was noted to be younger (p = 0.03) and 4 times more likely to be obese (p = 0.03) compared with the CO-HCFA group (Table 2). Obesity was not observed at a substantially higher rate in the CO group compared with the CO-HCFA group. The CDIs in CO group were >5 times more likely to be associated with IBD compared with CO-HCFA and ≈6.5 times more likely when compared with the HO group; only the latter comparison approached significance (p = 0.094). Compared with HO patients (p =0.001), CO and CO-HCFA patients were statistically less likely to have had antibacterial drugs before symptom onset (p = 0.01).

Discussion

This study demonstrates possible relationships between CDI, IBD, and obesity. By comparing a relatively low-risk group of patients with CDI to those with more traditional risk factors, we sought to identify an association between obesity and CDI. This association was underscored by the hypothesis that in a group without exposure to health care facilities, the statistical significance of other risk factors such as obesity and IBD may be increased. Under the categories created by SHEA-IDSA guidelines, case-patients with CO CDI were 4 times more likely to be obese compared with the community-onset health care
facility–associated group, and almost 2 times as likely to be obese as the general population of Massachusetts. Like IBD, obesity may be associated with a higher risk of CDI.

The relationship between IBD and CDI is evolving. Issa et al. (16) and Rodemann et al. (17) demonstrated that ≈80% of IBD patients who acquired CDI did so in outpatient settings, and another series of inpatients showed IBD patients had CDI onset within an average of 1 day of admission, compared with 4 days for other CDI case-patients. IBD patients received a greater number of antibacterial drugs, had greater exposure to health care facilities, and were frequently administered immunosuppressive drugs that could have increased their risk of infection (18). However, there is biologic plausibility that IBD may create an intestinal environment hospitable for CDI, independent of antibacterial drugs and immune modulators.

Studies have demonstrated that the increased incidence of CDI and colonization in IBD patients may be mediated by a derangement of gut flora (19). Evolving literature suggests that the community of microorganisms living in symbiosis with the human host affects energy metabolism, alters responses by innate immunity, and can determine outcomes of host pathogen interactions (20, 21). The diversity and the composition of the gut microbiobial community determine the effectiveness of its symbiosis with the host (22). Changes in fecal microbiome have been demonstrated in recurrent cases of CDI associated with antibacterial drug use (9). This defect is also noted in obese patients and those with IBD (23). The similarities in alterations of normal microbial symbiosis in both IBD and obesity may explain why obese patients may be at risk for acquiring CO CDI. Greenblum et al analyzed fecal samples from a cross section of volunteers and examined gene-level and network-level topological differences in intestinal microbiomes associated with obesity and IBD. Obesity and IBD were linked with enzyme level variations and topographical changes, suggesting low diversity environments (23).

Aside from the overall decrease in richness of phyotypes of bacterial species, certain host conditions appear to be associated with specific changes in the intestinal microbiota and up or down-regulation of certain bacterial species. The development of CDI appears to be linked to the loss of the ability of the indigenous intestinal species to resist colonization by additional invasive pathogens (9). In particular, a decrease in the relative proportion of the phylum Bacteroidetes to that of Firmicutes has been associated with CDI. Manges et al. found that these changes could be driven by antibacterial drugs and health care exposure (24). IBD and obesity manifest similar changes in the fecal microbial community (25). Obesity may provide a milieu with increased susceptibility for invasion and infection by C. difficile.

Higher BMI has been associated with a greater chance by trauma patients of acquiring health care–associated infections, including CDI. In a recent retrospective case control study, Bishara et al. demonstrated a higher BMI in all hospitalized patients with CDI compared with inpatient controls (p<0.001) (26). This observation was particularly notable because case-patients and controls had above average BMIs, suggesting that there may be an even more drastic association in the general population. In addition, Bishara et al. noted this relationship between BMI and obesity without differentiation in the probable sites of acquisition (26). We were unable to show a difference in obesity between the CO and HO groups, implying that either there is an inherent difference between patients with health care–associated onset and those with hospital onset, or that our study was not statistically powered to detect the association. Because we could show no notable differences between CO-HCFA and HO in regression analysis except for antibacterial drug use, we believe that our study was limited by our sample size.

### Table 1. Patient characteristics associated with cases of community onset, community-onset health care–associated, and health care onset cases of Clostridium difficile infections

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Community onset, n = 59</th>
<th>Community-onset health care–associated, n = 32</th>
<th>Health care onset, n = 41</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>57.8 (22–96)</td>
<td>63.7 (23–95)</td>
<td>61.6 (21–96)</td>
<td>0.31*</td>
</tr>
<tr>
<td>Male sex, no. (%)</td>
<td>27 (46)</td>
<td>10 (31)</td>
<td>23 (56)</td>
<td>0.106</td>
</tr>
<tr>
<td>Medication use, no. (%)</td>
<td>31 (52.5)</td>
<td>11 (34.4)</td>
<td>33 (80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prior antibiotic use</td>
<td>24 (41)</td>
<td>18 (56)</td>
<td>19 (46)</td>
<td>0.363</td>
</tr>
<tr>
<td>Physical status/illness, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese (body mass index &gt;30)</td>
<td>20 (34)</td>
<td>4 (13)</td>
<td>13 (32)</td>
<td>0.078</td>
</tr>
<tr>
<td>Immunocompromised†</td>
<td>14 (24)</td>
<td>11 (34)</td>
<td>11 (27)</td>
<td>0.551</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14 (24)</td>
<td>9 (28)</td>
<td>7 (17)</td>
<td>0.519</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>10 (17)</td>
<td>1 (3)</td>
<td>1 (2)</td>
<td>0.018</td>
</tr>
<tr>
<td>Required inpatient admission, no. (%)</td>
<td>41 (69)</td>
<td>29 (91)</td>
<td>39 (95)</td>
<td>0.002</td>
</tr>
<tr>
<td>Average days stayed</td>
<td>7.7</td>
<td>8.2</td>
<td>18.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Required intensive care unit stay</td>
<td>11 (27)</td>
<td>8 (28)</td>
<td>24 (61)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* p value for 1-way analysis of variance.
† Immunocompromised group included all malignancies, innate and acquired immune conditions such as HIV/AIDS, and congenital immune defects. This group excluded comorbidities listed separately.
Use of antiulcer medication has been identified as a risk factor for CDI in the community (27). There was no difference in the rate of antiulcer medication use among the 3 subgroups in this cohort. This may reflect local prescribing practices of physicians, because inpatients and outpatients were equally likely to be exposed to these medications. Case-patients who had HO CDI were more likely to have been in an ICU during the study admission. Overall, this trend and the increased incidence of prior antibacterial drug use in this group may represent a higher severity of illness in this cohort. The greater likelihood of nosocomial acquisition of disease could be caused by longer lengths of hospitalization (28). Most CO–HCFA case-patients had a history of prior hospitalization. Long-term facilities, day surgery centers, and outpatient hemodialysis sites appear to also serve as potential sites of increased transmission of CDI outside the hospital.

The main limitation of this study is related to the lack of data for true prevalence of risk factors in each group, because we compared cases with each other on the basis of the location of onset and not to controls. Hence, the trends observed require further validation with prospective analysis to establish whether there is a true association between obesity and CDI as noted in the CO cohort. The analysis is also limited by the retrospective design and, as mentioned before, the relatively small sample size. In addition, data collection was dependent on chart extraction, and hence dependent on provider documentation. Since cases were defined by patient samples with positive diagnostic assays, this study did not differentiate between patients who were colonized and those with active disease. This may have overestimated true disease prevalence, as has been demonstrated (29). However, because only non-formed fecal samples are accepted for analysis at our laboratory, it is likely that the majority of the cases represented true disease.

Conclusions

Translational research could help elaborate the dimensions of the interaction of the intestinal microbiota with C. difficile in obese patients. It would also be of interest to establish if there is a dose response between BMI and risk for CDI acquisition. Further, it is critical to establish whether obesity is a risk factor for high rates of C. difficile colonization, as is IBD; if that risk factor is established, prospective observations would improve understanding of whether obesity plays a role in the acquisition of CDI, or alters severity of disease and risk for recurrence. Last, the examination of the CO–HCFA group in this study underscores the importance of increased infection control at ancillary health care facilities and surveillance for targeting high-risk patients who were recently hospitalized.

Acknowledgment

We thank Kalpana Gupta for her insight into the subject matter and aid in the editing of this work.

Dr Leung is a resident in physical medicine and rehabilitation at University of Michigan. His primary research interest is health care-associated infections in long-term care patients.

References


Address for correspondence: Nahid Bhadelia, Section of Infectious Diseases, Boston Medical Center, 850 Harrison Ave, Dowling 3N, Room 3104, Boston, MA 02118-4001; email: nbhadeli@bu.edu
Previous findings have suggested that the nosocomial transmission capacity of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is lower than that of other MRSA genotypes. We therefore performed a 6-month (June 1–November 30, 2011) nationwide study to quantify the single-admission reproduction number, $R_0$, for LA-MRSA in 62 hospitals in the Netherlands and to compare this transmission capacity to previous estimates. We used spa typing for genotyping. Quantification of $R_0$ was based on a mathematical model incorporating outbreak sizes, detection rates, and length of hospital stay. There were 141 index cases, 40 (28%) of which were LA-MRSA. Contact screening of 2,101 patients and 7,260 health care workers identified 18 outbreaks (2 LA-MRSA) and 47 secondary cases (3 LA-MRSA). $R_0$ values indicated that transmissibility of LA-MRSA is 4.4 times lower than that of other MRSA (not associated with livestock).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of nosocomial infections and leads to considerable illness, death, and health care costs (1,2). The worldwide epidemiology of MRSA has changed as MRSA originating in the community has increased. These community-associated MRSA (CA-MRSA) strains are replacing their hospital-associated counterparts in hospitals in the United States; the major dominant clone is MRSA strain USA300 (3). In recent years, another MRSA clone, which originated in the community and is associated with exposure to livestock, has emerged in different countries worldwide, including the United States (4,5). Even more worrying, countries with a historically low prevalence of MRSA, like the Netherlands and Denmark, have seen an increase in livestock-associated MRSA (LA-MRSA), belonging to clonal complex 398 (5). In the Netherlands, LA-MRSA accounted for 39% of all new MRSA isolated in 2011 (6). Yet almost all isolates have been detected through screening, and in 2009, nine infections were caused by MRSA sequence type 398 (7). Invasive infections caused by LA-MRSA include endocarditis, osteomyelitis, and ventilator-associated pneumonia (8,9).

It has been suggested that in the Netherlands, this MRSA genotype has a lower capacity than other genotypes for nosocomial transmission (10,11). The lower transmission rates might result from differences in human host characteristics or from a lack of pathogen adaptation to the human host, which could change over time (12). In a previous study in the Netherlands in 2005, we quantified the transmission capacity, expressed as the single-admission reproduction number per hospital admission, $R_0$, and obtained values of 0.16 for LA-MRSA and 0.68 and 0.98 for MRSA not associated with livestock (hereafter referred to as other MRSA) (10). We therefore performed a nationwide study to quantify $R_0$ for LA-MRSA in hospitals in the Netherlands and to compare this transmission capacity to our previous estimates.

**Methods**

**Data Collection**

Medical microbiologists and infection control practitioners in all 91 hospitals in the Netherlands were contacted and asked to collect data concerning MRSA outbreaks and the results of subsequent contact screening retrospectively during June–August 2011 and prospectively during September–November 2011. A standardized Web page was used for data collection. An index case-patient was defined...
as a hospitalized patient colonized or infected with MRSA and treated without use of barrier precautions. Age, sex, and number of days hospitalized from MRSA detection through discharge were obtained. According to the guidelines in the Netherlands, identification of a MRSA index case-patient initiates contact screening among contact patients and health care workers (HCWs) (13). The numbers of screened patients and HCWs and the number of secondarily colonized patients and HCWs were obtained. A secondary case-patient was defined as a patient with MRSA with a spa type identical or related to that from the index case-patient, detected during contact screening of a patient or HCW. Newly identified MRSA carriers with MRSA spa types that were unrelated to that of an index case-patient were considered incidental findings. The study was approved by the medical research ethics committee of the University Medical Center Utrecht.

MRSA Genotyping
For all MRSA isolates, single-locus DNA sequencing of the repeat region of Staphylococcus protein A gene (spa typing) was performed by the national reference laboratory of the Netherlands (National Institute for Public Health and the Environment [RIVM]), as described (14), by use of the Ridom StaphType program (www.ridom.de) to allocate spa types. MRSA isolates were considered to be associated with livestock if they had a livestock-associated spa type: t011, t034, t108, t567, t571, t588, t753, t779, t898, t899, t943, t1184, t1197, t1254, t1255, t1451, t1456, t1457, t2123, t2287, t2329, t2330, t2383, t2582, t2748, t2971, t2974, t3013, t3014, t3053, t3146, or t3208 (15–17). All other spa types were considered to not be associated with livestock. To identify potentially unknown livestock spa types, we used Bionumerics 5.1 (Applied-Maths, Sint Maartens-Latem, Belgium) to create a spa-based minimal spanning tree of spa types considered livestock-associated and the spa types of index cases (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/11/12-1085-Techapp1.pdf). Genes encoding for Panton-Valentine leukocidin (PVL), LukS-PV, and LukF-PV were identified by the reference laboratory, as described (18).

Model
To estimate the strain-specific transmission capacity $R_s$ value, we used a previously described mathematical model based on queuing theory (19). $R_s$ is defined as the average number of secondary cases caused by 1 primary case (the index case) when other patients are susceptible during a single hospital admission of the primary case-patient (20). In this model, 3 rates determine the spread of MRSA in the hospital setting: the rate at which the MRSA strain spreads, the rate at which MRSA colonization of a patient is detected (e.g., microbiological cultures), and the rate at which a colonized patient can no longer be detected. The model predicts that the distribution of the number of patients colonized at the time of detection of the index case is geometrically distributed. The parameter of the geometric distribution of detected outbreak sizes was determined by using maximum-likelihood estimations. Small detected outbreak sizes could correspond to either low transmission potential or high detection rate.

Patients with MRSA remain colonized during their hospital stay; therefore, the infectious period ends at the time of discharge. Genotype-specific discharge rates were calculated from admission and discharge data for index case-patients admitted to participating hospitals during the study period. The detection rate was based on all blood, respiratory tract, and wound cultures conducted during 2011 at the University Medical Center Utrecht. The upper detection limit consists of all these cultures divided by the total number of patient days in 2011. By combining the detection and discharge rate with the parameter of geometric distribution, we could calculate $R_s$. Details about the model are included in the online Technical Appendix.

Statistical Analyses
Categorical variables were assessed 2-sided by using $\chi^2$ or Fisher exact tests, as appropriate; a cutoff value of $p<0.05$ was applied for significance. Continuous variables were analyzed by using the Mann-Whitney U test. Confidence intervals were calculated by using the profile-likelihood method. To test whether our assumption of a geometrical distribution of the detected outbreak sizes is justified by the data, we performed the Anderson-Darling goodness-of-fit test. Data were analyzed by using SPSS for Windows version 20.0 (IBM Corp., Armonk, NY, USA). Further details about the statistical methods used are included in the online Technical Appendix.
Results

A total of 62 (69%) of the 91 hospitals in the Netherlands participated in the study, yielding data for 372 months of MRSA policy. During the 6-month study period, 158 MRSA index case-patients were identified in 57 hospitals, and none were identified in the other 5 hospitals. These numbers imply that, on average, in each hospital an index case was detected every 2.5 months. Two index case-patients were excluded because subsequent contact screening was not performed, and 15 index case-patients were excluded because barrier precautions were implemented on the day of admission. For these 15 index case-patients, contact screenings of 55 patients and 293 HCWs had identified 1 MRSA-colonized HCW with an unrelated MRSA genotype. For the remaining 141 index case-patients, 9,361 contacts (2,101 patients and 7,260 HCWs) were screened.

In total, 65 spa types were identified among the 141 index cases; the most common were t011 (n = 25 [18%]), t008 (n = 12 [9%]), and t002 (n = 7 [5%]). A total of 40 (29%) isolates had spa types indicative of LA-MRSA; the most prevalent were t011 (n = 25), t034 (n = 6), and t108 (n = 6) (Table 1). Luk-PV genes, indicative of PVL, were detected in 24 (18%) of 131 isolates investigated, all categorized as not being LA-MRSA strains. Among 12 MRSA spa type t008 isolates, PVL positivity was detected in 8 (67%) (Table 1), and among 10 (7%) MRSA isolates, the presence of PVL was undetermined.

Mean age among all index case-patients was 53 years. Among patients with LA-MRSA and other MRSA genotypes, no significant differences were found except for sex (Table 2). Among index case-patients with LA-MRSA genotypes, 83% were male, compared with 56% case-patients with other MRSA (p = 0.004). No statistically significant differences were found in length of hospital stay (p = 0.222) and number of days in hospital without barrier precautions (p = 0.503) between index case-patients with LA-MRSA and patients with other MRSA genotypes (Table 2).

Among 141 postexposure screenings, MRSA carriers were identified for 18 (13%) case-patients, yielding 39 newly identified colonized patients and 34 newly identified colonized HCWs with MRSA. Screening of index case-patients with LA-MRSA identified 15 (21%) carriers, and screening of index case-patients with other MRSA identified 58 (79%) carriers. Of these 73 MRSA carriers, 47 (64%) were colonized with a MRSA spa type that was identical to that of the corresponding index case-patient; 3 patients had spa types matching those of 2 index case-patients with LA-MRSA, and 44 had spa types matching those of 16 index case-patients with other MRSA. Transmission of MRSA (i.e., outbreaks) was documented for 18 index patients; the largest outbreak consisted of 12 secondary cases (8 patients and 4 HCWs, spa type t1081), and most outbreaks (11 [61%] of 18) consisted of only 1 secondary case (Figure). Contact screening for 1 index case-patient with LA-MRSA (t011) revealed 1 outbreak consisting of 3 patients with a MRSA genotype (t067) that was not LA-MRSA. These newly identified cases of MRSA carriage were considered to...

| Table 1. Genotypes of MRSA from index and secondary case-patients, the Netherlands* |
|-----------------------------------------------|------------------|------------------|------------------|
| spa-type | No. (%) with PVL | No. index case-patients, n = 141 | No. outbreaks, n = 18 | No. (%) secondary case-patients, n = 47 |
| LA-MRSA | | | | |
| t011 | 0/24 | 25 | 1 | 2 (4) |
| t034 | 0/5 | 6 | 0 | 0 |
| t108 | 0/5 | 6 | 1 | 1 (2) |
| t899 | 0/2 | 2 | 0 | 0 |
| t2330 | 0/1 | 1 | 0 | 0 |
| Other MRSA | | | | |
| t008 | 8/12 (67) | 12 | 0 | 0 |
| t002 | 1/7 (14) | 7 | 2 | 4 (2) |
| t032 | 0/5 | 5 | 1 | 6 (13) |
| t064 | 0/5 | 5 | 1 | 5 (11) |
| t1081 | 0/3 | 5 | 3 | 14 (31) |
| t888 | 0/3 | 4 | 0 | 0 |
| t038 | 0/3 | 3 | 1 | 1 (2) |
| t267 | 0/3 | 3 | 0 | 0 |
| t001 | 0/1 | 2 | 0 | 0 |
| t018 | 0/2 | 2 | 0 | 0 |
| t179 | 0/2 | 2 | 1 | 1 (2) |
| t447 | 0/2 | 2 | 1 | 1 (2) |
| t1430 | 0/2 | 2 | 0 | 0 |
| t1469 | 0/2 | 2 | 0 | 0 |
| Singletons | 14/42 (33)† | 45 | 6† | 12 (24) |

aPVL, Panton-Valentine leukocidin; MRSA, methicillin-resistant Staphylococcus aureus; LA-MRSA, livestock-associated MRSA.

†PVL positive: t022, t040, t044, t054, t131, t131, t138, t437, t657, t690, t791, t852, t2815, t3523, t7277.

††spa types causing outbreaks: t003, t088, t113, t1399, t7277, t9634.
be not associated with the index case with an LA-MRSA genotype.

During 2011, a total of 6,819 blood, 4,828 respiratory tract, and 1,132 wound cultures were performed. For the upper limit of detection, we used only 1 culture per patient per day, yielding 11,903 relevant cultures, divided by the number of patient-days (241,319) (online Technical Appendix Table A).

The ratio between detection and discharge rates did not differ much between patients with LA-MRSA and other MRSA (online Technical Appendix Table A). The parameter for geometric distribution for LA-MRSA and other MRSA is also provided in the online Technical Appendix. There was no reason to reject the hypothesis of a geometrically distributed outbreak size for LA-MRSA; but the hypothesis was rejected for other MRSA (p<0.05).

Based on the genotype-specific ratio between detection and discharge rates, $R_{s}$ values were 0.43 (95% CI 0.32–0.56), 0.12 (95% CI 0.03–0.31), and 0.52 (95% CI 0.38–0.69) for all 27 genotypes, LA-MRSA, and other MRSA, respectively. According to these $R_{s}$-values, the transmissibility of LA-MRSA was considered 4.4 times lower than that of other MRSA (0.38–0.69). The $R_{s}$ value for PVL-positive strains was 0.31 (95% CI 0.14–0.58).

### Discussion

Using data from 62 hospitals in the Netherlands, comprising 372 months of MRSA policy, we determined that livestock-associated MRSA genotypes, compared with other MRSA genotypes, are 4.4 times less likely to spread in the hospital. Our findings in this study add substantial knowledge to findings from our previous study of outbreaks in the Netherlands in 2005 (10,11). The current study included a larger cohort of hospitals and genotyping of all isolates. In our previous study, we compared *sma1* non-typeable MRSA to other MRSA genotypes without further genotyping. The genotyping demonstrates the heterogeneity in index cases with MRSA not associated with livestock. Moreover, in the current study, we collected more detailed patient information, such as admission and discharge dates and the number of days that index and secondary case-patients were treated without barrier precautions, which enabled more precise estimation of parameters. Absence of significant differences in age, length of hospital stay, or number of days not spent in isolation between index case-patients with LA-MRSA and those with other MRSA reduces the possibility that the differences in transmission capacity resulted from differences in patient characteristics. The only difference was that LA-MRSA index case-patients were more likely to be male, reflecting sex distributions among pig farmers and veal calf farmers.

For this study, we made several assumptions. First, no differentiation was made between patients and HCWs. Both are at risk for colonization with MRSA; however, infectious period and infectivity may differ. Second, all carriers were assumed to be equally infectious; whereas, superspreaders could play a major role in the transmission of MRSA in certain outbreaks. The consequences of these assumptions have been discussed in detail elsewhere (10).

This study has several limitations. For this model to work, MRSA outbreaks must be rare and rigorous screening must be performed after the identification of an index case. If multiple outbreaks of the same genotype occur on the same ward, $R_{s}$ would be an overestimation. Here, *spa* typing was used to identify cases of transmission between index and secondary case-patients. Among LA-MRSA, 63% were *spa* type t011; whereas, other MRSA consist of many different *spa* types. The high prevalence of LA-MRSA in pig-dense areas combined with the homogeneity of *spa* types could lead to an actual overestimation of these transmission events (and the estimated $R_{s}$ values of LA-MRSA).

LA-MRSA comprise a well-defined set of *spa* types, most commonly t011, t034, and t108; whereas, other MRSA comprise a highly heterogeneous group with hospital-associated genotypes and PVL-positive, community-associated genotypes (21). Almost 25% of all other MRSA were PVL positive, which is considered a characteristic of community-associated MRSA. Although 25% seems high, the actual incidence of index case-patients with PVL-positive MRSA was 24 in 379 hospital months, comprising an average of 1 index case per 16 months per hospital. In contrast to LA-MRSA and hospital-associated MRSA, there are no established risk factors in the Netherlands for colonization with CA-MRSA, and unknown carriers of these genotypes will not be screened when admitted to hospital (13). Although another study from the Netherlands reported a high number of PVL-positive isolates in MRSA-colonized patients without risk factors as described in the national guidelines (13,22), our findings demonstrate that PVL-positive strains do not constitute a major risk for health care settings in the Netherlands because the introduction rate and the $R_{s}$ in the absence of barrier precautions ($R_{s}$ for PVL-positive strains 0.31, 95% CI 0.14–0.58) are low. Nevertheless, if admission rates increase, outbreaks could emerge despite $R_{s}$ values <1 (20).

### Table 2. Characteristics of index case-patients with LA-MRSA and other MRSA genotypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LA-MRSA, n = 40</th>
<th>Other MRSA, n = 101</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{s}$ (95% CI)</td>
<td>0.12 (0.03–0.30)</td>
<td>0.52 (0.38–0.69)</td>
<td>NA</td>
</tr>
<tr>
<td>Age, y</td>
<td>56</td>
<td>52</td>
<td>0.337</td>
</tr>
<tr>
<td>Male, no. (%)</td>
<td>33 (83)</td>
<td>57 (56)</td>
<td>0.004</td>
</tr>
<tr>
<td>Medial length of stay, d</td>
<td>13</td>
<td>10</td>
<td>0.222</td>
</tr>
<tr>
<td>Median days not in isolation</td>
<td>5</td>
<td>6</td>
<td>0.503</td>
</tr>
</tbody>
</table>

*MRSA, methicillin resistant *Staphylococcus aureus*; LA-MRSA, livestock-associated MRSA; $R_{s}$, single-admission reproduction number; NA, not applicable.
spa type t1081 was associated with the highest number of outbreaks and with most secondary cases. This spa type has also been associated with outbreaks in nursing homes across the Netherlands. For spa type t1081, the MIC for cefoxitin (data not shown) is low (3 mg/L [range 3–8 mg/L]), hampering laboratory detection during routine procedures, which might have contributed to the high number of secondary cases found with this spa type.

Whole-genome analyses of multiple sequence type 398 S. aureus strains suggests that LA-MRSA originated from methicillin-susceptible S. aureus that crossed species barriers from humans to livestock, where it acquired resistance traits (23). It has been hypothesized that the transition from humans to animals was associated with the loss of several human immune evasion genes, carried on phage φSa3, which may prevent human niche adaptation of LA-MRSA (24). Whether this loss is associated with the lower $R_e$ remains to be determined.

The epidemiology of CA-MRSA in Europe differs markedly from that in the United States; >50% of community-acquired S. aureus infections in Europe are caused by a few PVL-positive clones (25). There is a paucity of data on the nosocomial transmission capacity of CA-MRSA. In hospitals in the Netherlands, though, the estimated $R_e$ of CA-MRSA, consisting of a heterogeneous group of genotypes, was estimated to be 0.07 (95% CI 0.00–0.28) (26), and in the hospitals participating in the present study, the $R_e$ value of PVL MRSA strains was 0.31, 95% CI 0.14–0.58. The differences between Europe and the United States regarding the epidemiology of PVL-positive CA-MRSA, therefore, remain unexplained.

Current guidelines in the Netherlands recommend MRSA screening for all patients with professional exposure to livestock, and many hospitals treat such patients in isolation while screening results are pending (i.e., preemptive isolation). In a previous multicenter study in the Netherlands, though, the estimated $R_e$ of livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) strains replacing traditional nosocomial MRSA strains? Clin Infect Dis. 2008;46:787–94. http://dx.doi.org/10.1086/528716


Dr Hetem is a physician specializing in clinical microbiology at the University Medical Center in Utrecht, the Netherlands. His research interests are the epidemiology of MRSA and the decolonization of S. aureus.


Address for correspondence: David J. Hetem, Department of Clinical Microbiology, University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands; email: d.j.hetem@umcutrecht.nl
CTX-M β-Lactamase–producing *Klebsiella pneumoniae* in Suburban New York, New York, USA

Guiqing Wang, Tiangui Huang, Pavan Kumar Makam Surendraiah, Kemeng Wang, Rashida Komal, Jian Zhuge, Chian-Ru Chern, Alexander A. Kryszuk, Cassidy King, and Gary P. Wormser

CTX-M extended-spectrum β-lactamase (ESBL)–producing *Klebsiella pneumoniae* isolates are infrequently reported in the United States. In this study, we analyzed nonduplicate ESBL-producing *K. pneumoniae* and *Escherichia coli* clinical isolates collected during 2005–2012 at a tertiary care medical center in suburban New York City, USA, for the presence of *bla*<sub>CTX-M</sub>, *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>* genes. Despite a high prevalence of *bla<sub>CTX-M</sub>* genes in ESBL-producing *E. coli* since 2005, *bla<sub>SHV</sub>* and *bla<sub>TEM</sub>* genes were not detected in *K. pneumoniae* until 2009. The prevalence of CTX-M–producing *K. pneumoniae* increased significantly over time from 1.7% during 2005–2009 to 26.4% during 2010–2012 (p<0.0001). CTX-M-15 was the dominant CTX-M genotype. Pulsed-field gel electrophoresis and multilocus sequence typing revealed high genetic heterogeneities in CTX-M–producing *K. pneumoniae* isolates. This study demonstrates the recent emergence and polyclonal spread of multidrug resistant CTX-M–producing *K. pneumoniae* isolates among patients in a hospital setting in the United States.

CTX-M enzymes are a group of class A extended-spectrum β-lactamases (ESBLs) that are rapidly spreading among *Enterobacteriaceae* worldwide (1). Since the initial isolation of CTX-M-1 from a European patient in the late 1980s (2), >130 CTX-M allelic variants have been described (http://www.lahey.org/Studies/other.asp#table1). These CTX-M variants have been divided into 5 major phylogenetic groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, or CTX-M-25 on the basis of their amino acid sequences (1,2).

During the past decade, CTX-M enzymes have become the most prevalent ESBL enzymes in clinical *Enterobacteriaceae* isolates, especially in ESBL-producing *Escherichia coli* in Europe, Asia, and South America (1,3). By contrast, SHV- and TEM-type ESBL enzymes are primarily found in ESBL-producing *K. pneumoniae* and *E. coli* clinical isolates in North America (3). In the United States, CTX-M–like ESBL-producing *Enterobacteriaceae* was first reported in 2003, when CTX-M enzymes were detected in 9 *E. coli* clinical isolates from 5 US states (4). The spread of CTX-M type ESBL in *Enterobacteriaceae*, however, was not appreciated until 2007 when a Texas study showed a high prevalence of CTX-M ESBL in *E. coli* clinical isolates recovered during 2000–2005 (5). Since then, CTX-M–producing *E. coli* isolates have been documented in dispersed US geographic regions (3,6,7). CTX-M enzymes are now the predominant ESBL type in *E. coli* clinical isolates in Texas (5), Pennsylvania (6), Illinois (8), and New York (9,10).

CTX-M–type ESBL enzymes have also been reported in the United States in some non-*E. coli Enterobacteriaceae* species, such as *Klebsiella* spp. (5,11,12), *Proteus mirabilis* (5,11), *Enterobacter* spp. (5), *Salmonella* spp. (13), *Shigella* spp. (14), and *Morganella morganii* (5). Nevertheless, CTX-M–type ESBL have been principally detected and reported in *E. coli* clinical isolates. To date, <50 CTX-M–producing *K. pneumoniae* isolates have been described in the United States, and the epidemiologic and microbiological data provided have been limited (5,11,12,15–18). The implications of CTX-M–producing *K. pneumoniae* for laboratory detection, patient care, and public health in the United States remain to be elucidated.

In this study, we investigated the prevalence of SHV–, TEM–, and CTX-M–encoding genes in a large collection of ESBL-producing *K. pneumoniae* and *E. coli* clinical isolates from a tertiary care medical center in suburban New York City in Westchester County, New York, over an
8-year period (2005–2012). Microbiological characteristics of CTX-M ESBL-producing K. pneumoniae isolates were examined, and certain clinical/epidemiologic features of patients with these isolates were analyzed.

Materials and Methods

Bacterial Isolates and Phenotypic Detection of ESBLs

Nonduplicate K. pneumoniae clinical isolates were recovered from patient specimens during January 2005–July 2012 at the clinical microbiology laboratory of Westchester Medical Center. These included 208 blaKPC-negative non-K. pneumoniae carbapenemase (non-KPC) ESBL-producing or third-generation cephalosporin-resistant K. pneumoniae isolates and 228 KPC (blaKPC-positive)–producing K. pneumoniae isolates. In addition, 163 nonduplicate ESBL-producing E. coli clinical isolates from the same period were also analyzed for comparison. Isolates were randomly selected to span the entire study year with an approximately equal number of isolates per quarter; only 1 isolate from each patient was chosen and tested. The center is a 643-bed academic tertiary-care medical center in Westchester County, New York. The Institutional Review Board of New York Medical College approved this study.

The bacterial isolates were identified and evaluated for antimicrobial drug susceptibility with the MicroScan Walk-Away 96 system (Siemens, Sacramento, CA, USA). ESBL production was phenotypically confirmed by a double-disk or broth microdilution method for suspected ESBL isolates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (19). The antimicrobial drug susceptibility of CTX-M–producing K. pneumoniae isolates against selected antimicrobial drugs was also assessed with standardized CLSI disk diffusion and Etest methods. Bacterial isolates were refrigerated on nutrient agar slants or were frozen (-80°C) in MicroBank cryovials containing 20% glycerol. As the DNA template in the PCR assays, 2–3 μL of the boiled cell suspension was used. PCR amplification of blCTX,M, blASHV, blTEM, and blKPC genes in K. pneumoniae and E. coli clinical isolates was performed by using a consensus primer pair specific to each type of β-lactamase as described (20–22). A multiplex PCR was developed and used for simultaneous detection of blCTX,M (551 bp) and blTEM (972 bp) genes. Two PCRs were performed for blASHV-ESBL and blKPC, respectively. PCRs were carried out by using the HotStart DNA polymerase master mix (QIA-GEN, Germantown, MD, USA) with 30–35 cycles at an annealing temperature of 50°C for blCTX,M and blTEM, and 52°C for blASHV and blKPC. PCR products were analyzed by agarose gel electrophoresis or by using the QIAxcel system (QIAGEN). The specificity of PCR amplicons on representative isolates was confirmed by DNA sequencing.

DNA Sequencing

For DNA sequencing, PCR products were purified by using the PCR Purification kit (QIAGEN) or the ExoSAP-IT PCR Clean-up kit (Affymetrix, Cleveland, OH, USA), according to the manufacturer’s instructions. The purified DNA amplicons were sequenced by using an ABI Prism BigDye Terminator (version 1.1) cycle sequencing ready reaction kit on the ABI Prism 3730xl or ABI 3500xl DNA Analyzers (Applied Biosystems, Foster City, CA, USA) in-house, or by a commercial facility (GeneWiz, South Plainfield, NJ, USA). The CTX-M, TEM, and SHV gene sequences were compared with sequences in GenBank by using the NCBI basic local alignment search tool (www.ncbi.nlm.nih.gov/BLAST).

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed by using primers and conditions as described by Diancourt et al. (23). PCR products from ESBL–producing K. pneumoniae isolates representing each CTX-M genotype was performed as described (24). The MLST database maintained by the Pasteur Institute (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Pulsed-field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) on CTX-M ESBL–producing K. pneumoniae isolates representing each CTX-M genotype was performed as described (24). The GelCompare II software, (version 2.0; Applied Maths, Austin, TX, USA) was used to calculate the Dice similarity coefficients and generate dendrograms by cluster analysis with the unweighted-pair group method using average linkages. Pulsotype designations were assigned at the ≥80% profile similarity level.

Results

CTX-M in ESBL–producing, non-KPC K. pneumoniae Clinical Isolates

Of the 121 ESBL–producing K. pneumoniae isolates originally recovered during 2005–2009, blASHV and blTEM genes were detected in 102 (84.3%) and 61 (50.4%) of 121 isolates, respectively (Table 1). Overall, 25 CTX-M-type
ESBL K. pneumoniae were identified. However, none of the 81 K. pneumoniae isolates from 2005 through 2008 was positive for blaCTX-M genes. CTX-M–type ESBL was first detected in 2 (5.0%) of 40 K. pneumoniae isolates from 2009. The prevalence of K. pneumoniae isolates carrying the CTX-M–encoding genes increased to 6 (17.6%) of 34 in 2010 and 12 (34.3%) of 35 in 2011. The level remained high (27.8%, 5/18) in the first 7 months of 2012. Overall, only 2 (1.7%) of 121 ESBL-producing K. pneumoniae isolates from 2005 through 2009 carried the blaCTX-M genes, compared with 23 (26.4%) of 87 isolates from 2010 through 2012 (p<0.0001, Fisher exact test), indicating the rapid emergence and spread of CTX-M enzymes among ESBL-producing K. pneumoniae clinical isolates since 2009.

**CTX-M in ESBL-producing E. coli Clinical Isolates**

One hundred sixty-three ESBL-producing E. coli clinical isolates from 2005 through 2012 were analyzed by PCR for detection of blaESBL genes of the SHV, TEM, and CTX-M types (Table 2). Unlike the situation with K. pneumoniae, blaCTX-M genes were detected in ESBL-producing E. coli isolated as early as 2005. Overall, 89 (54.6%) of 163 ESBL E. coli isolates from the 8-year period carried blaCTX-M genes. CTX-M was the leading ESBL type in all years examined except 2008. The blaCTX-M genes from 47 (52.8%) of 89 CTX-M–producing E. coli isolates were sequenced. CTX-M-15 was determined in 45 (95.7%) of 47 CTX-M–producing E. coli isolates analyzed. CTX-M-1 and CTX-M-3 genotypes were each found in 1 ESBL E. coli isolate.

**CTX-M in KPC-producing K. pneumoniae Clinical Isolates**

Two hundred twenty-eight KPC-producing K. pneumoniae isolates from 2005 to 2012 were examined by PCR for detection of blaKPC gene. All K. pneumoniae isolates were positive for the blaKPC gene by PCR as described (22). None was positive for the blaCTX-M gene. and certain microbiological characteristics of the isolates are shown in Table 3, Appendix (wwwnc.cdc.gov/EID/article/19/11/12-1470-T3.htm). Mean patient age was 56 years, and 13 (52%) of the patients were males. Sixteen patients (64%) had bacteriuria. CTX-M–producing K. pneumoniae isolates were recovered from 13 (52%) patients within 72 hours of hospital admission; however, 18 (72%) of these patients had been hospitalized in the 8 months before the current admission.

The blaCTX-M genes from all 25 CTX-M ESBL–producing K. pneumoniae isolates from 2009 through 2012 were sequenced. CTX-M-15 was identified in 19 (76.0%) and was the dominant CTX-M genotype. The remaining 6 isolates were determined to be CTX-M-3 (n = 4), CTX-M-1 (n = 1), and CTX-M-2 (n = 1), respectively. Twenty-four (96.0%) had coexisting blashv β-lactamases, which were predominantly non-ESBL blashv11 (n = 15) and blashv1 (n = 5). Four additional K. pneumoniae carried ESBL-type blashv β-lactamases, including blashv12 (n = 1), blashv2 (n = 1), and blashv3 (n = 2). Seventeen (68.0%) were positive for TEM-type β-lactamases, and all were confirmed to be blaTEM1.

The antimicrobial drug susceptibilities of CTX-M–producing K. pneumoniae isolates are summarized in Table 4. Of the 25 CTX-M–producing K. pneumoniae isolates examined in this study, only 12% (n = 3) and 36% (n = 8) of isolates were susceptible to ciprofloxacin and gentamicin, respectively. Low susceptibility rates were also observed for piperacillin/tazobactam (36%), tetracycline (20%) and trimethoprim/sulfamethoxazole (4%). Twenty-three of the 25 (92%) isolates tested were susceptible to carbapenems. Notably, the 2 carbapenem-resistant K. pneumoniae isolates (PK30 and PK107) carried blashv11 and blatem1. One of these K. pneumoniae isolates also showed resistance to colistin with an MIC of 64µg/mL. Both patients died of complications associated with bloodstream and respiratory tract infections. Three of 22 CTX-M–producing K. pneumoniae isolates examined by Etest were nonsusceptible to tigecycline (MICs 3 µg/mL, 3 µg/mL, and 8 µg/mL).

All 25 CTX-M–producing K. pneumoniae isolates examined were resistant to cefotaxime, and all but 1 isolate

---

**Table 1. Detection of blaESBL Genes of the SHV, TEM, and CTX-M types in 208 ESBL-producing Klebsiella pneumoniae clinical isolates, 2005–2012**

<table>
<thead>
<tr>
<th>Year</th>
<th>No. isolates tested</th>
<th>blaSHV (%) positive isolates</th>
<th>blaTEM (%) positive isolates</th>
<th>blaCTX-M (%) positive isolates</th>
<th>CTX-M type (no. isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>22</td>
<td>20 (90.9)</td>
<td>7 (31.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>21</td>
<td>15 (71.4)</td>
<td>11 (52.4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>17</td>
<td>11 (64.7)</td>
<td>10 (58.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>21</td>
<td>19 (90.5)</td>
<td>10 (47.6)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>40</td>
<td>37 (92.5)</td>
<td>23 (57.3)</td>
<td>2 (5.0)</td>
<td>CTX-M-15 (2)</td>
</tr>
<tr>
<td>2010</td>
<td>34</td>
<td>31 (91.2)</td>
<td>9 (26.4)</td>
<td>6 (17.6)</td>
<td>CTX-M-15 (4), CTX-M-2 (1), CTX-M-3 (1)</td>
</tr>
<tr>
<td>2011</td>
<td>35</td>
<td>32 (91.4)</td>
<td>13 (36.1)</td>
<td>12 (34.3)</td>
<td>CTX-M-15 (9), CTX-M-3 (2), CTX-M-1 (1)</td>
</tr>
<tr>
<td>2012</td>
<td>18</td>
<td>16 (88.9)</td>
<td>8 (44.4)</td>
<td>5 (27.8)</td>
<td>CTX-M-15 (4), CMX-M-3 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>181 (87.0)</td>
<td>91 (43.8)</td>
<td>25 (12.0)</td>
<td></td>
</tr>
</tbody>
</table>

*ESBL, extended-spectrum β-lactamase.*
showed higher MICs of cefotaxime than of ceftazidime. The 50% minimum inhibitory concentration (MIC<sub>50</sub>) for cefotaxime among these isolates was >256 µg/mL. By contrast, the MIC<sub>90</sub> and 90% inhibitory concentration for ceftazidime were 16 µg/mL and 128 µg/mL, respectively. Two CTX-M–producing *K. pneumoniae* isolates (8.0%) were susceptible (MIC ≤4 µg/mL) and 5 isolates (20%) were intermediate in susceptibility (8 µg/mL) to ceftazidime according to the 2010 revised CLSI breakpoints (Figure 1). In addition, we determined the susceptibilities of 22 CTX-M–producing *K. pneumoniae* isolates against cefotaxime and ceftazidime by using the standard disk diffusion method. All CTX-M–producing *K. pneumoniae* isolates examined were resistant to cefotaxime by disk diffusion (mean inhibitory zone size 8.3 mm; range 6–13 mm). Two of these isolates were susceptible (≥21 mm) and 5 had intermediate (18–20 mm) susceptibility to ceftazidime by disk diffusion (Table 3). The disk diffusion results showed a category agreement with the Etest MIC of 100% for cefotaxime and 90.9% for ceftazidime with 2 minor errors.

### PFGE and MLST Analysis of CTX-M–producing *K. pneumoniae*

Of 17 representative CTX-M–producing *K. pneumoniae* isolates analyzed by PFGE, 8 different pulsotypes (PF1–8) were identified with Dice coefficients of ≥80% similarity (Figure 2). Ten of 17 *K. pneumoniae* isolates examined belonged to 3 major groups (PF3, PF4, PF5) with 3–4 isolates in each group. The remaining pulsotypes contained only 1 or 2 *K. pneumoniae* isolates. No clear temporal relationship was shown among the highly related isolates.

MLST was performed on 18 CTX-M–producing *K. pneumoniae* isolates. These isolates were selected to represent different CTX-M genotypes, pulsotypes, antimicrobial susceptibility profiles, and years of isolation. Twelve STs were recognized for the *K. pneumoniae* isolates examined (Table 3). Notably, all 3 CTX-M group 1, non–CTX-M-15 *K. pneumoniae* isolates analyzed (KP38, PK107, and PK135) had ST11, whereas 10 different STs (ST15, ST16, ST17, ST48, ST147, ST252, ST258, ST280, ST392, and ST437) were identified for the 14 CTX-M-15 *K. pneumoniae* isolates. Isolate F351 was the only non–CTX-M-1 group *K. pneumoniae* isolate identified in this study and was determined to be a separate group (ST792) by MLST. Of the 14 CTX-M–producing *K. pneumoniae* isolates evaluated simultaneously by DNA sequencing, PFGE and MLST, a high genetic divergence was demonstrated by the detection of 4 CTX-M genotypes (CTX-M-1, CTX-M-2, CTX-M-3, and CTX-M-15), 8 pulsotypes (PF1–8) and 11 STs (ST11, ST15, ST16, ST17, ST48, ST147, ST252, ST280, ST392, ST437, and ST792) (Figure 2).

### Discussion

CTX-M ESBL–producing *E. coli*, especially ST131 strains, have emerged in recent years in several US states (5–7, 25, 26). In this study, we detected *bla*<sub>CTX-M</sub> genes in ESBL-producing *E. coli* strains isolated from patients at a tertiary care medical center in suburban New York City as early as 2005. Eighty-nine (54.6%) of 163 ESBL-producing *E. coli* isolates in the study period (2005–2012) carried *bla*<sub>CTX-M</sub>. Our findings confirm the emergence and dominance of CTX-M enzymes in ESBL-producing *E. coli* since the mid-2000s in the New York City metropolitan area (9, 10).

Despite this high prevalence of CTX-M in ESBL-producing *E. coli* since 2005, none of 81 ESBL-producing *K. pneumoniae* isolates recovered from patients at the same tertiary care medical center from 2005 through 2008 was positive for *bla*<sub>CTX-M</sub>. CTX-M–type ESBL was first detected in *K. pneumoniae* isolates from our institution in 2009. The percentage of *K. pneumoniae* isolates carrying *bla*<sub>CTX-M</sub> has increased significantly since then. During 2010–2012, *bla*<sub>CTX-M</sub> genes were identified in 23 of 87 (26.4%) ESBL-producing *K. pneumoniae* isolates. These data demonstrate the rapid emergence and spread of CTX-M ESBL–producing *K. pneumoniae* in our patients. To date, CTX-M–producing *K. pneumoniae* has been recognized in several US states, including Texas (2004–2007, n = 11) (5, 12), Nebraska (2005, n = 1)

<table>
<thead>
<tr>
<th>Year</th>
<th>No. isolates tested</th>
<th><em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;</th>
<th><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;</th>
<th><em>bla</em>&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>CTX-M type (no. isolates/total no. isolates sequenced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>20</td>
<td>6 (30.0)</td>
<td>4 (20.0)</td>
<td>7 (35.0)</td>
<td>CTX-M-15 (5/5)</td>
</tr>
<tr>
<td>2006</td>
<td>16</td>
<td>1 (6.3)</td>
<td>3 (18.8)</td>
<td>12 (56.3)</td>
<td>CTX-M-15 (6/6)</td>
</tr>
<tr>
<td>2007</td>
<td>24</td>
<td>4 (16.7)</td>
<td>9 (37.5)</td>
<td>10 (50.0)</td>
<td>CTX-M-15 (4/6), CTX-M-1 (1/6), CTX-M-3 (1/6)</td>
</tr>
<tr>
<td>2008</td>
<td>20</td>
<td>5 (25.0)</td>
<td>10 (50.0)</td>
<td>6 (30.0)</td>
<td>CTX-M-15 (5/5)</td>
</tr>
<tr>
<td>2009</td>
<td>22</td>
<td>0 (0)</td>
<td>12 (54.5)</td>
<td>13 (59.1)</td>
<td>CTX-M-15 (5/5)</td>
</tr>
<tr>
<td>2010</td>
<td>20</td>
<td>1 (5.0)</td>
<td>9 (45.0)</td>
<td>13 (65.0)</td>
<td>CTX-M-15 (6/6)</td>
</tr>
<tr>
<td>2011</td>
<td>21</td>
<td>3 (14.3)</td>
<td>9 (42.9)</td>
<td>11 (52.3)</td>
<td>CTX-M-15 (8/8)</td>
</tr>
<tr>
<td>2012</td>
<td>20</td>
<td>2 (10.0)</td>
<td>10 (50.0)</td>
<td>13 (65.0)</td>
<td>CTX-M-15 (6/6)</td>
</tr>
</tbody>
</table>
| Total| 163                 | 22 (13.5)           | 66 (40.5)           | 89 (54.6)           | has been recognized in several US states, including Texas (2004–2007, n = 11) (5, 12), Nebraska (2005, n = 1)
(15), Pennsylvania (2007, n = 5) (11), and 1 isolate in 2007 each from California, Massachusetts, Michigan, New Jersey, New York, Washington, and Wisconsin (12). In addition, a few CTX-M *K. pneumoniae* isolates have been reported from 2 collections of the SMART surveillance program with isolates recovered during 2008–2009 (16) and 2009–2010 (18). No CTX-M was detected in US ESBL-producing *K. pneumoniae* isolates collected before 2000 (3), with all CTX-M–producing *K. pneumoniae* isolates recovered from US patients in or after 2004. Therefore, we speculate that the emergence and spread of *bla*<sub>CTX-M</sub> in *K. pneumoniae* are recent evolutionary events that most likely occurred in the mid- to late-2000s in the United States.

The particular CTX-M enzyme type in ESBL-producing *K. pneumoniae* and *E. coli* varies geographically. CTX-M-15, which belongs to the CTX-M-1 group, is the most prevalent CTX-M allele with a worldwide distribution (1,2,26). CTX-M-14, which belongs to the CTX-M-9 group, is another common variant that is highly prevalent in some European and Asian countries (27–30), whereas CTX-M-2 in the CTX-M-2 group and CTX-M-8 seem to be dominant in South America (1,31). In the United States, CTX-M-15 is the most frequently detected genotype among CTX-M–producing *K. pneumoniae* isolates, followed by CTX-M-14 (5,11,12). CTX-M-2 group and CTX-M-8 group ESBL-producing *K. pneumoniae* each was identified in 1 isolate (16).

Our data provide strong evidence for the recent, rapid emergence, and polyclonal spread of the CTX-M-1 group, especially CTX-M-15 ESBL-producing *K. pneumoniae* in a US hospital setting. In this study, 24 (96.0%) of 25 *bla*<sub>CTX-M</sub>-positive *K. pneumoniae* were categorized as group 1 CTX-M, including isolates encoding CTX-M-15 (n = 19), CTX-M-1 (n = 1), and CTX-M-3 (n = 4). Similarly, group 1 CTX-M was detected in 47 (100%) of 47 *bla*<sub>CTX-M</sub>-positive *E. coli* isolates. In addition, 1 *K. pneumoniae* isolate had the CTX-M-2 genotype. No CTX-M-14 was detected in these *K. pneumoniae* and *E. coli* isolates. CTX-M-14 has been reported in *E. coli* ESBL isolates in several US states, including geographically adjacent Pennsylvania (6). CTX-M-14 has also been reported in *K. pneumoniae* isolates in the Calgary Healthcare Region of Canada (32). Why CTX-M-14 is absent in the ESBL-producing *E. coli* and *K. pneumoniae* isolates from the New York, NY, metropolitan area is unknown. Because CTX-M-15–producing *K. pneumoniae* isolates may exhibit significantly higher resistance rates to ciprofloxacin and pipercillin-tazobactam than CTX-M-14–producing isolates (27,28), CTX-M genotypes and their antimicrobial drug profiles should be monitored among CTX-M–producing *E. coli* and *K. pneumoniae* isolates in regions where they are emerging.

We investigated the genetic relatedness of CTX-M–producing *K. pneumoniae* isolates by PFGE and MLST. Of the 17 representative isolates examined by PFGE, 8 different pulsotypes were determined. Similarly, 12 MLST STs were identified for the 18 CTX-M–producing isolates analyzed. Our data, in combination with findings from other groups (1), suggest that CTX-M–producing *K. pneumoniae* isolates are genetically heterogeneous. The emergence and polyclonal spread of CTX-M–producing *K. pneumoniae* likely occurred among isolates with diverse genetic backgrounds. This hypothesis contrasts with findings regarding KPC-producing *K. pneumoniae*: a clonal spread of KPC-producing *K. pneumoniae* isolates belonging to the ST258 lineage was observed by us (33) and Pournaras et al. (34).

In clinical strains, CTX-M–encoding genes have commonly been located on plasmids that vary in size from 7 kb to 160 kb (2). Plasmid-mediated transmission of CTX-M genes in *Enterobacteriaceae* that involves several motile

---


<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. isolates tested</th>
<th>No. (%) susceptible isolates</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>MIC range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime†</td>
<td>22</td>
<td>0 (0.0)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>16–&gt;256</td>
</tr>
<tr>
<td>Cefazidime†</td>
<td>22</td>
<td>2 (9.1)</td>
<td>0.094</td>
<td>0.125</td>
<td>0.047–2.0</td>
</tr>
<tr>
<td>Pip/Tazo</td>
<td>25</td>
<td>9 (36.0)</td>
<td>64</td>
<td>&gt;64</td>
<td>16–264</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>25</td>
<td>23 (92.0)</td>
<td>≤2</td>
<td>&lt;2</td>
<td>≤2–4</td>
</tr>
<tr>
<td>Meropenem†</td>
<td>22</td>
<td>21 (95.5)</td>
<td>0.25</td>
<td>1.5</td>
<td>0.19–6.0</td>
</tr>
<tr>
<td>Imipenem†</td>
<td>22</td>
<td>20 (90.1)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>≤1–2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>25</td>
<td>3 (12.0)</td>
<td>≤16</td>
<td>≤32</td>
<td>≤16–≤32</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>25</td>
<td>8 (32.0)</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤4–≤8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>25</td>
<td>5 (20.0)</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>≤4–≤8</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>25</td>
<td>1 (4.0)</td>
<td>&gt;2/38</td>
<td>&gt;/38</td>
<td>&lt;2/38–&lt;2/38</td>
</tr>
<tr>
<td>Tigecycline‡‡</td>
<td>22</td>
<td>19 (86.4)</td>
<td>1</td>
<td>3</td>
<td>0.75–8</td>
</tr>
<tr>
<td>Colistin†§</td>
<td>22</td>
<td>21 (95.5)</td>
<td>0.25</td>
<td>0.38</td>
<td>0.19–64</td>
</tr>
</tbody>
</table>

*‡*MICs were determined by Etest.  
†*n* = 25; MIC<sub>50</sub>: 50% minimum inhibitory concentration; MIC<sub>90</sub>: 90% minimum inhibitory concentration; Pip/Tazo, piperacillin/tazobactam; TMP/SMX, trimethoprim/sulfamethoxazole. MICs were determined by the MicroScan system, except for certain antimicrobial agents that were tested by Etest as specified.  
‡Susceptibility defined by Clinical Laboratory and Standards Institute breakpoints for *Acinetobacter baumannii* (19).
genetic elements has been described (2, 35). Given the dominance of CTX-M-15 genotypes among genetically heterogeneous K. pneumoniae isolates, our study also implies the probable horizontal transfer of a genetic element carrying \( \text{bla}_{\text{CTX-M}} \) among K. pneumoniae isolates.

Of the 12 STs determined for the CTX-M ESBL–producing K. pneumoniae isolates, ST11, ST15, ST17, ST48, ST147, and ST258 have been reported in CTX-M–positive K. pneumoniae in Spain, Hungary, or Korea (28, 37, 38). Among these, only ST17 was reported among CTX-M–producing K. pneumoniae isolates in Canada (39). In this study, we determined the STs among CTX-M–producing K. pneumoniae isolates in the United States and document the existence of 6 STs (ST16, ST252, ST280, ST392, ST437, ST792) in CTX-M–producing K. pneumoniae not previously described.

The CTX-M–producing K. pneumoniae isolates evaluated in this study showed several notable epidemiologic, clinical, and microbiological features. First, most CTX-M–producing isolates were recovered from patients with bacteriuria, which is similar to that observed for infections caused by CTX-M–producing E. coli in New York, NY, (9, 10). Although CTX-M–producing K. pneumoniae was isolated in clinical specimens collected within 72 hours of hospitalization in about half of the patients, 18 (72%) of 25 patients had been hospitalized in the prior 8 months. This factor highlights the potential for acquiring CTX-M–producing K. pneumoniae in health care settings and differs from the experience with CTX-M–producing E. coli that are associated with infections arising in the community setting unrelated to exposure to health care facilities (26). Second, the CTX-M–producing K. pneumoniae study isolates exhibited high rates of resistance to gentamicin (68%), trimethoprim-sulfamethoxazole (96%), and tetracycline (80%), in addition to resistance to ciprofloxacin (88%) and piperacillin-tazobactam (64%) as described previously in Europe and Asia (27, 28, 37). Whether such high rates of resistance are associated with the dominant spread of CTX-M-15–producing, rather than CTX-M-14–producing, K. pneumoniae, in these patients is not known. The coexistence of CTX-M ESBL and TEM-1 and SHV-type \( \beta \)-lactamases in these isolates may have also contributed to the observed high rate of antimicrobial drug resistance. All except 1 of our CTX-M–positive K.

Figure 1. MIC distribution for cefotaxime (CTX) and ceftazidime (CAZ) in CTX-M extended-spectrum \( \beta \)-lactamase–producing Klebsiella pneumoniae clinical isolates from a tertiary care medical center, in suburban New York, New York, USA, 2005–2012 (n = 22). The MICs were determined by Etest.

Figure 2. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns showing the genetic relatedness of CTX-M extended-spectrum \( \beta \)-lactamase (ESBL)–producing Klebsiella pneumoniae isolates from patients in suburban New York, NY, USA (n = 17). Eight PFGE pulsetypes (PF1–8) were identified with ≥80% similarity, which is marked by the vertical line. The corresponding CTX-M genotype, sequence type (ST), if available, and year of isolation for each isolate are listed on the right side of the dendrogram.
*K. pneumoniae* isolates produced SHV- and CTX-M–type ESBLs. These findings have clinical implications for selecting empiric antimicrobial drug therapy when infection caused by ESBL-producing *K. pneumoniae* is suspected. The rapid emergence of such CTX-M–producing *K. pneumoniae* isolates, mainly in US hospitals, is also raising new concerns for public health and infection control practice. Third, none of the 228 KPC-producing *K. pneumoniae* isolates examined carried bla<sub>KPC</sub>. Coexistence of bla<sub>KPC</sub> and bla<sub>CTX-M</sub> has only been reported in KPC-producing *K. pneumoniae* in China (40). Whether certain genetic mechanisms prevent KPC-producing *K. pneumoniae* from acquiring bla<sub>CTX-M</sub> is unclear.

This study reveals the rapid emergence and polyclonal spread of CTX-M–producing *K. pneumoniae* in patients in Westchester County, New York. A limitation of our study is that the clinical isolates were collected from patients at a single tertiary-care medical center. Investigations of CTX-M–producing *K. pneumoniae* isolates from a variety of geographic regions should be undertaken to clarify the epidemiology and clinical and public health effects of the emergence of CTX-M–producing *K. pneumoniae* in the United States.

**Acknowledgments**

We thank Ira Schwartz and Maria Aguero-Rosenfeld for helpful advice and generous support. We are also indebted to the technologists at the Westchester Medical Center Clinical Microbiology Laboratory for saving the study isolates and for their technical contributions.

This study was supported by an intramural grant of New York Medical College (No. 46-356-1) sponsored by the Castle-Krob Foundation.

Dr. Wang is the chief of microbiology at the Westchester Medical Center and assistant professor at New York Medical College. His primary research interests include the epidemiology, mechanisms, and molecular diagnosis of emerging antimicrobial drug resistance and tick-borne diseases.

**References**


Address for correspondence: Guiqing Wang, Department of Pathology Clinical Laboratories, Westchester Medical Center, Macy Pavilion, Rm 11-04, 100 Woods Rd, Valhalla, NY 10595, USA; email: guiqing_wang@nymc.edu
The health care system in Papua New Guinea is fragile, and surveillance systems infrequently meet international standards. To strengthen outbreak identification, health authorities piloted a mobile phone–based syndromic surveillance system and used established frameworks to evaluate whether the system was meeting objectives. Stakeholder experience was investigated by using standardized questionnaires and focus groups. Nine sites reported data that included 7 outbreaks and 92 cases of acute watery diarrhea. The new system was more timely (2.4 vs. 84 days), complete (70% vs. 40%), and sensitive (95% vs. 26%) than existing systems. The system was simple, stable, useful, and acceptable; however, feedback and subnational involvement were weak. A simple syndromic surveillance system implemented in a fragile state enabled more timely, complete, and sensitive data reporting for disease risk assessment. Feedback and provincial involvement require improvement. Use of mobile phone technology might improve the timeliness and efficiency of public health surveillance.

Papua New Guinea has been described as a fragile state (1). Health care systems in such settings are characterized by limited infrastructure, lack of equity, management capacity issues, and inadequate disease information (1). In Papua New Guinea, insufficient investment by government, weak management and leadership capacity, and an inadequate number of health care personnel play a crucial role in the suboptimal performance of the health care system (2). Despite these limitations, the country is working toward reaching the minimum requirements of disease surveillance for the International Health Regulations (IHR 2005) (3).

Health indicators for Papua New Guinea illustrate some of the country’s challenges: 87% of the population lives in rural areas, the number of primary health care facilities has decreased by 40% over 20 years (2), and only 3% of roads are paved. The average life expectancy is 53 years, and the maternal mortality rate of 733/100,000 live births is likely underestimated. Communicable diseases remain the primary causes of illness and death in all age groups, and outbreaks are frequently reported. Lack of health system access and preparedness are particular problems in remote, rural settings (4,5), whereas migration to informal, periurban settlements and weak infrastructure have been identified as risk factors for disease outbreaks in urban areas (6). When compared with other countries in the region, Papua New Guinea often sees more severe effects from outbreaks of commonly occurring pathogens, particularly in remote settings (4,7–11). Special populations, such as internally displaced persons, may be particularly vulnerable to disease outbreaks.

The Papua New Guinea National Health Information System (NHIS) monitors trends for public health syndromes (12); in recent years, the Hospital Based Active Surveillance (HBAS) system has been the cornerstone of surveillance for suspected cases of measles, poliomyelitis, and neonatal tetanus (13). However, the surveillance system for diseases targeted for elimination or eradication is not achieving globally established performance targets (14), and systems for the timely monitoring of endemic diseases, such as diarrheal diseases, are also weak (15). Syndromic surveillance offers a useful adjunct to
diagnosis-based disease surveillance in developing countries (16) and has recently been successfully implemented in the Pacific region (17). These systems can be used to detect outbreaks early, to follow the magnitude and geographic distribution of outbreaks, to monitor disease trends, and to provide reassurance that an outbreak has not occurred (1).

The use of mobile technology to support the achievement of health objectives has the potential to transform service delivery globally (18). Electronic reporting of infectious disease surveillance data has been shown to improve both timeliness and completeness of reporting (19). Health information systems are potential beneficiaries of mobile health solutions for accelerating vital event monitoring in the Asia-Pacific region (20). In recent years, greater competition within the communications sector has dramatically increased mobile phone network coverage in Papua New Guinea (21). After the delayed detection of serious outbreaks with high mortality rates in rural areas (4,5,9), including an ongoing nationwide cholera outbreak for which the timeliness of surveillance was poor, Papua New Guinea health authorities piloted a mobile phone–based syndromic surveillance system (MOPBASSS) for timely outbreak detection. We describe the system, evaluate its attributes, and determine whether it met its objectives.

Materials and Methods

System Descriptions

Health System

Papua New Guinea’s population is unevenly distributed among 4 regions; almost 40% of the population lives in the highlands region. The country’s 20 provinces operate within a decentralized health system (22). National health authorities have overall responsibility for health care policy and standards, providing technical advice, coordination of the health information system, health planning, and data systems (22). Primary health care is the responsibility of provincial governments, and provincial hospitals report to the national level (2). Health services are provided through a system of community aid posts, health centers and subcenters, and district and provincial hospitals, as well as a national referral hospital.

NHIS

Since 1989, national health authorities in Papua New Guinea have monitored the performance of the health system by using the computerized NHIS (23). By 2002, this passive system was centrally managed and regarded as providing quality data for health care monitoring and planning, with links across all health system levels (12). Data relevant to health management and disease control programs are collected monthly on paper-based health records from each health center and sent through the district health office to the provincial health office, where the data are entered into a database. The system then calculates percentages using census population data as denominators to provide analysis of disease outbreak and trends (12). Hard-copy and electronic data are sent to the national level, where they are re-entered and cleaned before being integrated into the national system. While reporting completeness is strong (24), data timeliness and accuracy are not (25).

HBAS System

Since the late 1990s, a hospital-based surveillance system has been in use in Papua New Guinea (13) and has monitored suspected cases of measles, neonatal tetanus, and acute flaccid paralysis (AFP). This zero-reporting system, in which designated reporting sites report even if there are 0 cases, is driven by surveillance officers from the provincial health authorities, who visit the provincial hospitals to review registers and discuss recent patient illness manifestations (signs and symptoms) with the treating clinicians. The forms are compiled monthly and become the documentary evidence to determine if surveillance targets are met and whether poliomyelitis can be excluded as the cause of AFP cases (13). The sensitivity of this system is suboptimal, and global performance targets are not routinely met (14).

Events-based Surveillance System

Information about events (e.g., disease outbreaks, clusters of deaths in humans or animals) that are a potential risk to public health is collected, verified, and assessed by using ad hoc reports transmitted through the health system but also by recording rumors and reports identified through informal channels. Documentation of risk assessments began in 2009.

MOPBASSS

The MOPBASSS used in Papua New Guinea was tested in 2 health centers in Port Moresby in 2010, then piloted nationwide during epidemiologic weeks 17–26 during 2011. A 2-stage randomization process first selected the participating provincial, then district, outpatient settings (3 provincial hospitals and 7 district health centers) to participate as reporting sites. The pilot intervention included the provision of data collection tools, a 1-day on-site training, sample collection materials, guidelines, and mobile phones. Ethical approval to conduct the pilot was granted by the Medical Research Advisory Council of Papua New Guinea (MRAC 10.23).

The MOPBASSS information flow is detailed Figure 1. Table 1 lists the system objectives and syndromes under surveillance.
Mobile Phone–based Surveillance

Public Health Event Detection

MOPBASSS data were extracted from the online database, and analyses were performed to describe outbreak detection and user experience. Comparisons were made between MOPBASSS and the NHIS, HBAS, and measles laboratory databases and included the average reporting delay (in days), the completeness of reporting, and the number of measles cases (a frequently reported syndrome common to all 4 systems). The accuracy of data transcription from forms to the database could only be measured at the site that provided usable data at the pilot evaluation meeting. Where no surveillance feedback bulletins were available to make data comparisons with existing systems at week 26, comparisons were made by using data from the next available bulletin so that data could be compared across all systems. Qualitative and quantitative methods were used to evaluate the system, using established frameworks (26,27).

Nine facilities submitted weekly data through MOPBASSS during the pilot phase. Clinical staff from the 10 sites and public health staff from the 3 provinces participated in focus group discussions. Ten stakeholders (77%) completed the self-administered evaluation survey. Attributes associated with public health event detection were defined as follows:

- **Sensitivity**—the number of measles cases reported through MOPBASSS compared with the HBAS and NHIS.
- **Timeliness**—the average number of days reporting delay through MOPBASSS compared with the NHIS.
- **Validity**—the accuracy of the system to detect outbreaks, measured by comparing reports across systems, including laboratory surveillance data.
- **Data quality**—the completeness of information recorded in the online database as reported by stakeholders compared with data in the paper-based collection tool.
- **Representativeness**—the extent to which the system accurately described the distribution of acute public health events in the population.

System Experience

Qualitative investigations were conducted to describe the system and stakeholder experience by using standardized, self-administered questionnaires and stakeholder focus group discussions conducted by persons experienced in the methodology. Stakeholders included surveillance focal points from the 10 sites (outpatient nurse coordinators and 1 pediatrician), disease control staff from the 3 provincial health offices, and national surveillance staff. Data collection included information on training, the online database, case investigation and diagnosis, reporting using mobile phones, and surveillance guidelines. Attributes associated with system experience were defined as follows:

- **Acceptability**—the self-reported willingness of stakeholders to further engage with MOPBASSS, as well as indirect measures, including the timeliness and completeness of reporting.
- **Stability**—the consistency of the system in providing access to public health intelligence, measured by the number of times the system was unable to provide access to data.
- **Usefulness**—the extent to which stakeholders reported MOPBASSS contributes to public health.
- **Portability**—user perceptions on how easily the system could be established in another setting.
• Costs—the US dollar amount to establish the piloted system.

Results

Public Health Event Detection

Sensitivity
Using NHIS as reference, we found that MOPBASSS was more sensitive at detecting measles cases than the HBAS (95% vs. 26%) (Table 2). However, the low number of notifications for the condition “prolonged fever” in MOPBASSS compared with a similar syndrome (malaria) reported in the NHIS indicate the sensitivity for detection of this syndrome may be low.

Timeliness
The MOPBASSS average weekly reporting delay was 2.4 (range 0–52) days (Figure 2), compared with 84 days for the NHIS. Of the 156 MOPBASSS weekly reports, 105 (67%) were submitted on the expected Monday; of these, 57 reports (37%) were submitted by the expected time of 11:00 AM. Seven sites (87%) received weekly feedback at least once in the 10-week pilot period; 1 site never received feedback.

Validity
The limited microbiological investigation of acute public health events made it difficult to assess the absolute validity of the system. However, the laboratory confirmation of dengue fever virus infection in patients that met the case definition for suspected dengue hemorrhagic fever (DHF) indicates the syndromic data for this condition in this time and place was valid.

Data Quality
Although data transcription from the paper-based data collection forms into the phone reporting template and transfer to the database was high quality (98% accuracy), data quality associated with the use of clinical case definitions was not as accurate. The proportion of weekly reports where 0 cases were notified for all syndromes decreased during the pilot, starting at 50% in the first weeks and declining to 15% by the last week of the pilot, which may indicate that clinicians became better at identifying or reporting syndromes. Few data-sending errors occurred, and verification processes ensured no outbreak investigations were instigated erroneously.

Representativeness
Because the participating surveillance sites were the referral health facilities within their respective districts, it is conceivable that only more severe public health events would be reported through these facilities. The 2-stage randomization process to select participating sites and the number of participating sites provides some indication that the timeliness and completeness of reporting that was achieved through MOPBASSS may be generalizable to other provinces. Given that nongovernment facilities in Papua New Guinea are frequently managed more effectively than their government-run counterparts, it is conceivable that this system could function equally well in nongovernment health facilities.

System Experience

Acceptability
All stakeholders reported an interest to continue participation; all but 1 stakeholder reported MOPBASSS was working effectively to detect acute public health events. Stakeholders reported the system was fast, simple, effective, and reliable and enabled the timely initiation of verification, assessment, and response processes. Participation in MOPBASSS was not associated with an excessive time burden, and the program complemented existing systems. Data management was considered simple; it is contracted to the private sector, which removes many person-dependent steps for health authorities. Timely access to data through the Internet-based database was beneficial for national staff, but data access was challenging for provincial staff. The high completeness of reporting through MOPBASSS (70% vs. 40% for HBAS) (Figure 2) and the timeliness and sensitivity of the new system may also reflect its acceptability. The relative validity for outbreak reporting was high; all outbreaks that were identified through alternative systems were also identified through MOPBASSS.
on several occasions, landline telephone or high-frequency radio might have been the preferred option for providing the initial report for selected conditions.

**Stability**

The system was highly stable during the pilot period, with no reported issues with the online database. The subscriber identification module card from 1 of the 10 mobile phones was misplaced, and reporting ceased at this site for 6 months (week 13 to week 39) until it was reported, the card replaced, and the phone returned. This site also had the weakest mobile phone network coverage.

**Usefulness**

MOPBASSS data was largely used by national health authorities to support inferences about disease patterns that would not have been possible without it; however, stakeholders reported these data were not widely used at the provincial level. Of the 8 clinical sites surveyed, most found feedback either very useful (62%) or useful (25%). Despite some issues with data accuracy, the system provided a certain degree of reassurance that cholera was not circulating at reporting sites during the nationwide outbreak and was considered a measure of the satisfaction of public health decision-makers within the national health authorities.

During the pilot program, MOPBASSS outputs were increasingly used for risk assessments at the weekly surveillance meeting of the national health authorities. The system also facilitated international data reporting to regional monitoring systems. Two weeks after training was conducted at the first established site, a case of hemorrhagic fever was identified, reported, and investigated, which enabled the laboratory confirmation of 3 cases of DHF, which is rarely reported in Papua New Guinea. Similarly, a week after training at a district site, 3 persons meeting the case definition for AFP were identified and reported (not through the mobile phone system); <20 cases of AFP are reported annually from the 20 provinces. Of the 18 cases of suspected measles reported from provincial hospital sites, none were fully investigated.

**Portability**

Stakeholders perceived that the simplicity of the reporting system would likely contribute to its portability and that the system could easily be established among vulnerable populations, such as internally displaced persons or refugees. The system could enable national health authorities to support provincial authorities to work with partners to rapidly establish postdisaster surveillance, particularly if data collection tools were integrated with those of the NHIS.

**Costs**

The pilot intervention cost approximately US$45,000, excluding staff costs. Half the cost was for software development, including the phone template and secure online database. The remaining costs were for investigation materials, mobile phones, and field missions to establish the system. There was no cost to the data provider and no requirement for phone credit.

**Discussion**

Creativity and flexibility are crucial when implementing programs that overcome the obstacles and constraints within fragile states (I). Establishing MOPBASSS during the nationwide cholera outbreak in Papua New Guinea may have enhanced the program’s acceptability because the threat level and the perceived value of early detection were high, but the program’s attributes likely contributed. We have shown that MOPBASSS reporting was more timely, complete, and sensitive than reporting through existing systems. MOPBASSS reporting was simple, effective, reliable, and acceptable and enabled the routine, systematic, and ongoing reporting of syndromes of public health importance from the district level.

Before the pilot, DHF fever was rarely reported in Papua New Guinea, despite frequent evidence of dengue transmission (28). While an association between training and the identification of DHF is not causal, it is likely that the surveillance training, the inclusion of hemorrhagic fever into a routine data collection, and the increased availability of rapid diagnostic tools contributed to the early identification and timely implementation of community level control measures during this outbreak. However, because DHF was not reported through the MOPBASSS, these findings highlight the importance of surveillance training more broadly rather than an advantage of MOPBASSS. In addition, the pre-positioning of dengue rapid diagnostic kits enabled a timely preliminary diagnosis.

---

**Table 2. Notifications for suspected measles cases during MOPBASSS pilot at 3 provincial hospitals, Papua New Guinea, May–September 2011**

<table>
<thead>
<tr>
<th>Hospital</th>
<th>MOPBASSS (clinician based)</th>
<th>HBAS (health office based)</th>
<th>NHIS (clinician based)</th>
<th>Fully investigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6 (100)</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>11 (85)</td>
<td>4 (31)</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>18 (95)</td>
<td>5 (26)</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are no. (%). NHIS was used as reference. MOPBASSS, mobile phone–based syndromic surveillance system; HBAS, Hospital Based Active Surveillance system; NHIS, National Health Information System.
ties that no acute watery diarrhea outbreaks were occurring during the outbreak of dengue fever before confirmatory testing and may have demonstrated the utility of rapid tests as an adjunct to MOPBASSS.

The implementation of new surveillance systems can be associated with early confusion regarding clinical case definitions (29). During the MOPBASSS reporting pilot, problems were noted with the use of several case definitions, including “prolonged fever,” which gave a lower than expected yield when compared with malaria notifications through the NHIS. Whereas establishing a functional weekly reporting system was the main priority for the pilot, diagnostic accuracy and appropriate use of case definitions can be strengthened through training (30) and should be an area of ongoing focus.

Regional measles elimination surveillance standards stipulate that ≥80% of measles cases should be fully investigated (31); however, Papua New Guinea investigates only ≈5% of suspected measles cases. Despite the ability of the MOPBASSS program to provide more timely data on suspected measles, including instant pop-up messages on phone screens reminding clinicians to investigate when selected conditions such as measles are reported, none of the measles cases identified during the pilot were fully investigated. Laboratory support to MOPBASSS when aberrations are detected requires strengthening and may benefit from greater involvement of subnational laboratory staff and the provision of rapid tests for selected conditions.

Geographic representativeness is particularly critical for outbreak detection systems in settings with dispersed populations and challenges to health system access and referral (32). Most (87%) of the Papua New Guinea population lives in rural areas, where health system infrastructure and human resources can be limited in general and specifically for outbreak reporting (33). When the pilot commenced, cholera had spread widely across the country, but outbreaks had not been identified at participating sites (9). The system provided reassurance to national health authorities that no acute watery diarrhea outbreaks were occurring because low case numbers were reported from participating sites during a multijurisdictional and unpredictable cholera outbreak.

Strong linkage between clinical and public health authorities for outbreak detection is emphasized in the regional strategy for strengthening preparedness for emerging diseases (34). Syndromic surveillance systems have demonstrated their capacity to strengthen linkage between clinical services and public health authorities (29). Such linkages are traditionally weak in Papua New Guinea (35), but participation in this pilot program appeared to bring these stakeholders into closer working relationships. MOPBASSS provided opportunities for outpatient nurse clinicians to demonstrate innovation, coordination, and leadership capabilities in making the system work in each setting. These clinicians may be drivers of stronger collaboration on outbreak identification and response with subnational public health authorities. Improving the access to timely data by subnational staff will further reduce barriers to timely public health response and increase ownership of the system, a crucial step toward greater sustainability.

Our evaluation has several limitations. It was not independent, which could introduce measurement bias, and the short intervention period limited our ability to evaluate the flexibility and sustainability of the system. Establishing baseline data for syndromes of public health importance cannot be achieved in a short pilot but might possible with sustained MOPBASSS implementation to capture seasonal and cyclical trends and more informed interpretation of possible aberrations. Data accuracy was only measured at the site that provided data for transcription auditing, but the high accuracy at this site, combined with the user-reported simplicity of the reporting tools and the lack of transcription errors identified during acute public health event verification processes, indicates that data accuracy problem did not affect system sensitivity. Contracting data management to the private sector is simple but can be expensive in a resource-limited setting, so the lack of cost-benefit analysis is also a limitation of our review.

The utility of health information systems to provide data in disasters is being investigated (36). We did not measure the flexibility of the system formally, but we believe little additional time, personnel, or funds would be required to accommodate future modifications, such as what types of data are collected and how many data providers are needed for increased population coverage and detection or tracking of low-frequency events. The potential for strengthening health information systems by using mobile phones is not limited to public health event detection; other programs may benefit from the timely sharing of key program data. Adapting the available technology to remotely load data collection templates would enable greater flexibility and would enable
additional disease control programs to develop reporting templates for the same mobile device.

Outbreak identification systems that rely on clinician reporting have previously demonstrated their effectiveness (37). Automated reporting may decrease the burden on health care and public health workers and enable more complete reporting of potential cases of public health importance (16). Data reporting was successfully achieved in MOPBASSS for 2 main reasons: 1) the responsibility for reporting was given to the outpatient department setting that sees clinical cases and was coordinated by a designated leader, and 2) automated reporting system was simple and easy to use. Providing peripheral-level staff with regular feedback is universally recognized as strengthening surveillance programs, as demonstrated by the positive influence of feedback on reporting completeness in the NHIS (24). Further consideration is required to ensure feedback can be improved to strengthen the system more consistently and explore how technology may facilitate this process.

Acknowledgments

The authors are grateful to the outpatient staff that contributed to the system at the 10 sites and for the support of health authorities from the National Capital District and staff from 6-mile and 9-mile clinics for the pretesting of the system.

Mr Rosewell is an epidemiologist at the World Health Organization Country Office in Papua New Guinea. His research interests include strengthening outbreak detection and response systems in resource-limited settings, the subject of his PhD at the School of Public Health and Community Medicine, University of New South Wales.

References


Address for correspondence: Alexander Rosewell, World Health Organization, Port Moresby, Papua New Guinea; email: arosewell@yahoo.com
Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia


The source of human infection with Middle East respiratory syndrome coronavirus remains unknown. Molecular investigation indicated that bats in Saudi Arabia are infected with several alphacoronaviruses and betacoronaviruses. Virus from 1 bat showed 100% nucleotide identity to virus from the human index case-patient. Bats might play a role in human infection.

Since Middle East respiratory syndrome (MERS) was described in September 2012, over 90 cases have been reported worldwide, 70 from Saudi Arabia. The incidence of infection with the causative agent, a betacoronavirus (MERS CoV) (1), has not been determined; however, the mortality rate among those who received clinical care is ≈65% (2). Although instances of human-to-human transmission have been documented between case-patients and others in close contact (including hospital patients sharing rooms, family members, and medical personnel), the sources of infection for most patients remain unknown. Because of sequence similarities between β-CoVs identified in bats and those of MERS CoV isolated from humans, a bat reservoir has been posited (3–5). Although neither detection of MERS CoV in bats nor contact of human MERS patients with bats have been reported, a role for bats in human infection cannot be excluded because contact can be indirect (mediated through another animal vector or fomites).

Author affiliations: Ministry of Health, Riyadh, Saudi Arabia (Z.A. Memish, S.F. Fagbo, R. AlHakeem, A.A. Al Rabeeah); Columbia University, New York, New York, USA (N. Mishra, V. Kapoor, A. Kapoor, T. Briese, W.I. Lipkin); EcoHealth Alliance, New York (K.J. Olival, J.H. Epstein, P. Daszak); Ministry of Health, Bisha, Saudi Arabia (M. Al Asmari); and EcoHealth Alliance, Dhaka, Bangladesh (A. Islam)

DOI: http://dx.doi.org/10.3201/eid1911.131172

The Study

In October 2012 and April 2013, three agencies collected samples from bats in regions where MERS cases had been identified (Figure 1). The agencies are the Ministry of Health of Saudi Arabia, the Center for Infection and Immunity of Columbia University, and EcoHealth Alliance.

During the October investigation, the team interviewed the family of an index case-patient in Bisha and collected samples from bats <12 km from his home, in an abandoned date palm orchard, and <1 km from his place of employment, a hardware store that fronted a garden and date palm orchard. Although neither family members nor employees recalled seeing bats, the team observed roosting bats and guano in abandoned wells and ruins within 12 km of his home and insectivorous bats at dusk in the garden behind his store. Over 3 weeks, 96 bats representing 7 species (Rhinopoma hardwickii, Rhinopoma microphyllum, Taphozous perforatus, Pipistrellus kuhlii, Eptesicus bottae, Eidolon helvum, and Rosettus aegyptiacus) were captured in mist nets and harp traps, then released after visual speciation and collection of morphometric measurements; wing punch biopsy samples; blood; throat swab samples; and rectal swab samples or fecal pellets. Samples were collected into viral transport medium or lysis buffer.

During the 3-week April investigation, fecal samples were collected on tarps laid out at bat roosting sites in and around Bisha, Unaizah, and Riyadh. Representative animals at each roosting site were captured, identified morphologically, and released after wing punch biopsy samples were collected for speciation by DNA analysis. Samples were collected into cryovials.
All samples were stored in liquid nitrogen and conveyed to Riyadh for storage at −80°C before being transported to Columbia University in New York in dry nitrogen. The October 2012 shipment was inadvertently opened at customs in the United States and sat at room temperature for 48 hours before transfer to Columbia University; at arrival, all samples had thawed. The April 2013 samples arrived intact. Total nucleic acid was extracted from samples by using the NucliSENS easyMAG system (bioMérieux, Durham, NC, USA) and subjected to 8 PCRs with primers and protocols designed to amplify regions within the helicase, RNA-dependent RNA polymerase (RdRp), and nucleocapsid or envelope proteins of CoVs (6–9). Products were sequenced and analyzed for similarity to GenBank database entries by using the BLASTn and BLASTx programs (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Primer sequences are shown in Table 1. The identity of bat species yielding specific viral products was determined by amplifying and sequencing a fragment of the cytochrome B gene (10). All visual classifications of species were confirmed except for that of *T. perforatus* bats. There is no reference sequence for *T. perforatus* bats in GenBank. However, because the closest reference sequence was from *T. nidiventeris* bats, at 84% identity we presume that the product represents bona fide *T. perforatus* bat cytochrome B gene sequence. Representative cytochrome B sequences have been uploaded to GenBank (accession nos. KF498635–KF498641).

Table 1. PCRs and primers used in CoV detection

<table>
<thead>
<tr>
<th>PCRs (reference)</th>
<th>Primers, 5′→3′</th>
<th>Nested fragment size, region (primer locations on the reference genome)†</th>
<th>Type of CoV (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested pan-CoV-I (6)</td>
<td>PLQ-F1, CGTGGGIAACWARYTBCCWCYTICARBRGG PLQ-R1, GGGCTCAGTCATCGACATGAGG GCGGCWCCWCYGNGTAAATT PLQ-R2, GGGWAWCCCACTGTYGTGAYRTC</td>
<td>≈400 nt, RdRp (18310–187450)</td>
<td>α-CoV (8), β-CoV (1)</td>
</tr>
<tr>
<td>Nested pan-CoV-II (7)</td>
<td>WT-CoV-F1, GGTGGAGYAYTCHAAARTGGA WT-CoV-R1, CAACATCAGWYRAATCATATA WT-CoV-R2, GAYTAYYCHAAARTGGAAGAGG WT-CoV-F3, GAYTAYYCHAAARTGGAAGAGG</td>
<td>≈430 nt, RdRp (15260–15700)</td>
<td>α-CoV (5), β-CoV (2)</td>
</tr>
<tr>
<td>Hemi-nested RdRp-sequencing assay (9)</td>
<td>EMCD-SeqRdRp-Rev, GCAWGCNADVGTCTAATTAGG EMCD-SeqRdRp-Fwd, GTCTATWAGTCGAATAAGATAGRG EMCD-SeqRdRp-Rnest, CACTTACGRTARCTCCAWCCCA</td>
<td>≈230 nt, RdRp (15048–15290)</td>
<td>α-CoV (2), β-CoV (1)</td>
</tr>
<tr>
<td>Hemi-nested N-sequencing assay (9)</td>
<td>EMCD-SeqN-Fwd, CTTCTGCTACATGGAAGCCCA EMCD-SeqN-Rev, GATGCGGTTGCGAACACAAAC EMCD-SeqN-Fnest, TGACCCCCAAGATCCACAGAC</td>
<td>≈280 nt, N seq (29,549–29,860)</td>
<td>NA</td>
</tr>
<tr>
<td>Nested CII-pan-CoV-I (6)</td>
<td>NM-CoV-2F1, ACWGTCTCAGGCCWCCWGGG CTCCAGGCGCMTGCGCCGGGG NCM-CoV-2F2, GTCTCAGGCCWCCWCCGGNNAC GGCAGCGTGWGCWGRTCTCNACRTA NCM-CoV-2R1, AGCTGWCGWGGGRTCTCGGCIACRTANAC</td>
<td>≈355 nt, helicase (17,060–17,410)</td>
<td>β-CoV (2)</td>
</tr>
<tr>
<td>Nested CII-MERS-RdRp</td>
<td>NM-HCOV-F1, GTCTATGACTAGTACGTCGACCT NHCOV-F2, AGAGCTCGCAGTCTGGACCG NM-HCOV-F2, AGAGCTCGCAGTCTGGACCG NM-HCOV-R1, ACCCTAAAGATGCAGGTGATTAAC NM-HCOV-R2, TGCGGATTAACACCTTTTGAC</td>
<td>≈190 nt, RdRp (15068–15249)</td>
<td>β-CoV (1, MERS-CoV)</td>
</tr>
<tr>
<td>Hemi-nested CII-MERS N sequence</td>
<td>NM-Seq-F1, ACTTCTCTCGTGATAGGACGACG NM-Seq-R1, GGAAGTTTGCGCTGATATCTC NM-Seq-R2, GGAGGTTTGGCTACATCTTGGCT</td>
<td>≈170 nt, N seq (29545–29713)</td>
<td>NA</td>
</tr>
<tr>
<td>upE and ORF1b real-time assays (8)</td>
<td>upE-Fwd: GCAACCGCGGATCGTCAATT upE-Prb: FAM-CTCTCTCAGATAATCCGGCGGAGCTCAG-TMARA upE-Rev: GGCTCTACGCGCAGGAAAAACATA ORF1b-Fwd: TCTGATGTTAGGATGGTCATGAT ORF1b-Prb: FAM-CCGCAGTCATTGAAGTGCACAGGT-CAATG-TMARA ORF1b-Rev: TGCACCCCATGGAAATCTCATTTG</td>
<td>Upstream of E gene and ORF 1b</td>
<td>NA</td>
</tr>
</tbody>
</table>

*CoV, coronavirus; MERS, Middle East respiratory syndrome; RdRp, RNA-dependent RNA polymerase; NA, not applicable; ORF, open reading frame.†Primer locations are based on human β-CoV 2c EMC/2012, complete genome (GenBank accession no. JX869059).
Table 2. CoVs detected in bats, Saudi Arabia*

<table>
<thead>
<tr>
<th>Bat family, genus, species</th>
<th>Location</th>
<th>October 2012</th>
<th>April 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. bats</td>
<td>Throat swab</td>
</tr>
<tr>
<td>Emballonuridae Taphozous perforatus</td>
<td>Bisha ruins</td>
<td>29</td>
<td>29 (0)</td>
</tr>
<tr>
<td>Pteropodidae Eidolon helvum</td>
<td>Bisha town center</td>
<td>25</td>
<td>25 (0)</td>
</tr>
<tr>
<td>Rhinopomatidae Rh. hardwickii</td>
<td>Naqi and Old Naqi</td>
<td>36</td>
<td>36 (0)</td>
</tr>
<tr>
<td>Rh. microphyllum</td>
<td>Old Naqi</td>
<td>1</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Vespertilionidae Eptesicus brettei</td>
<td>Bisha ruins</td>
<td>1</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Pipistrellus kuhlii</td>
<td>Bisha ruins</td>
<td>1</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Rhinopomatidae Rh. hardwickii</td>
<td>Greater Bisha area</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vespertilionidae T. perforatus</td>
<td>Bisha ruins</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>T. perforatus</td>
<td>Greater Unaizah area</td>
<td>9</td>
<td>9 (0)</td>
</tr>
<tr>
<td>Vespertilionidae P. kuhlii</td>
<td>Greater Riyadh area</td>
<td>5</td>
<td>5 (0)</td>
</tr>
</tbody>
</table>

*CoV, coronavirus; MERS, Middle East respiratory syndrome; NA, not applicable.
†Based on BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

CoV sequences were amplified from 220 of 732 roost feces samples and 7 of 91 rectal swab samples or fecal pellets. A product obtained by PCR amplification of nucleic acid from a fecal pellet of a T. perforatus bat captured in October 2012 in Bisha showed 100% nt identity to the human β-CoV 2c EMC/2012 cloned from the index case-patient in Bisha. A phylogenetic analysis of CoVs obtained in this study is shown in Figure 2. CoV sequences have been uploaded in GenBank (accession nos. KF493884–KF493888).

Conclusions

A wide range of CoV species are circulating among bats in Saudi Arabia. Although the prevalence of CoVs was high (~28% of fecal samples), MERS CoV was found in only 1 bat. A 3.5% MERS CoV infection rate (n = 29; 95% CI 0–20%) in T. perforatus bats is low compared with that for severe acute respiratory syndrome–like CoV in rhinolophid bats in China (10%–12.5%) but consistent with CoV prevalence among bats in Mexico (4). Furthermore, the sensitivity for viral nucleic acid detection in samples collected in October 2012 was probably reduced because of failure in cold chain transport. Whereas 219 (32%) of 675 of fecal pellets collected in April revealed a CoV sequence by PCR, only 8 (5%) of 148 of rectal swab samples or fecal pellets collected in October were positive by the same assays. We were unable to recover additional sequences beyond the 190-nt RdRp fragment represented in Figure 2 but are confident in the fidelity of the finding. First, although RdRp is a conserved portion of the CoV genome, there is no precedent for 100% identity of a bat CoV to human MERS CoV sequence. Second, when this work began we did not have cultured MERS CoV, human MERS samples, or MERS CoV cDNA in the...
laboratory at Columbia University where samples were removed directly from the tubes in which they were collected in the field for nucleic acid extraction, PCR, and sequence analysis. Third, the only MERS-positive signal was obtained in PCR analysis of the *T. perforatus* bat captured in Bisha near the home and workplace of the MERS index case-patient used to generate the human β-CoV 2c EMC/2012 sequence.

Bats are reservoirs of several viruses that can cause human disease, including rabies, Hendra, Nipah, Marburg, severe acute respiratory syndrome CoV, and probably Ebola viruses (11–14). Cross-species transmission from bats to humans can be direct, through contact with infected bats or their excreta, or facilitated by intermediate hosts (15). Bat CoVs are typically host specific; however, MERS-related CoVs have reportedly been found in many bat families, including Vespertilionidae, Molosidae, Nycteridae, and now Emballonuridae (sheath-tailed bats) in Africa, the Americas, Asia, and Europe. We sampled only a small sample of bats in Saudi Arabia. Nonetheless, given the rarity of MERS CoV sequences detected by our survey and the broad distribution of MERS cases throughout the Middle East, we speculate that there are probably other hosts. Future work should investigate additional bat and other wildlife species and domestic animals for CoV infection and potential linkage to human disease.

**Acknowledgments**

We thank Rebecca Hersch and George Amato for cytochrome B gene sequencing, Lorenzo Uccellini and Steven Sameroff for assistance with nucleic acid extraction, Kawathar Muhammad for sample coordination, and Meera Bhat and Ellie Kahn for assistance with manuscript preparation.

Work in the Center for Infection and Immunity and EcoHealth Alliance was supported by the National Institute of Allergy and Infectious Diseases, the US Agency for International Development (PREDICT), and the Defense Threat Reduction Agency.

Dr Memish is deputy minister for public health, director of the WHO Collaborating Center for Mass Gathering Medicine in the Ministry of Health, and professor in the College of Medicine of Alfaisal University in Riyadh. His research interests include emerging infectious diseases, infection control, and preventive medicine.
MERS CoV in Bats, Saudi Arabia

References


Address for correspondence: W. Ian Lipkin, Center for Infection and Immunity, Columbia University, 722 West 168th St, New York, NY 10032, USA; email: wil2001@columbia.edu
Hantavirus Pulmonary Syndrome Outbreak, Brazil, December 2009–January 2010

Ana Cláudia Pereira Terças, Marina Atanaka dos Santos, Marta Gislene Pignatti, Mariano Martinez Espinosa, Alba Valéria Gomes de Melo Via, and Jaqueline Aparecida Menegatti

An outbreak of hantavirus pulmonary syndrome occurred in the Sobradinho Indian settlement of the Kayabí ethnic group in northern Mato Grosso during December 2009–January 2010. We conducted a retrospective study to clarify the outbreak’s epidemiologic and clinical characteristics. Results suggest a relationship between the outbreak and deforestation and farming expansion in indigenous areas.

Hantavirus pulmonary syndrome (HPS) was first identified in 1993 in the semi-arid southwestern region of the United States known as the Four Corners (1,2). This manifestation occurred in the form of an outbreak of the Sin Nombre virus in a community of Navajo Indians.

HPS is associated with American wild rodents of the family Cricetidae. Members of the subfamily Sigmodontinae serve as rodent reservoirs of hantaviruses, and persons become infected mainly through inhaling rodent secretions and aerosolized excreta (3–5). Propitious ecologic conditions such as social, economic, and spatial factors facilitate the initiation and maintenance of the disease and determine its emergence (6–7).

In Brazil, areas of deforestation and environmental change, which have resulted from economic growth and agricultural production in the past 20 years, has had an effect on the number of HPS cases (8,9). In Mato Grosso, the first HPS cases were recorded in 1999 in the city of Campo Novo do Parecis, where the Castelo dos Sonhos

Author affiliations: Federal University of Mato Grosso, Cuiabá, Brazil (A.C.P. Terças, M.A. dos Santos, M.G. Pignatti, M.M. Espinosa); University Center Cândido Rondon, Cuiabá (A.C.P. Terças); and State Secretary of Health of Mato Grosso, Cuiabá (A.V.G. de Melo Vía, J.A. Menegatti)
Hantavirus Pulmonary Syndrome, Brazil

The other 6 infected persons who did not live in house 3 would go to this house on a daily basis, and these persons exhibited unspecified signs and symptoms. As a consequence, they underwent serologic testing. Four tested persons were positive for IgG; 2 tested positive for IgM/IgG (Figure 2). In the family with the deceased mother, a 19-year-old girl and a 4-year-old boy were infected.

A 38-year-old woman, who lived with 7 patients with symptoms, died on January 11; she exhibited the same initial symptoms and reported insufficient breathing before her death. She received no assistance and was buried inside a hut in accordance with her cultural traditions.

Cases occurred equally in male and female patients. Patient ages ranged from 1 to 38 years, with an average of 13.7 years (Table 1). A total of 14 signs and symptoms were reported; the largest proportions of patients experienced fever (100%), dry cough (72.2%), and abdominal pain (66.7%) (Table 2). The clinical manifestations were recorded from December 30, 2009, through 28 January 28, 2010. Thus, the interval between the cases did not exceed the disease incubation period, which may vary from 4 to 55 days (7).

During this outbreak, pulmonary disease developed in 6 patients, and 5 survived. The symptoms preceding the death of 1 patient were recorded by her husband, who drew attention to her breathing difficulty and intense sudoresis. These symptoms could have been signs of circulatory shock.

The time between the onset of the symptoms and hospitalization was, on average, 3.17 days (median3) (Table 2). The duration of hospitalization ranged from 4 to 10 days, with a median of 4 days and an average of 6.40. The death rate in this outbreak was 10% lower than the state rate (33.3%) and the national rate (44.4%) for 2010 HPS outbreaks.

The hantaviruses known to circulate in this area are the strains Castelo dos Sonhos (in Oligoryzomys utiariensis rats) and the Laguna Negra (in Calomys aff. callosus mice) (9,13–15). These are typically responsible for cases of HPS in Mato Grosso, in southern Pará State (Castelo dos Sonhos), and in the cities near the Xingu Indigenous Park.

In the outbreak described here, no PCR or sequencing was done to confirm the strain.

In all of these cases, the home was the likely environment where infection occurred. However, other situations in which persons are at risk for infection include the following: harvesting and transportation of grains (30.0%) on plantations, house cleaning in a wilderness area (100.0%), contact with wild rodents and their excreta (100.0%), and contact with persons with HPS (90.0%).

Figure 1. Xingu Indigenous Park and the Sobradinho Indian settlement, Mato Grosso State, Brazil.

Figure 2. Genogram for residents of house 3 and other persons infected during the hantavirus outbreak in the Sobradinho Indian settlement, January 2010, Mato Grosso State, Brazil. HPS, hantavirus pulmonary syndrome.
Patients have become infected during housecleaning, when hantavirus rodent excreta could have been swept into the air. This supposition is supported by the fact that the infection was detected in the woman who did the cleaning and in children and adolescents who were also in the house. Other risky situations include agricultural activities, the management and storage of grains, and the direct contact with wild rodents and their excreta.

Conclusions

Disease awareness and information campaigns targeted toward the prevention of hantaviruses in the Xingu Indigenous Park should be intensified, given the risk of the potential presence of infected rodents in other Indian settlements. As HPS has become recognized in Brazilian indigenous areas, new studies should be conducted to evaluate the serum prevalence among indigenous peoples. Such surveillance will allow identification of the possible reservoirs and the prevalence of hantaviruses in the area.

Acknowledgments

We thank the inhabitants of the Sobradinho Indian settlement in Xingu Indigenous Park, the health team members of Xingu Indigenous Park, especially Claudimari Slavieiro, Marilúcia Marques dos Santos e Pólo Base Diauarum/Convênio Fundação Nacional de Saúde e Universidade de São Paulo and José Ferreira de Figueiredo from Distrito Sanitário Especial Indígena Cuiabá; State Health Secretary of Mato Grosso; the laboratory professionals at the Mato Grosso laboratory; the professional staff and researchers at the Evandro Chagas Institute/Fundação Oswaldo Cruz–Pará; the technical team for hantaviruses and rodent-associated diseases at Fundação Nacional de Saúde and the Federal University of Mato Grosso.

Dr Terças teaches biomedicine at the University Center Cândido Rondon in Cuiabá, Mato Grosso, Brazil. She also conducts research in the Laboratory of Hantaviruses and Rickettsioses, Oswaldo Cruz Institute. She is especially interested in diagnosis, epidemiology, and prevention of hantavirus infections and emerging diseases.

References


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) male</th>
<th>No. (%) female</th>
<th>No. (%) total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>2 (22.2)</td>
<td>–</td>
<td>2 (11.1)</td>
</tr>
<tr>
<td>5–10</td>
<td>3 (33.3)</td>
<td>3 (33.3)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>11–15</td>
<td>3 (33.3)</td>
<td>3 (33.3)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>16–20</td>
<td>1 (11.1)</td>
<td>–</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>–</td>
<td>3 (33.3)</td>
<td>3 (16.7)</td>
</tr>
<tr>
<td>Criterion of confirmation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory</td>
<td>9 (100.0)</td>
<td>8 (88.9)</td>
<td>17 (94.4)</td>
</tr>
<tr>
<td>Clinical and epidemiologic</td>
<td>–</td>
<td>1 (11.1)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Hospital stay</td>
<td>3 (33.3)</td>
<td>3 (33.3)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>Observations at house</td>
<td>2 (22.2)</td>
<td>3 (33.3)</td>
<td>5 (27.8)</td>
</tr>
<tr>
<td>Never left Indian settlement</td>
<td>4 (44.4)</td>
<td>3 (33.3)</td>
<td>7 (38.9)</td>
</tr>
<tr>
<td>Evolution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cure</td>
<td>9 (100.0)</td>
<td>8 (88.9)</td>
<td>17 (94.4)</td>
</tr>
<tr>
<td>Death</td>
<td>–</td>
<td>1 (11.1)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Total</td>
<td>9 (50.0)</td>
<td>9 (50.0)</td>
<td>18 (100.0)</td>
</tr>
</tbody>
</table>

*HPS, hantavirus pulmonary syndrome; –, none. Source: Health State Secretary of Mato Grosso, 2011.


Address for correspondence: Ana Claudia Pereira Terças, Centro Universitário Cândido Rondon, Av Beira Rio 3001, Cuiabá Mato Grosso 78000-000, Brazil; email: enf_anacnp@hotmail.com

Sign up for Twitter and find the latest information about emerging infectious diseases from the EID journal. @CDC_EIDjournal
Increased Incidence of Campylobacter spp. Infection and High Rates among Children, Israel

Miriam Weinberger, Larisa Lerner, Lea Valinsky, Jacob Moran-Gilad, Israel Nissan, Vered Agmon, and Chava Peretz

During 1999–2010, the annual incidence of Campylobacter spp. infection in Israel increased from 31.04 to 90.99 cases/100,000 population, a yearly increase of 10.24%. Children <2 years of age were disproportionally affected; incidence in this age group (356.12 cases/100,000 population) was >26-fold higher than for the 30–<50 age group.

Campylobacter spp. have become the leading cause of foodborne infections in many industrialized countries, despite extensive control efforts (1). Recent studies suggest that Campylobacter spp. infection in Israel may also be on the rise (2), in contrast to a substantial decrease in the incidence of Salmonella spp. infection, from 86.9 cases/100,000 population in 1995 to 44.0 cases/100,000 population in 2009 (3). We examined recent trends of Campylobacter spp. infection in Israel, with a focus on age- and sex-specific rates of infection.

The Study

Campylobacteriosis is a reportable disease in Israel. Microbiology laboratories countrywide passively submit human isolates from all sources to the National Campylobacter Reference Laboratory, Israeli Ministry of Health, Jerusalem, for confirmatory testing. Species are identified by using standard methods (4). The reporting system and laboratory methods did not change during the study period of January 1, 1999–December 31, 2010. For this study, patients’ date of birth and sex were retrieved using special permission by using identification numbers, which were subsequently replaced by unique numbers to retain patient anonymity. An infection episode was defined as the isolation of Campylobacter spp. from a single patient from any clinical source. Annual incidence rates for the study period were calculated by dividing the number of annual infection episodes by the population size retrieved from the Israeli Bureau of Statistics (5). The average age-specific annual incidence rate was calculated on the basis of the 12 annual incidence rates obtained for the study period. Because incidence counts and rates follow a Poisson distribution, Poisson regression models accounting for overdispersion were used to study annual trends of the incidence rate (dependent variable) for all isolates and for 2 major Campylobacter species, C. jejuni and C. coli; the calendar year was the independent variable. Poisson models were also used to study the effects of sex and age group on the incidence rates, adjusted for annual trends. All model effects were expressed by incidence rate ratio (IRR) and 95% CI. SAS software version 9.2 (SAS Institute, Cary, NC, USA) was used for all analyses. The study was approved by the Assaf Harofeh Medical Center local ethics committee.

During the study period, the Campylobacter Reference Laboratory confirmed 47,253 episodes of Campylobacter spp. infection. Most (>99%) infections were C. jejuni (37,062 episodes, 78.43%) and C. coli (10,092 episodes, 21.36%); the remaining <1% were C. fetus (25 episodes), C. upsaliensis (6 episodes), C. lari (2 episodes), or unidentified species (66 episodes). Bacteremia was noted for 331 (0.7%) episodes.

During the 12 study years, the annual incidence rate of all laboratory-confirmed Campylobacter spp. infection episodes increased 2.93-fold, from 31.04 to 90.99 cases/100,000 population. A similar increase was observed for C. jejuni (2.87-fold, 24.59 to 70.54 cases/100,000) and C. coli (3.06-fold, 6.38 to 19.54 cases/100,000). The linear annual increase in the incidence rate for the entire study period was 10.24% (95% CI 8.46–12.06) for all episodes, 10.07% (95% CI 8.42–11.74) for C. jejuni episodes, and 10.73% (95% CI 8.19–13.33) for C. coli episodes. A sharp rise in the annual increase rate, from 8.22% (95% CI 4.88–11.68) to 18.97% (95% CI 12.95–25.31), was noted between 1999–2006 and 2007–2010 (period I and period II) (Table).

Complete patient age and sex data were available for 38,092 (80.63%) of all infection episodes, including 29,931 (80.76%) C. jejuni infection episodes and 8,083 (80.09%) C. coli infection episodes. The annual incidence trends of Campylobacter spp. infection for the subgroup with complete demographic data were similar to those described for the entire group. Further age- and sex-related analyses were completed for episodes for which complete demographic data was available. IRR was 1.36 (95% CI 1.22–1.52) for male...
sex compared with female sex, adjusted for annual trends; similar elevated rates for male sex were found for *C. jejuni* (IRR 1.39, 95% CI 1.25–1.54) and *C. coli* (IRR 1.28, 95% CI 1.10–1.50) and for the 2 study periods (Figure 1; Table).

The age-specific average annual incidence rate formed an asymmetric, U-shaped curve. The highest average annual incidence rate occurred during the first decade of life (135.44 cases/100,000 population), and more specifically, during the first and second years of life (363.12 and 348.80 cases/100,000 population, respectively). The lowest average annual incidence rate occurred in the eighth decade of life (26.44 cases/100,000 population).

Six age groups were established for comparison of incidence rates; the age group of 30–<50 years was used as reference. The average annual incidence rate of infection in the age group 0–<2 years (356.12 cases/100,000 population) was 26.27 (95% CI 18.70–36.99) times higher than for the reference group (13.63 cases/100,000 population), adjusted for annual trends. Differences in incidence between the other age groups and the reference age group were smaller, ranging from an IRR of 1.42 (95% CI 0.92–2.20) for the 50–<70-year group to an IRR of 5.50 (95% CI 3.91–7.75) for the 2–<10-year age group. Similar IRRs for the respective age groups were found for infection caused by the 2 major *Campylobacter* species and throughout the study periods (Figure 2; Table).

### Conclusions

We found a sharp increase in the incidence of *Campylobacter* spp. infection in Israel, with rates tripling within just 12 years. This trend was observed for the 2 major *Campylobacter* species, *C. jejuni* and *C. coli*, and affected all age groups; the highest infection rates were seen during the first 2 years of life. Infection rates were substantially higher among children <2 years of age compared with rates for other Western countries (6,7) but were comparable to that reported for New Zealand (8). The difference in incidence...
between this and the other age groups, forming a U-shaped curve, is more characteristic of rates for developing countries and is believed to be indicative of repeated exposure to *Campylobacter* spp. in early childhood that results in the acquisition of protective immunity at older age (9). Similar trends have also been described for defined subpopulations in the United Kingdom (10).

The global disproportional burden of campylobacteriosis among young children is far from being understood (11). A recent study could not show increased exposure to known risk factors in young children compared with other age groups (12). Increased susceptibility because of immature immune systems, environmental contamination, cross-contamination in the kitchen, hand-to-mouth behavior, and overreporting have all been implicated.

The rapid increase and high incidence of campylobacteriosis in Israel resemble that of New Zealand (13). A food source of *Campylobacter* spp. infection in Israel has not been elucidated; however, during the study period, poultry meat sales markedly transitioned from mainly frozen to mainly fresh or chilled products (S. Dolev, pers. comm.). Similar trends were implicated for the rising incidence in New Zealand and were successfully mitigated by supervising fresh poultry sales (13). However, toddlers who do not consume poultry had the highest incidence of *Campylobacter* spp. infection for both countries (8).

Our study was conducted using a large and comprehensive national database of laboratory-confirmed *Campylobacter* spp. infections that has a high rate of species characterization. However, laboratory-confirmed infections represent only a small portion of diarrheal diseases (14,15). Moreover, young children may be more likely to receive medical care and have stool cultured (14,15).

In conclusion, the rapid increase in *Campylobacter* spp. incidence in Israel illustrates the need for an urgent national intervention plan. In particular, high infection rates among young children should prompt intensive research efforts to discover the routes of exposure.

**Acknowledgment**

We thank Sergio Dolev for his comments regarding poultry meat sale trends in Israel.

Dr Weinberger is the head of the Infectious Diseases Unit at Assaf Harofeh Medical Center, Zerifin, Israel and a senior lecturer at the Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel. Her primary research interest is the epidemiology of zoonotic infections, with a focus on foodborne infections.

**References**

Increased Incidence of Campylobacter spp., Israel


Address for correspondence: Miriam Weinberger, Infectious Diseases Unit, Assaf Harofeh Medical Center, Zerifin 70300, Israel; email: miriw@netvision.net.il
Two Novel Arenaviruses Detected in Pygmy Mice, Ghana

Karl C. Kronmann,1 Shirley Nimo-Paintsil,1 Fady Guirguis, Lisha C. Kronmann, Kofi Bonney, Kwasi Obiri-Danso, William Ampofo, and Elisabeth Fichet-Calvet

Two arenoviruses were detected in pygmy mice (Mus spp.) by screening 764 small mammals in Ghana. The Natal multimammate mouse (Mastomys natalensis), the known Lassa virus reservoir, was the dominant indoor rodent species in 4 of 10 sites, and accounted for 27% of all captured rodents. No rodent captured indoors tested positive for an arenavirus.

Lassa fever is an arenavirus infection transmitted to humans from rodents in a limited geographic region of western Africa. Nosocomial outbreaks have been recorded in Sierra Leone, Liberia, Guinea, and Nigeria: the countries best known to report Lassa fever (1). Most cases reported in travelers have originated in these 4 countries (2). However, cases have been reported from other countries in the region, including 1 caused by a previously undescribed strain of Lassa virus (LASV) after the case-patient traveled through Ghana (3). In addition, infection of humans with an arenavirus other than LASV has recently been recognized in southern Africa (4).

LASV has a bisegmented genome: the nucleoprotein (NP) and glycoprotein (GP) genes are on the small RNA segment, and the polymerase (L) and matrix protein (Z) genes are on the large RNA segment. LASV circulates in rodent populations even when infections in humans are not occurring, providing a source for subsequent outbreaks among humans.

Arenaviruses have species-specific reservoirs, and studies in Sierra Leone and Guinea found Mastomys natalensis to be the only rodent reservoir for LASV (5,6). In Guinea, M. natalensis abundance and viral prevalence rates in rodents have been associated with LASV seroprevalence among humans (6). Using a risk map model, we selected 10 sites in Ghana to examine rodent populations and arenavirus carriage rates.

The Study

The study was performed in accordance with a protocol approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, and the Institutional Review Board and Institutional Animal Care and Use Committee of the US Naval Medical Research Unit No. 3.

Seven sites were selected from areas of high predicted risk and 3 from areas of low predicted risk (Figure 1). A village was then selected for each site according to 3 criteria: a human population between 500 and 2,000; distance >20 km from any urban center or major road; and willingness to participate. All field work was scheduled during the rainy seasons of 2010 and 2011, when viral prevalence rates in rodents have been shown to be higher (7).

Traps were baited and set overnight in houses and outside in fields and woods along trap lines. Rodents captured were necropsied on site by using field biosafety level 3 procedures (8). Morphologic data, blood, and organs were collected from each animal for species identification and viral testing. 

A total of 16 species of rodents and 1 genus of shrews were identified among 764 captures (Table). Mastomys natalensis, the target species for the study, represented 27% (209/764) of captured species, and was outnumbered by another commensal rodent, Praomys daltoni, in 6 of 10 sites (Table). Trapping success was calculated for traplines set on 3 consecutive nights (no. trapped rodents × no. of sites × no. of nights) and was 9.2% (635/6895) for all locations; 23% indoors and 4.8% outdoors. Of rodents captured indoors, 98% (492/504) were Mastomys or Praomys species.

Total RNA was extracted from whole blood, or homogenized heart tissue when blood was not sufficient, in the biosafety level 3 facility at Noguchi Memorial Institute for Medical Research by using the RNasy Mini Kit (QIAGEN, Hilden, Germany) with QIAshredder columns and purified with on-column RNase-free DNase set (QIAGEN). The samples were tested for the presence of arenavirus GP gene RNA by using Power SYBR Green RNA-to-C1-Step PCR Kit (Applied Biosystems, Foster City, CA, USA) (9).

To confirm the first screening, a second PCR targeting the L gene was performed in the Mastomys and Mus spp. samples (10). Two Mus spp. captured outdoors in the villages
of Jirandogo (site 4) and Natorduori (site 1), respectively, were found to be positive for arenavirus RNA. For these 2 positive specimens, additional PCRs were performed by using primers OWS1+, OWS1000–, OWS2165A+, OWS2165B+, OWS2840A–, OWS2840B–, OWS2770+, OWS3400A–, and OWS3400– to acquire longer fragments of GP and NP genes (I/1). These fragments were sequenced on both strands, assembled, and aligned in MacVector (MacVector, Inc., Cary, NC, USA), then phylogenetically analyzed using PhyML (I/2). The viral and murine (cytochrome b) sequences were deposited in GenBank under accession nos. JX845167–JX845174. Voucher specimens are stored at Noguchi Memorial Institute for Medical Research and the United States Army Medical Research Institute for Infectious Diseases.

The phylogenetic position of the virus found in a Mus baoulei mouse, named Jirandogo for the village in which it was discovered, is unclear: Jirandogo clusters with the Nigerian LASV strain Lili Pinneo (lineage I) but with low branch support (20% bootstrap support) in the GP gene tree (Figure 2, panel A); is basal to all Lassa strains in the NP gene. At the nucleotide level, identity scores between Jirandogo and other published Lassa strains were 70.9%–74.6%, 71.6%–74.6%, and 76.9%–83.8% for GP, NP, and L, respectively. At the amino acid level, the scores ranged between 79.1%–84.2%, 82.0%–84.2%, and 93.8%–98.2% for GP, NP and L proteins, respectively. The other sequence, found in Mus mattheyi mice and named Natorduori after the village in which it was found, clusters with lymphocytic choriomeningitis virus in all 3 phylogenetetic trees.

**Conclusions**

This study aimed to provide data on the risk for Lassa fever in Ghana, a country situated between well-known Lassa fever–endemic regions, but with little known

---

**Figure 1.** Lassa virus risk map of Ghana showing 10 numbered study sites adapted from Fichet-Calvet and Rogers, Model 3 (1). a) Guinea savanna woodland; b) moist semideciduous forest; c) tropical rainforest. Solid black lines and letters indicate vegetation zones. A color version of this figure indicating high and low predicted risk for Lassa fever is available online (wwwnc.cdc.gov/EID/article/19/11/12-1491-F1.htm).

---

**Table.** Species distribution of small mammals captured by village in Ghana*  

<table>
<thead>
<tr>
<th>Species</th>
<th>No. animals captured, by study site (indoors/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Crocidura spp.</td>
<td>0/1</td>
</tr>
<tr>
<td>Gerbilliscus gambianus</td>
<td>0/1</td>
</tr>
<tr>
<td>Gerbilliscus kempi</td>
<td>0/0</td>
</tr>
<tr>
<td>Lemniscomys striatus</td>
<td>0/0</td>
</tr>
<tr>
<td>Lophuromys sikapusi</td>
<td>0/0</td>
</tr>
<tr>
<td>Mastomys erythroleucus</td>
<td>0/0</td>
</tr>
<tr>
<td>Mastomys natalensis</td>
<td>30/33</td>
</tr>
<tr>
<td>Mus baoulei</td>
<td>0/0</td>
</tr>
<tr>
<td>Mus mattheyi</td>
<td>1/12†</td>
</tr>
<tr>
<td>Mus minutoides</td>
<td>0/0</td>
</tr>
<tr>
<td>Mus musculusoides</td>
<td>1/1</td>
</tr>
<tr>
<td>Mus setulosus</td>
<td>0/0</td>
</tr>
<tr>
<td>Praomys daltoni</td>
<td>0/0</td>
</tr>
<tr>
<td>Praomys tullbergi</td>
<td>0/0</td>
</tr>
<tr>
<td>Rattus rattus</td>
<td>0/0</td>
</tr>
<tr>
<td>Taterillus gracillus</td>
<td>0/12</td>
</tr>
<tr>
<td>Uromys ruddi</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*Site names and coordinates: 1 = Natorduori (10°15’49” N 02°37’56” W), 2 = Bowena (09°32’82” N 01°37’56” W), 3 = Tearneba (10°23’70” N 00°21’56” W), 4 = Jirandogo (08°20’83” N 00°20’75” W), 5 = Amosomas (07°35’35” N 02°18’85” W), 6 = Ankaakar (08°10’56” N 01°47’35” W), 7 = Eshiwenwu (06°26’97” N 00°51’11” W), 8 = Dononga (10°37’16” N 01°25’31” W), 9 = Mangoase (07°58’11” N 01°39’79” W), 10 = Monkwo (07°40’45” N 00°37’98” W). † rodent with positive PCR for arenavirus.
disease itself. Our findings support the idea that Lassa virus is not widely prevalent in Ghana, and the Mastomys natalensis reservoir is not as common as in more highly endemic countries. In Ghana, 27% (209 of 764 captured rodents) were identified as Mastomys natalensis, compared with 54% (601/1123) in Guinea and 80% (82/103) in Mali (7,13). Overall, P. daltoni, a species not known to harbor LASV, outnumbered M. natalensis in Ghana, and was the predominant rodent species found indoors in 6 of our 10 sites.

Sequences of arenaviruses were detected in 2 species of pygmy mice, Mus baoulei and Mus mattheyi. Jirandogo, the sequence found in Mus baoulei, is phylogenetically close to LASV clade viruses. However, the maximum amino acid difference of 18% in NP between Jirandogo and LASV exceeds the 12% cutoff criteria, and therefore places it outside the LASV clade (14).

Arenaviruses other than LASV have recently been reported to cause human disease in Africa, but it is not known whether the viral sequences we found in Ghana are from viruses pathogenic to humans. Mus species in Africa are sometimes found indoors where exposure to humans is more likely, but most are found outdoors, including those from which we collected the 2 positive samples tested in our study. Infrequent cases in humans would be expected from arenavirus carriage in outdoor species, such as Mus.

It is notable that possible cases of Lassa fever have recently been reported in Ghana by the Ministry of Health from the high risk area near site 5 of our study (4). These reported cases in humans occurred ≈35 km from site 5, where Mastomys natalensis represented 15% of all rodents captured, and showed no evidence of arenavirus infection by PCR testing. The cases in humans were reported on the basis of the results of PCR tests, and further sequencing will be necessary to confirm the finding.

Although arenaviruses have species–specific reservoirs, recent work suggests host switching may be more common than previously believed (15). It is reassuring that no arenavirus was found in Mastomys natalensis species tested from Ghana, although 2 were detected in other rodent species from sites with high predicted risk. More

Figure 2. Phylogenetic trees depicting virus sequences found in rodents from the villages of Jirandogo and Natarduori, Ghana. Lineages of Lassa virus clade are indicated by Roman numerals on the right. For each virus, phylogenetic trees are shown for 3 genes: 2a, glycoprotein gene (partial 1,034 bp), 2b, nucleoprotein gene (partial 1,287 bp), and 2c, Polymerase gene (L partial, 340 bp). The analysis was performed using PhyML (11), with a general time reversible nucleotide substitution model and 100 bootstrap replicates. Branches highly supported by PhyML are indicated with bootstrap values >50. Scale bars indicate nucleotide substitutions per site.
information about geographic and temporal fluctuations in Mastomys natalensis rodent populations, the frequency of virus host-switching among rodents and the degree of arenavirus circulation is needed to better understand the implications of our findings for the risk for disease outbreaks from LASV or other arenaviruses in Ghana.

Acknowledgments

We thank the Ghana Health Service, in particular Elias Sory, for the strong support of this work in Ghana. We greatly appreciate the assistance and enthusiasm of the regional Ghana Health Service directors and local Ghana Health Service nurses. Stephen Gire provided valuable training on molecular testing and laboratory biosafety techniques. We greatly appreciate the assistance of LTC Nancy Merrill for her steady guidance in working with the IACUC. We are indebted to Naiki Puplampu, Clara Yeboah, Mba Mosore, Monica Owusu, Joseph Otchere, and Patrick Ben-Coffie for their help in the field.

Rodent trapping was authorized by the Forestry Commission of Ghana (permission no. FCWD/GH-01).

The study was supported by the Global Emerging Infections Surveillance and Response (GEIS) of the US Armed Forces Health Surveillance Center (AFHSC) (C0238_10_N3, C0435_11_N3, and C0687_12_N3) and European Marie Curie fellowship (PIEF-GA-2009-235164).

Dr. Kronmann is currently the head of infectious diseases at the Naval Medical Center in Portsmouth, Virginia. His research interests include tropical infectious disease and emerging infectious disease surveillance.

References


Address for correspondence: Karl C. Kronmann, Ghana Detachment, US Naval Medical Research Unit No. 3, American Embassy, Accra, Ghana; email: karl.kronmann@med.navy.mil
West Nile Virus, Texas, USA, 2012

Kristy O. Murray, Duke Ruktanonchai, Dawn Hesalroad, Eric Fonken, and Melissa S. Nolan

During the 2012 West Nile virus outbreak in Texas, USA, 1,868 cases were reported. Male patients, persons >65 years of age, and minorities were at highest risk for neuroinvasive disease. Fifty-three percent of counties reported a case; 48% of case-patients resided in 4 counties around Dallas/Fort Worth. The economic cost was >$47.6 million.

West Nile virus (WNV) first emerged in Texas, USA, in 2002 (1). Since then, the virus has become endemic, with ≥2,200 human cases reported in the state during 2002–2011 (2). In 2012, an unprecedented outbreak of WNV occurred in Texas; ≥1,900 cases were reported. The objective of this study was to understand the epidemiology of the 2012 WNV outbreak in Texas.

The Study

WNV infection is a reportable condition in Texas, with clinical cases passively reported by physicians to the local health departments, which in turn report to Texas Department of State Health Services (TxDSHS). We examined surveillance data for all reported cases for which symptom onset occurred during the 2012 calendar year, and we used descriptive statistics to describe the clinical features and demographic characteristics of reported case-patients. We calculated attack rates by sex, age, and race/ethnicity and incidence rates by county using population estimates for 2012 (3). Odds ratios (ORs), 95% CIs, and p values were calculated to determine differences in demographic variables between severe disease (WNV neuroinvasive disease [WNND]), which included encephalitis, meningoencephalitis, and meningitis) and less severe disease (uncomplicated WNV, which included encephalitis, meningitis, and meningoencephalitis) and less severe disease (uncomplicated WNV fever). Epi Info 7.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for all statistical calculations.

A total of 1,868 cases were reported to TxDSHS during the 2012 transmission season (Table), including 844 (45%) WNND cases and 89 deaths (case-fatality rate 5%). Dates of onset ranged from May 1, 2012, through December 6, 2012 (Figure 1). The outbreak peaked during week 33 (mid-August) with 225 reported cases, which is historically the same peak for all reported WNV cases in Texas during 2002–2011 (2). The median time from date of symptom onset to date of official report to TxDSHS was 27 days (range 6–274 days).

When examining the demographic characteristics of the reported cases, we found significant differences in sex, age, and race/ethnicity with regard to severity of disease. Overall, a higher percentage of male case-patients were reported (55%), and male case-patients were significantly more likely than female case-patients to have WNND (OR 1.5, 95% CI 1.2–1.8, p<0.001). Median age of all case-patients was 54 years (range 1 month–100 years). As each age category increased, the attack rates also increased (Table). Persons ≥65 years of age were significantly more likely than younger persons to have WNND (OR 2.1, 95% CI 1.8–2.6, p<0.001). The median age of the 89 case-patients who died was 79 years (range 25–100 years). When examining race/ethnicity of all cases, we observed the highest attack rate (11.1 cases/100,000 population) in white, non-Hispanics. However, minority populations were significantly more likely to have WNND (OR 1.9, 95% CI 1.6–2.4, p<0.001).

Of the 254 counties in Texas, 135 (53%) reported a WNV case (Figure 2). The overall incidence rate for the state was 7.8 cases per 100,000 population. Almost half of the cases were reported from the northeastern quadrant of the state, including the Dallas/Fort Worth metroplex (902 [48%] cases): Dallas (396 [21%]), Tarrant (259 [14%]), Collin (64 [3%]), and Denton (183 [10%]) counties. These 4 counties had a combined incidence rate of 16 cases per 100,000 population.

Conclusions

The 2012 WNV outbreak in Texas was unexpected in terms of the magnitude of virus transmission and number of human cases. We recently observed a 3-year pattern of increases in reported human cases in Texas, as seen in 2003, 2006, and 2009 (2). In 2012, the dramatic epidemic was consistent with this prior observation, with the 1,868 reported cases being more than double the historic high, which occurred in 2003 (735 cases). In addition to the dramatic increase in human cases in 2012, the state also reported an increase in equine cases (121 cases in 2012 compared with 6 cases in 2011). The exact factors that contributed to this epidemic are unknown and most likely complex, considering that successful transmission depends on supportive environmental conditions, vector abundance, avian reservoir and susceptible host abundance, pathogenicity of the virus, and sizeable populations of immunologically naïve reservoir species.

WNV more severely affects persons ≥65 years of age; deaths typically are reported in elderly persons (4,5).
During 2012, there was some media speculation that more cases of severe disease occurred in younger persons and that the circulating strain of virus possibly was more pathogenic than in prior years. Compared with Texas data for 2002–2011, we did not find any statistically significant differences in median ages of reported WNND or fatal cases in 2012 using the Kruskal-Wallis 1-way analysis of variance on ranks. Our findings from 2012 remain consistent with our experience from prior years; however, it remains critical to emphasize the importance of recognizing disease and testing persons of any age who have clinical signs and symptoms consistent with WNV infection.

The 2012 WNV outbreak in Texas greatly affected the state economically. On the basis of the acute medical care and productivity loss cost estimates provided by Barber et al. (6) (adjusted to 2012 USD), we crudely estimate the 2012 outbreak in Texas cost ≈ $47.6 million (range $14.5–$140.7 million; online Technical Appendix Table, wwwnc.cdc.gov/EID/articlepdfs/19/11/13-0768-Techapp1.pdf). In addition to these acute costs, the outbreak also required an increase in resources for mosquito control and public health efforts to respond to the epidemic. A recent study reported the cost of aerial spraying alone in Dallas County exceeded $1.6 million (7). The long-term economic impact of this outbreak also is expected to be substantial as a consequence of long-term rehabilitation and disability costs (8), possible risk for chronic kidney disease (9), and risk for premature death in severe cases (10).

The unprecedented 2012 outbreak confirms the need for continued vigilance for surveillance to enable timely implementation of control measures to prevent virus transmission. We expect Texas will continue to experience endemic levels of virus transmission with periodic epizootics. Considering the economic and physical costs

Table. Demographic characteristics and attack rates of all West Nile virus cases reported to the Texas (USA) Department of State Health Services during the 2012 outbreak

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All cases, no. (%, n = 1,868)</th>
<th>Attack rate*100,000 population</th>
<th>WNV fever, no. (%, n = 1,024)</th>
<th>WNV neuroinvasive disease, no. (%, n = 844)</th>
<th>Deaths, no. (%, n = 89)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>1,028 (55.0)</td>
<td>8.1</td>
<td>519 (50.7)</td>
<td>509 (60.3)</td>
<td>56 (62.9)</td>
</tr>
<tr>
<td>F</td>
<td>840 (45.0)</td>
<td>6.5</td>
<td>505 (49.3)</td>
<td>335 (39.7)</td>
<td>33 (37.1)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18</td>
<td>70 (3.8)</td>
<td>1.0</td>
<td>42 (4.1)</td>
<td>28 (3.3)</td>
<td>0</td>
</tr>
<tr>
<td>18–24</td>
<td>71 (3.8)</td>
<td>2.7</td>
<td>42 (4.1)</td>
<td>29 (3.4)</td>
<td>0</td>
</tr>
<tr>
<td>25–44</td>
<td>439 (23.5)</td>
<td>6.2</td>
<td>283 (27.6)</td>
<td>156 (18.5)</td>
<td>5 (5.6)</td>
</tr>
<tr>
<td>45–64</td>
<td>728 (39.0)</td>
<td>11.7</td>
<td>424 (41.4)</td>
<td>304 (36.0)</td>
<td>13 (14.6)</td>
</tr>
<tr>
<td>≥65</td>
<td>560 (30.0)</td>
<td>20.0</td>
<td>233 (22.8)</td>
<td>327 (38.7)</td>
<td>71 (79.8)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>1,273 (68.1)</td>
<td>11.1</td>
<td>738 (72.1)</td>
<td>535 (63.4)</td>
<td>54 (60.7)</td>
</tr>
<tr>
<td>Black</td>
<td>117 (6.3)</td>
<td>4.0</td>
<td>43 (4.2)</td>
<td>74 (8.8)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>White, Hispanic</td>
<td>318 (17.0)</td>
<td>3.2</td>
<td>134 (13.1)</td>
<td>184 (21.8)</td>
<td>22 (24.7)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>160 (8.6)</td>
<td>11.2</td>
<td>109 (10.6)</td>
<td>51 (6.0)</td>
<td>12 (13.5)</td>
</tr>
</tbody>
</table>

*Attack rates based on 2012 population estimates from the Texas State Data Center (3).
to persons severely affected, development of an effective vaccine is urgently needed to prevent disease. Until a vaccine becomes available, public health authorities will need to maintain their focus on surveillance, disease recognition, implementation of control measures, and public education about protective measures.

Acknowledgments

We thank Jim Schuermann, Nate Wolfe, and Alexandra Inghber for their assistance with data collection and preparation of this manuscript.

This project was generously supported in part by the Gillson Longenbaugh Foundation and the National Institutes of Health, National Institute of Allergy and Infectious Diseases (5R01AI091816-01).

Dr Murray is the associate vice-chair for research and associate professor of pediatrics in the Department of Pediatrics and National School of Tropical Medicine at Baylor College of Medicine and Texas Children’s Hospital. Her research focuses on vector-borne and zoonotic diseases.

References


Address for correspondence: Kristy O. Murray, Department of Pediatrics, National School of Tropical Medicine, 1102 Bates St, Suite 550, Houston, TX 77030, USA; email: kmurray@bcm.edu

Diphtheria

[Dif-ther’-ee-a]

From the Greek diphthera (leather), diphtheria is named for the tough pseudomembrane that forms in the patient’s throat. One of the earliest accounts of what may have been symptoms of diphtheria is found in Hippocrates work Epidemics III, written 2,500 years ago. Reports of epidemics of “throat distemper” began to appear in the 1500s, but before the 19th century, diphtheria was known around the world by many different names, such as Syrian ulcer, membranous angina, malignant croup, and Boulogne sore throat. In 1821, French physician Pierre Bretonneau described diphtheria’s unique clinical characteristics during an epidemic in southern France, when he named it diphtérite after the leathery texture of the pseudomembrane.

Sources


Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

DOI: http://dx.doi.org/10.3201/eid1911.ET1911
Mayaro Virus Infection, Amazon Basin Region, Peru, 2010–2013

Eric S. Halsey, Crystyan Siles, Carolina Guevara, Stalin Vilcarromero, Erik J. Jhonston, Cesar Ramal, Patricia V. Aguilar, and Julia S. Ampuero

During 2010–2013, we recruited 16 persons with confirmed Mayaro virus infection in the Peruvian Amazon to prospectively follow clinical symptoms and serologic response over a 12-month period. Mayaro virus infection caused long-term arthralgia in more than half, similar to reports of other arthritogenic alphaviruses.

Since the discovery of Mayaro virus (MAYV) in Trinidad in 1954, the etiologic agent of Mayaro fever has been identified in French Guiana, Suriname, Venezuela, Peru, Bolivia, and Brazil (1–9). The presumed primary vectors, Haemagogus mosquitoes, inhabit rural settings and tree canopies, a factor that may explain the relative paucity of cases and restricted endemicity. However, Aedes aegypti mosquitoes have been shown to be competent vectors of MAYV in the laboratory (10), suggesting that an urban-dwelling arthropod could be a vector of this virus over a wider scale. MAYV infection has been demonstrated in tourists returning from the Amazon region, highlighting not only the need to consider MAYV in febrile returned travelers, but also a possible role in global transmission (11).

Incapacitating chronic joint pain has been described with other arthritogenic alphaviruses (12), but little is known about the prognosis and serologic response over long periods after MAYV infection. Therefore, we conducted a prospective 1-year longitudinal study to determine the clinical manifestations and to describe the serologic response among humans with Mayaro fever in the Peruvian Amazon Basin.

The Study

Persons identified for this cohort were recruited in a passive febrile surveillance study in 15 health centers in 4 Peruvian cities: Iquitos, Yurimaguas, Chanchamayo, and Puerto Maldonado (Figure 1). Persons meeting the following criteria were recruited: age ≥5 years, oral/rectal temperature ≥38°C (or axillary ≥37.5°C), and no obvious focus of infection. Written consent was obtained from all adults and from a parent or guardian for participants <18 years of age; participants 8–17 years of age also provided written assent. The surveillance period of this study was December 6, 2010–April 30, 2012. Follow-up appointments continued for another year, through April 5, 2013. The institutional review boards of the US Naval Medical Research Unit No. 6 and the Peruvian Ministry of Health approved the protocol.

Compared with the day of the visit for acute illness (acute-phase visit), follow-up evaluations occurred at 20 days (range ±10 days), 3 months (±10 days), 6 months (±15 days), and 12 months (±30 days). At the acute-phase visit and at all follow-up visits, a blood sample was obtained.

For every participant, we attempted to determine the cause of infection by testing acute-phase serum for virus in Ae. albopictus (C6/36) and African green monkey kidney (Vero 76) cell culture (with immunofluorescence assay) and for viral nucleic acid by reverse transcription PCR (RT-PCR). Capture IgM and IgG ELISAs were performed at 1:100 dilution on the acute-phase and all follow-up samples to evaluate antibody responses to MAYV and other endemic arboviruses (i.e., Venezuelan equine encephalitis, Oropouche, group C, Guaroa, and dengue viruses) (6). Samples with detectable IgM or IgG were serially diluted and retested. Seroconversion was defined as a ≥4-fold increase in IgM titer between the acute-phase visit and the second visit. A Mayaro fever case was defined as IgM seroconversion or virus detected by isolation or by RT-PCR. In addition, we collected throat swabs from participants with pharyngeal erythema at the acute-phase visit and urine samples from the 3-month follow-up visit to determine the presence of MAYV with RT-PCR (13).

Of 2,094 febrile participants enrolled, 16 (0.8%) had Mayaro fever (Table 1). Of the 16 persons with Mayaro fever, 11 had MAYV isolated by the cell culture assays (11 in both Vero 76 and C6/36), 13 were MAYV positive by RT-PCR, and all had IgM ELISA seroconversion between the acute-phase and 20-day follow-up visits (Table 1). In all 16 participants, no IgM ELISA seroconversion occurred for endemic non-alphavirus viruses (i.e., Oropouche, group C, Guaroa, and dengue viruses). Four participants demonstrated IgM ELISA seroconversion against another alphavirus, Venezuelan equine encephalitis virus, but these 4 all had MAYV identified by immunofluorescence assay and by RT-PCR. Using RT-PCR, we did not detect MAYV in 2 acute visit throat swabs, any second-visit (20-day) serum samples, and any third-visit (3-month) urine samples.
Besides fever, the most common symptoms affecting participants in the acute stage of MAYV infection were malaise, headache, arthralgia, myalgia, and retro-orbital pain. The prevalence of these and other nonjoint signs and symptoms at the acute-phase and follow-up visits are available in the online Technical Appendix Table, wwwnc.cdc.gov/EID/articlepdfs/19/11/13-0777-Techapp1.pdf.

Although reports of joint pain waned in study participants by the second (20-day) visit, complaints increased at the chronic phase visit. The chronic joint pain often interfered with activities of daily living (Table 2).

Table 1. Demographic factors and laboratory findings at 5 encounters for patients with MAYV infection, Amazon Basin region, Peru, 2010–2013*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y/sex</th>
<th>Day of illness at enrollment</th>
<th>Isolation</th>
<th>RT-PCR†</th>
<th>Acute phase</th>
<th>Day 20</th>
<th>Month 3</th>
<th>Month 6</th>
<th>Month 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12/F</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28/F</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19/F</td>
<td>4</td>
<td>Neg</td>
<td>Neg</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11/M</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>41/F</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20/M</td>
<td>2</td>
<td>Neg</td>
<td>Neg</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>36/F</td>
<td>1</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>35/F</td>
<td>1</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>43/M</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>34/F</td>
<td>3</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>46/F</td>
<td>3</td>
<td>Neg</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>51/M</td>
<td>4</td>
<td>Neg</td>
<td>Neg</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>40/F</td>
<td>1</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>11/M</td>
<td>2</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>11/M</td>
<td>3</td>
<td>MAYV</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>64/M</td>
<td>2</td>
<td>Neg</td>
<td>MAYV</td>
<td>0; 0</td>
<td>6,400</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mayo virus, RT-PCR, reverse transcription PCR; Neg, negative; – visits not attended by the patient.
†Isolation and RT-PCR results are from the acute-phase visit.
‡For ELISA IgM and IgG results, endpoint titration values were determined. All serology values are expressed as inverse titers.
ankle, and knee also were mentioned, similar to the partici-
symptoms in the fingers. Involvement of joints of the wrist,
to 12 months, most of these studies identified persistent
sons. By using follow-up periods ranging from 1 month
were either solitary case reports or case series of <4 per-
2, 9–11, although all of these
9–11, although all of these

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 19, No. 11, November 2013

Table 2. Specific limitations and length of time of limitation caused by long-term joint pain in 16 patients with Mayaro virus infection, Peru, 2010–2013

<table>
<thead>
<tr>
<th>Patient</th>
<th>Occupation</th>
<th>Limitation secondary to long-term joint pain</th>
<th>Duration of limitation, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Student</td>
<td>Inability to write because of pain and stiffness in finger joints of both hands; inability to walk long distances because of pain in knees and ankles</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Office worker</td>
<td>No long-term limitations</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>Housewife</td>
<td>No long-term limitations at 3 mo; lost to follow-up at 6 mo</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>Student</td>
<td>No long-term limitations</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>Housewife</td>
<td>No long-term limitations</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>Soldier</td>
<td>No long-term limitations at 6 mo; lost to follow-up at 12 mo</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>Housewife</td>
<td>Limited ability to perform housework because of pain in hand and wrist joints</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>Secretary</td>
<td>Inability to remain seated for long periods</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>Electrician and other manual labor</td>
<td>Limited ability to climb electric poles because of pain and stiffness in both hands and elbows</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>Teacher</td>
<td>Inability to lecture standing up for prolonged periods because of pain in feet and knees</td>
<td>12</td>
</tr>
<tr>
<td>11</td>
<td>River boat cook</td>
<td>Limited ability to cook because of pain in both hands, wrists, and knees at 3 mo; lost to follow-up at 6 mo</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>Teacher</td>
<td>Difficulty writing because of pain in both hands and arms and pain in shoulders with movement</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td>Teacher</td>
<td>Inability to lecture standing up for prolonged periods because of pain in hands, knees, and ankles</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>Student</td>
<td>Inability to write because of pain and stiffness in finger joints of both hands</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>Student</td>
<td>Moderate pain when playing sports, such as basketball</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>Driver</td>
<td>No specific limitations noted</td>
<td>NA</td>
</tr>
</tbody>
</table>

*NA, not applicable.

Conclusions

This study demonstrated that persons with acute Mayaro fever often have many nonspecific symptoms but may continue to have chronic joint pain for at least 1 year after acute illness. Our study offers physicians valuable prognostic data to share with patients. It also indicates the need to consider MAYV infection in patients with seronegative arthritis (i.e., negative rheumatoid factor and antinuclear antibodies) in regions to which MAYV is endemic.

Previous reports have documented persistent joint pain after MAYV infection (2–5, 9, 11), although all of these were either solitary case reports or case series of ≤4 persons. By using follow-up periods ranging from 1 month to 12 months, most of these studies identified persistent symptoms in the fingers. Involvement of joints of the wrist, ankle, and knee also were mentioned, similar to the participants in our study.

Long-term manifestations of infection with other alphaviruses have been more robustly characterized, with persistent arthralgia being commonly described. Follow-up of chikungunya virus–infected patients on Réunion Island revealed that >60% had joint pain >3 years after acute illness that most often affected the fingers, wrists, knees, and ankles (14). Sindbis virus infection in a cohort in Finland resulted in persistent arthralgia lasting at least a year in half of those infected, with ankles, fingers, and wrists being most often affected (15). One caveat of our study and other studies is the difficulty in definitively attributing persistent arthralgia solely to viral infection, although our participants’ limitations in activities of daily living were all described as starting after their acute Mayaro fever illness.

IgM seroconversion occurred in all of the participants in our study that were identified with either isolation or RT-PCR, consistent with another report that noted the reliability of serology in detecting MAYV infection (9). In our study participants identified by serology, both RT-PCR and culture were more sensitive than what others have found for Sindbis virus infection, for which 1 study found sensitivities of 7% and 1%, respectively (15). However, RT-PCR and culture were negative in the only 2 participants in our study who had Mayaro fever after day 3 of symptoms, suggesting a narrow window when these assays may be effective.

No effective vaccine or antiviral agent exists for the arthritogenic alphaviruses, and treatment relies mainly on supportive modalities, such as nonsteroidal anti-inflammatory medications (12). Our results offer evidence that MAYV, similar to other alphaviruses, may cause protracted joint symptoms and provide further impetus to the development of more effective preventive and treatment strategies.

Acknowledgments

We thank Brett Forshey for his critical reading of this manuscript and Karen Campos and Yojani Aguilar for their assistance with field work and follow-up in Yurimaguas.

This work was supported by the Armed Forces Health Surveillance Center’s Global Emerging Infections Systems Research Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
The authors have declared that no competing interests exist. The corresponding author had full access to all data in the study and final responsibility for the decision to submit this publication.

Dr Halsey is a board-certified infectious diseases physician, US Air Force officer, and Virology Department head at the US Naval Medical Research Unit No. 6 in Lima, Peru. His research interests include the clinical and epidemiologic aspects of flavivirus, alphavirus, and bunyavirus infections.

References


Address for correspondence: Eric S. Halsey, US Naval Medical Research Unit No. 6, 3230 Lima Pl, Washington, DC 20521-3230, USA; email: eric.halsey@med.navy.mil

EMERGING INFECTIOUS DISEASES®

Full text free online at www.cdc.gov/eid

The print journal is available at no charge to public health professionals.

UPDATE MY ADDRESS

☐ Yes, I still want the journal. Please send it to me at the address below.

Number on mailing label: (required)
Name:

Full mailing address: (BLOCK LETTERS)

Return:
Email: eideditor@cdc.gov
Fax: 404-639-1954
or mail to:
EID Editor
CDC/NCID/MS D61
1600 Clifton Rd, NE
Atlanta, GA 30333
USA
Evidence of Vaccine-related Reassortment of Rotavirus, Brazil, 2008–2010

Tatiana Lundgren Rose, Marcelle Figueira Marques da Silva, Mariela Martínéz Gomez, Hugo Reis Resque, Maria Yury Travassos Ichihara, Eduardo de Mello Volotão, and José Paulo Gagliardi Leite

Analysis of 27 rotavirus strains from vaccinated and unvaccinated children revealed reassortment events in 3 strains: a gene derived from a vaccine; a gene acquired from a circulating strain; and reassortment between circulating strains. Data suggest that the widespread use of this monovalent rotavirus vaccine may introduce vaccine genes into circulating human rotaviruses or vice versa.

Group A rotaviruses (RVAs) are a frequent cause of diarrhea in children. The RVA genome consists of 11 dsRNA segments that encode 6 structural (VP1–VP4, VP6, VP7) and 6 non-structural (NSP1–NSP6) proteins (1). The basis of a new classification system is phylogenetic analysis of 11 RVA genome segments, although binary classification is still used; genotyping is based on the coding genes for VP7 (G) and VP4 (P) (2).

Vaccination is considered effective in reducing the consequences of RVA. Two vaccines, Rotarix (Glaxo SmithKline, Brentford, UK) and RotaTeq (Merck & Co., Whitehouse Station, NJ, USA), are licensed in several countries. Both vaccines demonstrated broad protection against the most common RVA genotypes (3).

In Brazil, Rotarix, a monovalent attenuated human rotavirus vaccine for infants 6–24 weeks of age, was introduced in the National Immunization Programs in March, 2006. The vaccine is delivered in 2 doses, ≥4 weeks apart. In 2009, when vaccine coverage achieved 85.9%, reduction in mortality rates (22%) was observed (4). Shedding is not associated with increased gastroenteritis-like symptoms. Widespread use of Rotarix might reveal adverse reactions not observed in clinical trials, emphasizing the need for global surveillance (7).

In phylogenetic analysis of 11 genes, 10 genes clustered into 5 clades. In the remaining gene, NSP5, the circulating strains clustered into 4 groups (online Technical Appendix Figure 1, panel E, wwwnc.cdc.gov/EID/article/19/11/12-1407-Techapp1.pdf).

Strain ES15221–08, detected in an unvaccinated child, is genetically distinct and differs in origin from other G1P[8] circulating strains (online Technical Appendix Figures 1, 2). The VP1 gene (648 bp; online Technical Appendix Figure 2, panel A) in this sample showed 100% nt identity with the Rotarix strain. Strain MA19006–10 was genetically similar to the strain; however, the NSP5 segment (online Technical Appendix Figure 1, panel E) was closely related to a G1P[8] strain from Australia (JF490152). These 2 strains appear to have been generated by reassortment with this vaccine strain.

The Study

During 2008–2010, fecal samples were collected from 3,852 children; 702 specimens (18.2%) had RVA-positive ELISA results; 27 of those (3.8%) were characterized as G1P[8] by using reverse transcription-PCR. Eighteen of the 27 specimens were from vaccinated children (Table 1). Study methods were approved by Fiocruz Ethical Committee (No. 311/06).

RVA detection, genotyping, and sequencing were performed (5). Sequences were deposited in GenBank under accession numbers: JQ926436–JQ926600 and JX683535–JX683664. Sequences were compared with those in strains obtained from GenBank (including Rotarix JX943604.2–JX943614.2).

Strains analyzed belonged to the Wa-like genogroup (genotype 1); 26 strains showed a G1–P[8]–I1–R1–C1–M1–A1–N1–T1–E1–H1 genome constellation. One sample, collected from a child vaccinated with 1 dose during 2010 in Maranhão (MA) state (MA19030–10), contained the G1–P[8]–Ix–R1–Cx–M1–A1–N1–T3–E1–H1 constellation.

Nucleotide (nt) identity values between circulating strains in Brazil and the Rotarix strain ranged 76%–100% (Table 2). Three samples showed 100% nt identity with the Rotarix strain in ≥1 gene. The SE15901–08 strain, collected on day 7 after the first dose, showed 100% nt identity with all Rotarix strain gene segments and could represent vaccine shedding. Vaccine antigen excretion detected by ELISA achieved 80%, declining to 18%–24% when collected at day 30; 11%–16% of children shed the virus at day 45 (6). Shedding is not associated with increased gastroenteritis-like symptoms. Widespread use of Rotarix might reveal adverse reactions not observed in clinical trials, emphasizing the need for global surveillance (7).

In phylogenetic analysis of 11 genes, 10 genes clustered into 5 clades. In the remaining gene, NSP5, the circulating strains clustered into 4 groups (online Technical Appendix Figure 1, panel E, wwwnc.cdc.gov/EID/article/19/11/12-1407-Techapp1.pdf).

Strain ES15221–08, detected in an unvaccinated child, is genetically distinct and differs in origin from other G1P[8] circulating strains (online Technical Appendix Figures 1, 2). The VP1 gene (648 bp; online Technical Appendix Figure 2, panel A) in this sample showed 100% nt identity with the Rotarix strain. Strain MA19006–10 was genetically similar to the strain; however, the NSP5 segment (online Technical Appendix Figure 1, panel E) was closely related to a G1P[8] strain from Australia (JF490152). These 2 strains appear to have been generated by reassortment with this vaccine strain.

Author affiliations: Oswaldo Cruz Institute, Rio de Janeiro, Brazil. (T.L. Rose, M.F.M. Silva, M.M. Gomez, H.R. Resque, E. M. Volotão, J.P.G. Leite) and Federal University of Bahia, Salvador, Bahia, Brazil (M.Y.T. Ichihara)
Strain MA19030–10, detected in a child after 1 vaccine dose, was closely related to the MA group, but the NSP2 segment differed in origin from other MA samples (online Technical Appendix Figure 1, panel B) because it clustered with other Wa-like strains. The NSP3 gene belonged to genotype 3 because it was 99.2% (nt) similar to the AU-1 prototype strain (DQ490535.1). These results suggested reassortment events between Wa-like and AU-1 like co-circulating strains.

NSP2 and NSP3 genes from sample MA19030–10 and NSP5 genes from sample MA19006–10 differed from each other and from those of their respective clusters. Samples MA19006–10 and MA19030–10 are genetically distinct and might have distinct evolutionary histories. Studies that included this genogrouping system were performed to prove the existence of inter-genogroup reassortment between human RVA genogroups or human and animal genogroups. The existence and effectiveness of heterogeneous genome constellations remains unclear, probably because it is caused by mechanisms that create protein sets that work better when kept together (8).

Phylogenetic analysis of the VP8* (aa 1–247) portion of VP4 encoding gene showed circulating strains (online Technical Appendix Figure 2, panel D) clustered into 2 lineages (P[8]-3 and P[8]-1). The alignment of the deduced aa sequences showed potential trypsin cleavage sites at arginine 240 and 246 conserved in all samples. All circulating strains contained 91.3%–100% identical aa residues to the Rotarix strain in VP8* antigenic epitopes; no changes were observed on epitopes 8–2 and 8–4.

Two VP7 lineages (G1-I and G1-II) were identified. Samples closely related to the Rotarix strain belonged to G1-II; remaining strains belonged to G1-I. Comparing the regions defined as antigenic epitopes (7–1 and 7–2) for VP7 protein, we found ≥2 epitopes (aa 94, 123, 148, 217) were not conserved among circulating strains compared with the Rotarix strain. The following amino acids were conserved in the non-VP7/non-VP4 segments of either the Rotarix strain or other strains analyzed: cysteine residues involved in disulfide bonds at positions 6, 8, 85, and 285 in NSP2 protein; the leucine, isoleucine, aspartic acid, methionine and glutamine at positions 275, 281, 288, and 295 in the NSP3 protein; the N-linked glycosylation sites at positions 8 and 18, and cysteine residues at positions 63 and 71 in NSP4 protein; and serine residues at positions 153, 155, 163, and 165 in NSP5 protein.

**Conclusions**

This report characterizes the complete genome of G1P[8] strains in Brazil. Phylogenetic analysis showed that
sequences clustered consistently with the region of sample collection. Three strains circulating in Brazil were closely related to those in Rotarix; 1 of the 3 was 100% identical to Rotarix, likely representing shedding of this vaccine. Sequence analysis confirmed the presence of the Rotarix VP1–derived segment in 1 sample (ES15221–08), indicating an unreported reassortment event between the vaccine and a community strain.

The backbone of MA19030–10 sample was Wa-like, but the NSP3 segment exhibited a T3 genotype that was described for the AU-1 genogroup. This finding suggests that this strain derived its NSP3 gene from an AU-1-like strain through reassortment.

Changes in antigenic regions of VP4 and VP7 proteins have been associated with mutated RVA strains that spread (9,10). Comparison of the aa sequences of VP7 and VP8* of strains circulating in Brazil and the monovalent vaccine strain demonstrated that VP7 and VP8* of the circulating strains showed similar antigenic regions to those of the vaccine. No differences between strains from vaccinated and unvaccinated children were observed.

Considering the segmented RVA genome and that the Rotarix vaccine is an attenuated RVA human strain, it is expected that reassortants will arise and circulate among humans. The effects of such events are not known. This study described strains that originated from reassortment events between the Rotarix vaccine strain and strains detected in vaccinated and unvaccinated children. Improvement of RVA surveillance programs that include full genome sequencing analysis will strengthen the understanding of how vaccines will affect the RVAs circulating among humans, and how those events could affect the use of live vaccines, the frequency of RVA intra- and intergenogroup reassortment events under natural conditions, and the stability of RVA generated by such events.

**Acknowledgments**

The authors thank the PDTIS DNA Sequence Platform (RPT01A) and LATER/BioManguinhos, FIOCRUZ, Rio de Janeiro, Brazil; and the Secretary of Public Health State of Sergipe.
Maranhão, Espírito Santo, Bahia and Pernambuco. We also thank Alexandre Madi Fialho and Rosane Maria Santos de Assis for technical assistance.

This research was supported by federal funds from the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), the National Council for Scientific and Technological Development (CNPq), project PAPES VI/Fiocruz - CNPq, Oswaldo Cruz Institute (PROEP – Fiocruz - CNPq), the General Coordination of Public Health Laboratories – Secretary of Health Surveillance (CGLAB/SVS), project CAPES-MERCOSUL PPCP 023/2011, and Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State (FAPERJ). T.L.R. receives a post-doctoral scholarship from CNPq.

Dr Rose is a post-doctoral student at the Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Fiocruz, Brazil. Her research interests include RVA molecular epidemiology.

References


Address for correspondence: Tatiana L. Rose, Laboratory of Comparative and Environmental Virology, Pav Helio & Peggy Pereira, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Av Brazil 4.365, Manguinhos, Rio de Janeiro, 21040–360, Brazil; email: tatilrose@yahoo.com.br

Table of Contents

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 19, No. 11, November 2013

GovDelivery

Manage your email alerts so you only receive content of interest to you.

Sign up for an Online Subscription:
http://wwwnc.cdc.gov/eid/subscribe.htm

Emailed to you
Three Outbreak-causing Neisseria meningitidis Serogroup C Clones, Brazil


During 2003–2012, 8 clusters of meningococcal disease were identified in Rio de Janeiro State, Brazil, all caused by serogroup C Neisseria meningitidis. The isolates were assigned to 3 clonal complexes (cc): cc11, cc32, and cc103. These hyperinvasive disease lineages were associated with endemic disease, outbreaks, and high case-fatality rates.

The last epidemic of Neisseria meningitidis serogroup C meningococcal disease in Rio de Janeiro State, Brazil, occurred in 1994. It was caused by C:2b:P1.10 isolates that belonged to cluster A4 (1). Although the number of cases of serogroup C disease subsequently declined after a vaccination campaign, rates of serogroup C disease again began to increase in 2000. During 2003–2012, public health surveillance identified 8 clusters of serogroup C meningococcal disease in Rio de Janeiro State. We report the investigation of these meningococcal disease clusters and typing information of the causative agent.

The Study

Public health surveillance of meningococcal disease in Rio de Janeiro State is conducted by the Meningitis Advisory Committee of the State Department of Health, which uses data obtained from 2 surveillance sources: mandatory reports of meningococcal disease cases and reports of laboratory-confirmed N. meningitidis isolates collected by the Central Laboratory Noel Nutels and the Infectious Diseases State Institute São Sebastião, which are state reference laboratories, and 1 outsourced laboratory for bacterial meningitis (Cientificab Laboratory Products and Systems, Rio de Janeiro, Brazil). Chemoprophylaxis with rifampin is currently recommended for close contacts of persons with confirmed or suspected cases of meningococcal disease.

A cluster was defined as ≥3 cases of meningococcal disease with a clear epidemiologic link and with N. meningitidis of the same serogroup recovered from either a normally sterile site or detected by PCR. Reports of invasive meningococcal disease during 2000–2012 were obtained from the Meningitis Advisory Committee and analyzed by using EpilInfo (version 3.5.3; Centers for Disease Control and Prevention, Atlanta, GA, USA). This study was approved by the Ethical Committee of the Evandro Chagas Research Institute of the Oswaldo Cruz Foundation.

We identified 8 clusters involving 46 cases that occurred during 2003–2012; all were caused by serogroup C N. meningitidis (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/11/13-0610-Techapp1.pdf; Figure). N. meningitidis serogroup was determined by slide agglutination with specific rabbit antisera (BD Diﬁco, Sparks, MD, USA) or serogroup-specific PCR directly from cerebrospinal ﬂuid samples (2). Serotype and serosubtype were determined by immunoblot analysis at the National Meningitis Reference Center. Susceptibility to rifampin was determined by using E-test (bioMérieux, Marcy-l’Étoile, France).

The genetic lineage of N. meningitidis isolates recovered in culture (n = 11) or directly detected in cerebrospinal fluid samples (n = 24) was determined by multilocus sequence typing (MLST), and the antigenic proﬁle was determined by sequencing antigen-encoding genes: porB, porA (variable regions 1 and 2), and fetA variable region (3). A total of 122 serogroup C invasive isolates (C:2a [22]; C:2b [17]; C:4,7 [36]; C:19 [4]; C:23 [43]) recovered from 1990 through 2010 were also genotyped. Sequence types and alleles at antigenic loci were assigned by the N. meningitidis MLST database (www.mlst.net).

Serogroup C disease increased from 121 (26%) of 463 cases during 2000–2003, to 174 (44%) of 394 cases during 2004–2007, and to 499 (84%) of 594 cases during 2008–2012 (p<0.01). The case-fatality rate of serogroup C disease also increased during the same periods: 12%, 14%, 18%, 20%, 22%, 26%, 26%, 28%, and 32% respectively.

1The study was presented in part at the 18th International Pathogenic Neisseria Conference, September 9–14, 2012, Würzburg, Germany.
and 19% (p = 0.03), respectively. These serogroup C isolates were mainly represented by 4 serologic phenotypes: C:23:P1.14–6 (60%), C:4,7:P1.7,1 (15%), C2a:P1.5,2 (12%), and C:4,7:P1.19,15 (6%).

New cases associated with serogroup C meningococcal disease clusters occurred within an average of 15 days (range 1–30 days), and those infected had proximity to each other (same household, vicinity, daycare center, primary school, or workplace). The average age of patients was 13 years (range 10 months–51 years). The clinical signs and symptoms recorded when the person sought medical treatment were fever (98%), vomiting (80%), hemorrhagic rash (67%), headache (65%), neck stiffness (50%), impaired consciousness (41%), diarrhea (15%), abdominal pain (15%), convulsions (13%), sore throat (9%), and myalgia (6%). The overall case-fatality rate was 28% (13/46), ranging from 17% (cc103) to 44% (cc11) (Table); 6 (46%) of 13 deaths occurred as the person sought treatment. A program of vaccination with serogroup C polysaccharide vaccine was implemented twice, once in October 2003 (Paraty) and again in January 2008 (Armação de Búzios). Subsequently, 1 vaccinated person became infected in each locality (online Technical Appendix).

Isolates assigned to clonal complex (cc) 11, cc32, and cc103 were associated with the clusters of meningococcal disease (online Technical Appendix; Figure); all were rifampin-susceptible (MICs, 0.006–0.19 μg/mL). The results of genotyping the 122 invasive isolates collected from 1990 through 2010 are shown in the Table. The Table also indicates when the cluster-associated clones were first observed.

Conclusions
Clusters of meningococcal disease were a prominent feature of N. meningitidis infections in several countries during the 1990s (4,5). These meningococcal clusters have been associated with educational institutions and particular clones of serogroup C. Clusters and community outbreaks of serogroup C disease have recently been observed in Brazil with increasing frequency outside the person’s place of residence and involving teenagers and young adults, e.g., caused by the ST-3780 (cc103) isolates (6–8). A single cluster has been associated with the C:4,7:P1.19,15 phenotype (9).

Although the annual incidence rate remained stable (2–3 cases/100,000 population), clusters of meningococcal disease marked a change in the epidemiology of N. meningitidis infection during the 2000s in Rio de Janeiro State, while serogroup C disease and its case-fatality rate steadily increased. These clusters were caused by different clones, involved mostly children, and were accompanied by high case-fatality rates. The serogroup C clones found in this study seem to have emerged during the 2000s and are also now the major cause of endemic meningococcal disease. Some of these clones, namely, cc11 and cc32, have undergone capsular switching. For instance, the 2–2:P1.5–1,10–8:F3–6:ST-7816 (a single locus variant of ST-11) clone from 2009 was found to express a serogroup W capsule (10), and the 3–79:P1.7–1,1:F5–1:ST-639 clone was previously demonstrated to belong to serogroup B (3).
Chemoprophylaxis to control clusters has been ineffective in preventing new cases, possibly because transmission might have been occurring among social networks that did not receive chemoprophylaxis. In addition, it is not known whether chemoprophylaxis reduces risk in educational institutions (3). All of these clusters were potentially...

<table>
<thead>
<tr>
<th>Serogroup/year</th>
<th>Genotype and clonal complex (no. isolates)</th>
<th>Date of emergence of cluster-related clone (date of commencement of cluster)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C:2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1990</td>
<td>2:184.P1.5-1:2:2:F1-1:ST-5121 (1)</td>
<td></td>
</tr>
<tr>
<td>1997</td>
<td>2:60:P1.5:2:F3-6:ST-7849 (1)</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>2:184:P1.5-1:2:2:F1-1:ST-5121 (1)</td>
<td></td>
</tr>
<tr>
<td>C:2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1990</td>
<td>2:3:P1.18-1:3:F3-1:ST-8 (1)</td>
<td>No cluster associated with this clonal complex</td>
</tr>
<tr>
<td>2000</td>
<td>2:30:P1.5-2:10:F5-2:ST-153 (8)*</td>
<td></td>
</tr>
<tr>
<td>2002†</td>
<td>2:30:P1.5-2:10:F5-2:ST-153 (1)</td>
<td></td>
</tr>
<tr>
<td>C:4,7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1994</td>
<td>3:1:P1.19,15:F5-1:ST-7709 (1)</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>3:299:P1.7-1:1:F5-1:ST-639 (2)</td>
<td></td>
</tr>
<tr>
<td>2002†</td>
<td>3:1:P1.19,15:F5-1:ST-639 (3)</td>
<td></td>
</tr>
<tr>
<td>C:4,7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>3:294:P1.7-1:1:F5-1:ST-639 (1)</td>
<td></td>
</tr>
<tr>
<td>C:19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C:23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009†</td>
<td>2:23:P1.22,14-6:F3-9:ST-5122 (2)</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>2:23:P1.22,14-6:F3-9:ST-8730 (1)</td>
<td>No cluster associated with this strain</td>
</tr>
</tbody>
</table>

*No invasive C:2b isolate has been recovered since 2002.†The clone associated with the last epidemic of serogroup C disease in 1994.
vaccine preventable with monovalent serogroup C meningococcal vaccine, which was instituted in the state program of routine vaccination for children (<2 years) in October 2010. The implementation of molecular surveillance is advisable to both guide immunization programs and to monitor the effects of the immunization program and its consequences for the population biology of *N. meningitidis* associated with invasive disease.

Acknowledgments

We thank the staff of the National Meningitis Reference Center for serologic typing, the staff of the microbiology laboratories for providing meningococcal isolates, all health professionals involved in the investigation of communicable diseases, Mônica de A. F. M. Magalhães and Fabiane Bertoni for the digital map, and Julia Bennett and Keith Jolley for curation of the MLST database for *Neisseria meningitidis*.

This work was supported in part by a Fogarty International Center Global Infectious Diseases Research Training Program grant, National Institutes of Health, to the University of Pittsburgh (D43TW006592).

L.H.H. receives funding from the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases. He has received research support and lecture fees from Sanofi Pasteur; lecture fees from Novartis Vaccines; and Pfizer. Financial ties of L.H.H. with industry were terminated before he became a voting member of the Advisory Committee on Immunization Practices in July 2012.

Dr. Barroso is a consultant in communicable disease control and a researcher in public health at the Oswaldo Cruz Institute, Brazil. His research interests focus on meningococcal disease and bacterial epidemiology.

References


Address for correspondence: David E. Barroso, Laboratory of Epidemiology and Molecular Systematics, Oswaldo Cruz Institute, FIOCRUZ, Av. Brasil 4365, Rio de Janeiro, R.J., Brazil 21040-900; email: barroso@ioc.fiocruz.br

All material published in Emerging Infectious Diseases is in the public domain and may be used and reprinted without special permission; proper citation, however, is required.
Severe Plasmodium vivax Malaria in Pakistan


To compare the severity of Plasmodium vivax malaria with that of P. falciparum malaria, we conducted a retrospective cross-sectional study of 356 adults hospitalized with malaria (2009–2011) in Pakistan. P. vivax and P. falciparum accounted for 83% and 13% of cases, respectively; 79.9% of patients with severe malaria were infected with P. vivax.

Malariasis endemic to Pakistan and 64% and 36% of malaria cases are attributed to Plasmodium vivax and P. falciparum, respectively (1). The purpose of this study was to identify the complications of P. vivax among hospitalized malaria patients and compare the prevalence of these complications with those of P. falciparum malaria.

The Study

We conducted a retrospective cross-sectional study using convenience sampling at the Aga Khan University Hospital in Karachi, Pakistan. Participants were all adult patients (≥16 years of age) who were hospitalized with malaria during January 2009–December 2011. Reasons for hospitalization included intravenous antimalarial therapy, management of associated diagnoses, and complications. The following data on patients were retrieved through the hospital’s electronic and file records: age, sex, infecting Plasmodium species, malaria diagnosis methods, co-existing conditions, results of biochemical and microbiological investigations, radiographic findings, complications, hospital course, and outcome.

Records showed that Giemsa-stained peripheral blood smears, the malaria rapid diagnostic test (RDT), or both, were used for malaria diagnosis. The RDT used antibodies against P. falciparum histidine-rich protein 2 and P. vivax lactate dehydrogenase. For 45 case-patients for which results from peripheral blood smears and RDTs were discordant or unreliable, surface protein-specific PCR was performed by using stored patient blood samples to identify the Plasmodium species (2,3). Clinical syndromes were classified as severe on the basis of the World Health Organization’s 2010 severe falciparum malaria criteria (4).

Statistical analysis was performed by using SPSS version 20 (http://www-01.ibm.com/software/analytics/spss/). Averages, χ² test of independence, odds ratios with 95% CIs, and analysis of variance were computed when applicable.

Case-patients with prior co-morbid conditions were excluded from relevant subanalyses, for example, diabetes mellitus patients were excluded from hypoglycemia analysis. All analysis was also repeated after excluding all case-patients with associated infections and comorbid illnesses. The classification “comorbidity” included all conditions in the Charlson comorbidity index for mortality (5). The study was approved by the Aga Khan University’s Ethics Review Committee.

A total of 356 patients with malaria (mean ± SD age 42 ± 18 years) were hospitalized in the Aga Khan Hospital during 2009–2011. Among these, 296 (83.1%), 47 (13.2%), and 13 (3.7%) were found to have P. vivax infection, P. falciparum infection, and mixed infections (P. vivax and P. falciparum), respectively. Baseline patient demographics are given in Table 1. The proportion of P. vivax infection among hospitalized malaria patients increased from 75.0% in 2009 to 87.7% in 2011 (p < 0.02) (Figure 1, panel A).

One hundred thirty-nine (39.0%) patients had at least 1 complication by World Health Organization criteria (4), among which 111 (79.9%) patients had P. vivax infection. In 24 (51.0%) cases of P. falciparum infections and in 111 cases (37.5%) of P. vivax infections, respectively, severe malaria developed (p = 0.077). As shown in Figure 2, the proportion of severe malaria among P. vivax patients increased from 24.1% in 2009 to 43.2% in 2010 and 39.5% in 2011 (p = 0.02).

The most common complications in the patients are shown in Table 2. P. vivax and P. falciparum were responsible for comparable rates of pulmonary edema, the need for mechanical ventilation, coagulopathy, hypoglycemia, hemoglobinuria, metabolic acidosis, renal impairment, liver dysfunction, bleeding, and multi-organ dysfunction. Altered consciousness, anemia, and jaundice were associated with P. falciparum malaria. The mean platelet count for P. vivax patients was 55, significantly lower than that of P. falciparum patients (67.5; p = 0.001) and those with mixed infections (61; p = 0.024).

The mean hospital stay was 4.1 days for P. falciparum patients, 3.6 days for P. vivax patients, and 2.9 days for patients with mixed infections. Three P. vivax malaria patients experienced fatal acute myocardial infarctions. One patient, who had metastatic myeloma and P. falciparum malaria, died. The mortality rate was 2.1% for P. falciparum patients and 1.0% for P. vivax patients (p = 0.50).
Analysis was repeated after all patients with comorbid conditions were excluded (Table 1), which left 229 case-patients who had no illness other than malaria. Among these, 30 (13%) patients had *P. falciparum* infection, 189 (83%) had *P. vivax* infection, and 10 (4%) had mixed infection (Figure 1, panel B). In these patients, severe malaria appeared significantly more common in falciparum versus vivax malaria (53% and 33%, respectively, *p* = 0.029); however, 79.5% of the severe cases were caused by *P. vivax*. Hemoglobinuria and a higher mean creatinine level were more likely to occur with falciparum malaria than with vivax malaria (*p* < 0.02). Shock and secondary bacterial infections were no longer associated with *P. falciparum* infection. All other statistical associations held, although the strength of association varied.

**Conclusions**

A study of hospitalized malaria patients at the Aga Khan University Hospital during 1997–2001 showed that 51.8% of cases were caused by *P. vivax* and 46.5% by *P. falciparum*, with mortality rates of 1.5% and 2.0%, respectively (*p* = 0.029). Recent studies from elsewhere in Asia reported that 20%–40% of patients hospitalized with malaria had *P. vivax* malaria (*p* < 0.02). In our study, a much greater proportion of malaria cases were caused by *P. vivax* (83%), which was not unexpected because of the decreasing number of *P. falciparum* cases during the study period. Despite this high incidence of *P. vivax* malaria, the mortality rate found in our study is reassuring and stable at 1.0%.

The higher prevalence of jaundice, anemia, and hemo-

---

**Table 1. Demographic profile of study participants with *Plasmodium vivax* and *P. falciparum* malaria, Karachi, Pakistan, 2009–2011**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. vivax</em> Frequency (%)</th>
<th><em>P. falciparum</em> Frequency (%)</th>
<th>Mixed Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>98 (33)</td>
<td>12 (25)</td>
<td>6 (46)</td>
</tr>
<tr>
<td>M</td>
<td>198 (67)</td>
<td>35 (75)</td>
<td>7 (54)</td>
</tr>
<tr>
<td>Previously healthy adults</td>
<td>189 (64)</td>
<td>30 (64)</td>
<td>10 (77)</td>
</tr>
<tr>
<td>Concurrent illness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>49 (17)</td>
<td>4 (9)</td>
<td>0</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>37 (12)</td>
<td>2 (4)</td>
<td>3 (23)</td>
</tr>
<tr>
<td>Chronic kidney disease</td>
<td>10 (3)</td>
<td>3 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Co-existing infection†</td>
<td>34 (12)</td>
<td>5 (11)</td>
<td>0</td>
</tr>
<tr>
<td>Others‡</td>
<td>10 (3)</td>
<td>5 (11)</td>
<td>0</td>
</tr>
<tr>
<td>Total§</td>
<td>107 (38)</td>
<td>17 (36)</td>
<td>3 (23)</td>
</tr>
</tbody>
</table>

*§n = 356.
†Co-existing infections included dengue fever, urinary tract infection, enteric fever, and hepatitis C, diagnosed by appropriate serologic testing/culture.
‡Other conditions included chronic obstructive pulmonary disease, chronic liver disease, malignancy, and other conditions from the Charlson Comorbidity Index (5).
§Many patients had multiple comorbidities; therefore, the total does not sum the above.
globinuria seen with falciparum malaria in our study reflect the greater degree of hemolysis caused by *P. falciparum*. *P. vivax* has been reported elsewhere to cause a similar degree of anemia as *P. falciparum* (8). Differences in the level of endemic anemia between these study populations and may explain this discrepancy. Similar to our findings, another study reported the incidence of thrombocytopenia in hospitalized patients with vivax malaria as high as 96.3% (9). Pulmonary involvement has often been reported in complicated vivax malaria (7), as we found in our study. Hepatic dysfunction with jaundice has been reported in up to 57% of hospitalized patients with vivax malaria as high as 96.3% (9).

To estimate the true effects of severe disease with vivax malaria, researchers have recommended excluding comorbid conditions (7) and other infections (11). In this study, excluding concurrent illness enabled a stronger association between *P. falciparum* and severe malaria to emerge. Thus, *P. falciparum* caused a higher likelihood of specific complications such as central nervous system disturbance and hemolysis than did *P. vivax*. Yet, >80% of severe malaria still occurred in patients with *P. vivax* malaria.

Limitations of the study include its retrospective design, low power, and lack of PCR diagnostics for all the samples. Furthermore, the study findings reflect the malaria situation at a single urban tertiary care hospital, which cannot be generalized without knowing the denominator of all hospitalized malaria cases in the study area.

**Acknowledgments**

We acknowledge collaboration with Raymond A. Smego from the University of the Free State, Bloemfontein, South Africa, whose intellectual contribution to this study continued until his untimely death.

PCR studies for this project were funded by an Aga Khan University Research Council grant.

Dr Zubairi is an Associate Professor and Section Head in Pulmonary and Critical Care Medicine in the Department of Medicine, Aga Khan University Hospital, Karachi. His research interests are asthma, interstitial lung disease, and respiratory tract infections.

**References**


---

**Table 2. Comparison of complication rates in *P. falciparum* versus *P. vivax* infections, Karachi, Pakistan, 2009–2011**

<table>
<thead>
<tr>
<th>Complications</th>
<th>Case definition</th>
<th>No. (%) <em>P. falciparum</em> cases, n = 47</th>
<th>No. (%) <em>P. vivax</em> cases, n = 296</th>
<th>Odds ratio (CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO criteria†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altered consciousness‡</td>
<td>Disorientation or confusion</td>
<td>5 (10.6)</td>
<td>6 (2.0)</td>
<td>5.7 (1.7–19.7)</td>
<td>0.002</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>Plasma bicarbonate &lt;15 mmol/L</td>
<td>5 (10.6)</td>
<td>17 (5.7)</td>
<td>1.9 (0.7–5.6)</td>
<td>0.203</td>
</tr>
<tr>
<td>Pulmonary edema</td>
<td>Respiratory distress and bilateral</td>
<td>6 (12.8)</td>
<td>23 (7.8)</td>
<td>1.7 (0.7–4.5)</td>
<td>0.253</td>
</tr>
<tr>
<td>Abnormal spontaneous bleeding</td>
<td>Diffuse infiltrates on chest radiograph</td>
<td>1 (2.1)</td>
<td>16 (5.4)</td>
<td>0.4 (0.049–2.9)</td>
<td>0.336</td>
</tr>
<tr>
<td>Jaundice</td>
<td>Serum bilirubin &gt;3.0 mg/dL</td>
<td>12 (25.5)</td>
<td>28 (9.5)</td>
<td>3.3 (1.5–7.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td>Hemoglobin in urine</td>
<td>15 (31.9)</td>
<td>62 (20.9)</td>
<td>1.8 (0.9–3.4)</td>
<td>0.094</td>
</tr>
<tr>
<td>Shock</td>
<td>Systolic blood pressure &lt;80 mm Hg</td>
<td>4 (8.5)</td>
<td>5 (1.7)</td>
<td>5.4 (1.4–20.9)</td>
<td>0.007</td>
</tr>
<tr>
<td>Hypoglycemia‡</td>
<td>Blood glucose &lt;40 mg/dL</td>
<td>1 (2.1)</td>
<td>3 (1.0)</td>
<td>2.1 (0.2–20.9)</td>
<td>0.509</td>
</tr>
<tr>
<td>Renal impairment§</td>
<td>Serum creatinine &gt;3 mg/dL</td>
<td>2 (4.3)</td>
<td>10 (3.4)</td>
<td>1.3 (0.3–6.0)</td>
<td>0.761</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperpyrexia</td>
<td>Core body temperature &gt;40°C</td>
<td>4 (8.5)</td>
<td>32 (10.8)</td>
<td>0.8 (0.4–1.9)</td>
<td>0.416</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>Platelets &lt;150,000/mm³</td>
<td>39 (83.0)</td>
<td>272 (91.9)</td>
<td>0.4 (0.2–1.0)</td>
<td>0.051</td>
</tr>
<tr>
<td>Anemia</td>
<td>Hemoglobin &lt;7 mg/dL</td>
<td>5 (10.6)</td>
<td>58 (19.6)</td>
<td>0.5 (0.2–1.0)</td>
<td>0.141</td>
</tr>
<tr>
<td>Multiorgan dysfunction</td>
<td>Biochemical and/or radiographic evidence of ≥2 organs involved</td>
<td>5 (10.6)</td>
<td>21 (7.1)</td>
<td>5.0 (2.1–12.1)</td>
<td>0.000</td>
</tr>
<tr>
<td>Secondary infection</td>
<td>Radiographic/microbiological evidence of infection</td>
<td>9 (19.1)</td>
<td>2 (7.4)</td>
<td>2.9 (1.3–6.9)</td>
<td>0.009</td>
</tr>
<tr>
<td>Coagulopathy</td>
<td>Deranged PT/APTT</td>
<td>5 (10.6)</td>
<td>17 (5.7)</td>
<td>2.0 (0.7–5.6)</td>
<td>0.203</td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>ALT level &gt;normal</td>
<td>16 (44.4)</td>
<td>97 (40.9)</td>
<td>1.1 (0.5–1.9)</td>
<td>0.690</td>
</tr>
</tbody>
</table>

†WHO: World Health Organization; PT, prothrombin time; APTT, activated partial thromboplastin time. ALT, alanine aminotransferase.
‡Patients with preexisting diabetes were excluded from this count; n = 303.
§Patients with preexisting chronic kidney disease were excluded from this count; n = 343.


Address for correspondence: M. Asim Beg, Department of Pathology and Microbiology, The Aga Khan University, Stadium Rd, PO Box 3500, Karachi 74800, Pakistan; email: masim.beg@aku.edu
Infectious Shock and Toxic Shock Syndrome Diagnoses in Hospitals, Colorado, USA

Michael A. Smit, Ann-Christine Nyquist, and James K. Todd

In Colorado, USA, diagnoses coded as toxic shock syndrome (TSS) constituted 27.3% of infectious shock cases during 1993–2006. The incidence of staphylococcal TSS did not change significantly overall or in female patients 10–49 years of age but increased for streptococcal TSS. TSS may be underrecognized among all ages and both sexes.

First described in 1978, toxic shock syndrome (TSS) is a severe febrile illness now confirmed to be caused by exotoxin-producing strains of Staphylococcus aureus and Streptococcus pyogenes (1). Investigations based on extensive chart review and/or microbiology laboratory data suggest little or no decrease in overall TSS incidence and an increase in streptococcal TSS (2–4). Given the persistence and severity of TSS and the differences in its treatment from other causes of septic shock, its evolving epidemiology needs to be accurately monitored (5–7). To this end, we assessed International Classification of Diseases, Ninth revision, Clinical Modification (ICD-9-CM)–coded TSS cases in Colorado, USA.

The Study

In 2007, we queried the Colorado Hospital Association database for ICD-9-CM codes that identified diagnoses consistent with infectious shock or TSS unrelated to pregnancy or childbirth (8). The study population comprised Colorado residents 1–65 years of age, selected by ZIP code of residence, who were discharged from Colorado hospitals during 1993–2006. Presumptive cases of “infectious shock” were 1) TSS or meningococcal shock of any diagnostic code (040.82, 040.89, 036.3); 2) principal diagnosis of hypotension or shock (785.50, 785.59, 998.0, 458.0, 796.3) plus any secondary diagnosis of staphylococcal infection (038.1x, 041.1x, 482.4x); streptococcal infection (041.0x, 482.3x, 034.0, 038.1); scarlet fever (034.1); or bacteremia, septicemia, or other infection (code list available from authors); and 3) principal diagnosis of bacteremia, septicemia, staphylococcal infection, streptococcal infection, other infection, or scarlet fever, plus any secondary diagnostic code of shock (see above codes). Infectious shock was further grouped into 3 code categories: 1) TSS-specific code: code for TSS (040.82 or 040.89) in any diagnostic field; 2) possible TSS code: infectious shock code without a specific code for TSS but with a code for infection with S. aureus (038.11, 041.11, 042.41) or S. pyogenes (040.01, 482.31) or with scarlet fever (034.1); and 3) infectious shock code, not TSS: infectious shock not otherwise classified. TSS was further designated as “strep” if it was associated with any code for S. pyogenes; all other TSS cases were assumed to be caused by S. aureus and were designated as “staph.” All case definitions were based on ICD-9-CM codes assigned by the discharging hospital.

Annual population-based incidences during 1993–2006 were calculated as cases per 100,000 persons by using extrapolated estimates of population by age interval and sex based on the US 1990 and 2000 censuses (9). Annual numbers of TSS cases reported to the State of Colorado were obtained from the Colorado Department of Public Health and Environment and classified as either TSS associated with S. pyogenes (reporting began in 2000) or TSS (assumed otherwise to be associated with S. aureus infection).

Infectious shock incidence increased significantly from 1993 through 2006 (R² = 0.708, p<0.001 by linear regression). Of the 2,861 hospitalized persons with infectious shock, those assigned TSS-specific codes accounted for 411 (14.4%), and possible TSS codes constituted 371 (13.0%) (Figure 1). Of the 782 TSS-specific and possible TSS cases, 121 (15.5%) had a diagnostic code related to S. pyogenes; the remaining 661 (84.5%) cases were assumed by default to be associated with S. aureus. Case-fatality rates were significantly lower (p<0.001) for TSS-specific cases (5.6%) than for infectious shock, not TSS (29.3%). The incidence of TSS-specific (staph) cases did not change significantly from 1993 through 2006, whereas incidences for all other categories significantly increased. Beginning in 2000, an annual average of 54% (range 22%–100%) of TSS-specific (strep) and 16% (range 8%–26%) of TSS-specific (staph) cases were annually passively reported to the Colorado Department of Public Health and Environment.

The ages and sexes of patients assigned codes for TSS-specific and possible TSS (staph) is shown in Figure 2. Most cases occurred in female patients 10–49 years.

Author affiliations: Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA (M.A. Smit); University of Colorado School of Medicine, Aurora, Colorado, USA (A.-C. Nyquist, J.K. Todd); Colorado School of Public Health, Aurora (A.-C. Nyquist, J.K. Todd); and Children’s Hospital Colorado, Aurora (A.-C. Nyquist, J.K. Todd)

DOI: http://dx.doi.org/10.3201/eid1911.121547
years of age (peak 10-19 years). For both sexes, the proportion of cases was comparable for the <10-year and >49-year age groups.

The crude population-based incidence for TSS-specific (staph) cases in patients 1–65 years of age averaged 0.64 per 100,000 (95% CI 0.59–0.70). For female patients 10–39 years of age, the incidence averaged 1.18 per 100,000 (95% CI 0.94–1.41). We compared the annual incidence for TSS-specific (staph) cases in Colorado during 1993–2006 (this study) with estimated incidences from 2 previously reported periods in Colorado using medical record review for 1970–1982 and 1987–1997 (4,5). We found no significant difference in the annual incidence in female patients 10–39 years of age among the 3 periods (p = 0.134, analysis of variance).

Fifty-three case records (1.8% of total sample) of patients hospitalized during 1998–2006 at Children’s Hospital Colorado were identified with TSS-specific, possible TSS, or infectious shock not TSS codes and available for independent, blinded review using the Centers for Disease Control and Prevention’s staphylococcal and streptococcal TSS case definitions (10). Within this subset, our ICD-9-CM–based code definition for TSS-specific or possible TSS had a sensitivity of 86.5%, specificity of 75.0%, and positive predictive value of 88.9%.

Conclusions

Martin et al. reported that the incidence of septic shock increased from 1979 through 2000 in the United States (11). Using similar methods, we showed an increase in infectious shock in Colorado from 1993 through 2006 and estimated that the diagnosis of TSS accounted for as much as 27% of all cases of infectious shock. The TSS-specific incidence attributed to S. aureus has remained relatively constant in Colorado, although TSS attributed to S. pyogenes appears to be increasing. The latter has been noted in other studies as well (12,13). The observation that TSS accounts for a substantial proportion of all infectious shock is of clinical importance because TSS may respond to therapies (e.g., clindamycin, intravenous immune globulin, steroids) not ordinarily used for septic shock caused by other organisms, with more favorable outcomes as evidenced by significantly lower case-fatality rates for TSS noted in our study (5–8). For the current study period of 1993–2006, <20% of cases with a specific ICD-9-CM diagnosis of TSS (presumed staphylococcal) were reported to the state’s passive surveillance system. Passive surveillance systems may be of limited use if reported cases are not numerous enough to track trends (14). Our data show that the estimated incidence for staphylococcal TSS has not decreased significantly in Colorado since 1980 (3,4). Data recently published using similar methods also reported stable TSS incidence (15).

Our methods have important limitations. Our chart validation of coded definitions demonstrated reasonable sensitivity and positive predictive value for TSS-specific and possible TSS codes at a single institution; however, discharge coding among institutions is not necessarily consistent or precise and can result in ascertainment errors when applied to larger discharge populations. The TSS-specific code definition most likely underestimates the true incidence of less severe disease variants. Discharge
Infectious Shock and Toxic Shock Syndrome

databases contain little additional data that would facilitate risk factor assessment.

With these limitations, our results suggest that surveillance of TSS with a hospital discharge database provides significantly more sensitive case ascertainment than conventional passive reporting. Electronic definitions that use population-based databases could improve identification of TSS cases with a better understanding of its epidemiology. Given the clinical and management differences between shock caused by TSS, S. aureus, S. pyogenes, and other organisms, it is important to recognize TSS as a common cause of infectious shock in persons of both sexes and all ages.

Acknowledgments

We thank A. James Ruttenber for his mentoring and his contributor to this manuscript. We also thank Ken Gershman for providing Colorado Department of Health and Environment reporting data for this study.

This study design was reviewed and approved by the Colorado Multiple Institutional Review.

J.K.T. serves as a member of a Procter and Gamble product safety board.

Dr Smit is an assistant professor of pediatrics at the Warren Alpert Medical School of Brown University, Providence, Rhode Island. His research interests include epidemiology of infectious diseases, hospital infection prevention and control, antimicrobial stewardship, and global health.

References


Address for correspondence: James K. Todd, Children’s Hospital Colorado, 13123 East 16th Ave, B-276, Aurora, CO 80045, USA; email: james.todd@childrenscolorado.org
Human Bocavirus in Patients with Encephalitis, Sri Lanka, 2009–2010

Daisuke Mori, Udaya Ranawaka,
Kentaro Yamada, Shamen Rajindrajith,
Kazushi Miya, Harsha Kumara Kithsiri Perera,
Takashi Matsumoto, Malka Dassanayake,
Marcelo Takahiro Mitui, Hisashi Mori,
Akira Nishizono, Maria Söderlund-Venermo,
and Kamruddin Ahmed

We identified human bocavirus (HBoV) DNA by PCR in cerebrospinal fluid from adults and children with encephalitis in Sri Lanka. HBoV types 1, 2, and 3 were identified among these cases. Phylogenetic analysis of HBoV1 strain sequences found no subclustering with strains previously identified among encephalitis cases in Bangladesh.

Encephalitis is a serious infection causing high rates of illness and, in industrialized countries, has a case-fatality rate of 6.5%–12% (1,2). However, the situation in developing countries is largely unknown. Globally, the causes remain unrecognized in 60%–85% of encephalitis cases (1,2). Recently, human bocavirus (HBoV) has been implicated in causing life-threatening encephalitis in Bangladeshi children (3). In Sri Lanka, information about the causative agents of encephalitis is sparse. The aim of this study was to determine the occurrence of HBoV and other possible pathogens in children and adults with encephalitis admitted to a tertiary care hospital in Sri Lanka.

The Study

The study was conducted at Colombo North Teaching Hospital, Ragama, Sri Lanka, during July 2009–November 2010. A total of 233 patients (110 adolescents/adults ≥12 years of age and 123 children) were enrolled. Adolescents and adults were admitted to adult wards. Cerebrospinal fluid (CSF) samples were available from 191 patients. Criteria for enrollment were as follows: any combination of the triad of fever, headache, and vomiting, along with altered level of consciousness, seizures, focal neurologic deficits, altered behavior, and signs of meningeal irritation. Clinical and laboratory information was available for 164 patients. The male:female ratio for adolescents/adults was 1.3:1; ages ranged from 12 to 90 years (mean 42 years); For children, the male:female ratio was 0.7:1; ages ranged from 2 to 144 months (mean 48 months). The ethics committees of the University of Kelaniya and Oita University approved this study.

CSF samples were subjected to microscopic examination, total and differential leukocyte counts, bacterial culture, Gram staining, and measurement of protein and glucose. Blood was cultured for bacteria and examined for total and differential leukocyte counts, erythrocyte sedimentation rates, and hemoglobin and C-reactive protein levels.

Classical encephalitis-causing pathogens (Table) and diarrheagenic viruses, such as HBoV, rotavirus, astrovirus, norovirus, parechovirus, and human adenovirus (HAdV), were determined in CSF by PCR (online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/11/12-1548-Techapp1.pdf) (3–5). Anti-β2-methyl-d-aspartate receptor (NMDAR) encephalitis was diagnosed by on-cell Western analysis (6). For HBoV PCR-positive patients, HBoV types 1–4-specific IgG and IgM responses in CSF samples were measured by enzyme immunoassays (7).

Nucleotide sequences of all amplicons were determined to confirm the PCR products, to distinguish genotypes, and to perform phylogenetic analysis (3). BLAST analysis (www.ncbi.nlm.nih.gov/blast) was used to identify the viruses and genotypes. Multiple sequence alignment was conducted by using ClustalW2 (www.ebi.ac.uk/clustalw). The phylogenetic analysis was done with a neighbor-joining tree by using MEGA5 (www.megasoftware.net). A bootstrap analysis of 1,000 replicates was performed to test the reliability of the branching pattern.

The causes of encephalitis were type 2 dengue virus in 1 (0.5%) patient, human echovirus (HEcoV) type 9 or 25 in 2 (1%) patients, HBoV (Table) in 5 (3%), and HAdV 41 in 7 (4%); all were sole detections. None of the other viruses and no bacteria were detected. Samples positive for HBoV by primers designed from viral protein 1/2 also were positive by primers designed from nonstructural protein (NP) 1 gene. HEcoV was detected in 2- and 9-year-old children. HAdV 41 was not confined to children; ages of infected patients ranged from 13 months to 55 years. Of 81 CSF samples, anti-NMDAR encephalitis was detected in 2 (2%) adults (42 and 72 years of age). All patients in this study recovered and were discharged, except for one 13-month-old boy with HAdV 41 encephalitis who left the hospital against medical advice.
The severity of symptoms in the HBoV-positive patients did not differ from those of patients with other infections. None of the patients who had positive PCR results for HBoV1–3 had corresponding HBoV1–4 IgM or IgG in their CSF. Phylogenetic analysis (Figure) of the viral protein 1/2 gene showed that the Sri Lanka HBoV1 strains did not subcluster with encephalitis-associated Bangladesh strain, although they had 97%–98% nt identities. The Sri Lanka HBoV1 strains had 98%–99% nt identities among themselves and with other HBoV1 strains. The Sri Lanka HBoV2 strain was closely related to the Tunisia strain (96% nt identity). The Sri Lanka HBoV2 had 90%–91% nt identities with the Bangladeshi encephalitis-causing strains and 90%–96% nt identities with other HBoV2 strains. The Sri Lanka HBoV3 strain was closely associated with the cluster formed by viruses from the United Kingdom, Australia, Tunisia, and China and had 96%–97% nt identities with those strains. The sequence of NP1 gene is conserved and had 98%–100% nt identities among the Sri Lanka strains.

**Conclusions**

The study in Bangladesh suggested that HBoV-associated encephalitis might be restricted to malnourished children (3). However, our study demonstrates that HBoV also can be detected in well-nourished children and adults with encephalitis. How HBoV might trigger encephalitis is unclear. HBoV viremia has been documented, and the virus might therefore have the potential to cross the blood–brain barrier. The NP1 of HBoV inhibits interferon-β production by the host, suggesting evasion of the innate immune response during infection (8).

Unlike the Bangladesh study, where 2 of 4 encephalitis patients in whom HBoV was detected died (3), all patients in our study recovered. In addition to HBoV1 and HBoV2, we detected HBoV3 in a child with encephalitis, which to our knowledge, has not been reported as a cause of the disease. Although HBoV infections occur mainly in children, among the 5 Sri Lanka patients with HBoV encephalitis, 3 were adults or adolescents. None of the patients with HBoV encephalitis had HBoV IgM or IgG in their CSF, indicating...
how rapidly disease onset occurred and how little time the immune system had to respond. Generally, the specific seroprevalence rate of HBoV1 antibodies in infected persons is 59%, followed by HBoV2, 3, and 4 (34%, 15%, and 2%, respectively) (7).

Our detection rate of viruses as a cause of encephalitis was 7.5%, and adding anti-NMDAR encephalitis, the detection rate increased to 10%, which is similar to that of another study (9). Anti-NMDAR encephalitis is becoming a dominant cause of encephalitis in certain population (10); however, in Sri Lanka, it is 1%–4%, similar to other studies (11).

Dengue virus is the leading endemic cause of encephalitis in Brazil (12). This infection is also endemic to Sri Lanka and, before our study, dengue encephalitis was suspected but unconfirmed in the population. Enteroviruses frequently cause CNS infection, and the HECr09 9 and 25 found here are known to cause encephalitis (13).

Among the HAdVs, serotype F is mainly responsible for gastroenteritis, whereas encephalitis is caused mainly by serotypes B, C, and D (14,15). The large number of HAdV 41 encephalitis cases indicates a unique epidemiology in Sri Lanka.

Herpes simplex and varicella-zoster viruses are implicated as the major causes of encephalitis. However, these viruses were not responsible for encephalitis in our study or in the studies in Bangladesh. HBoV is dominant in both Bangladesh and Sri Lanka. The limitation of our study is that causation could not be proven by the presence of HBoV antibody during infection or the absence of HBoV DNA in the CSF when recovered. The HBoV DNA detected in our study may represent persistent DNA from past infection; however, history of recent respiratory or diarrheal infection was absent. Future studies using quantitative PCR and serology are warranted to better establish the etiologic role of HBoV infection and encephalitis.

Acknowledgments

We thank Danushka Nawaratne, Maliduwa Liyanage Harshini, Nayomi Fonseka, Pradeepa Harshani, Ranjan Premarathne, Wathsala Hathagoda, Lasanthi Weerasooriya, Aruna Kulatunga, Nilmini Tissera, Manel Cooray, Lilanthi de Silva, Manel Fernando, and Thirughanam Sekar for their cooperation. We also thank Lea Hedman for performing enzyme immunoassay for HBoV1-4.

This study was supported in part by a Research Fund at the Discretion of the President, Oita University (grant no. 610000-N5010) to K.A., the University of Helsinki Research Funds to...
Mr Mori is a medical technologist and is enrolled in the PhD program in the Department of Microbiology, Faculty of Medicine, Oita University, Japan. His research interest is molecular epidemiology of viruses.

References


Address for correspondence: Kamruddin Ahmed, Research Promotion Institute, Oita University, Yufu 879-5593, Oita, Japan; email: ahmed@oita-u.ac.jp
Building Influenza Surveillance Pyramids in Near Real Time, Australia

Craig B. Dalton, Sandra J. Carlson, Michelle T. Butler, Elissa Elvidge, and David N. Durrheim

A timely measure of circulating influenza virus severity has been elusive. Flutracking, the Australian online influenza-like illness surveillance system, was used to construct a surveillance pyramid in near real time for 2011/2012 participants and demonstrated a striking difference between years. Such pyramids will facilitate rapid estimation of attack rates and disease severity.

Data from several influenza surveillance systems are integrated in Australia each year (1–3) to create a timely and accurate picture of influenza activity. Each surveillance method has its strengths and limitations. The online national Flutracking surveillance system contributes to Australian influenza surveillance by providing weekly community-level influenza-like illness (ILI) attack rates not biased by health-seeking behavior and clinician-testing practices (4–7). The Flutracking surveillance system has been incorporated into the weekly national Australian influenza report since 2009 (3) to 1) compare ILI syndrome rates of vaccinated and unvaccinated participants to detect interpandemic and pandemic influenza and provide early confirmation of vaccine effectiveness or failure; 2) provide consistent surveillance of influenza activity across all jurisdictions and over time; and 3) enable year-to-year comparison of the timing, incidence, and severity of influenza.

In 2011, new questions were added to the Flutracking surveillance system to document health-seeking behavior and laboratory confirmation of influenza infection among participants. This enabled regular timely calculation of influenza surveillance pyramids to examine the proportion of participants with ILI that sought medical care, the type of medical care sought, and the proportion tested for, and confirmed to have, influenza infection. Surveillance pyramids provide a model for estimating the relative attrition as patients transition the multiple steps for an episode of illness to be registered in surveillance data (8). Flutracking data for 2011 and 2012 were used to investigate whether a near real time severity measure for circulating influenza strains could be determined.

The Study

The Flutracking surveillance system was in operation for 24 weeks in 2011 from the week ending May 8 to the week ending October 16, and 24 weeks in 2012 from the week ending May 6 to the week ending October 14. Recruitment methods in 2011 and 2012 were similar to those used in 2007–2010 (4).

The weekly survey questions in 2011 and 2012 were similar to those used in 2007–2010 (4). However, in 2011, the following questions were added to the weekly questionnaire:

Did participants reporting cough and fever seek health advice because of their illness? Response options for type of advice sought included an emergency department/after-hours service, general practitioner, 24-hour health advice telephone hotline, advice from other medical professional, or admitted as a hospital inpatient. Did a doctor or nurse tell the participant, who sought health advice, that they had influenza or another illness? Did you have an influenza test (for those who sought health advice)? If so, was it positive for influenza?

We compared participation numbers from 2006 through 2012 at national level. Surveillance pyramids were then produced for 6-week blocks for the weeks ending as follows: in 2011, May 8–June 12, June 19–July 24, July 31–September 4, and September 11–October 16; and in 2012, May 6–June 10, June 17–July 22, July 29–September 2, and September 9–October 14. The pyramid base comprised the number of participants reporting fever and cough over the 6-week period; the next layer was the subset of participants who sought medical advice (from a general practitioner, emergency department/after-hours service, as a hospital inpatient). The next layer was the number of participants who reported having a laboratory test for influenza or another illness. Did a doctor or nurse tell the participant, who sought health advice, that they had influenza or another illness? Did you have an influenza test (for those who sought health advice)? If so, was it positive for influenza?

DOI: http://dx.doi.org/10.3201/eid1911.121878
4,827 in 2008, 8,546 in 2009, 12,581 in 2010, 13,101 in 2011, and 16,046 in 2012. Among the 12,109 participants in 2011 and 14,467 participants in 2012 who completed at least 1 survey in the first 4 weeks of the survey each year, the median weekly participation rate for the remainder of each year was 95.8%. Of the 318,302 surveys completed in 2012, participants reported 10,379 (3.3%) episodes of fever and cough, and among 263,778 surveys completed in 2011, there were 8,009 (3.0%) reported episodes of fever and cough. Those who experienced the 8,009 episodes also reported 2,409 (30.1%) visits to general practitioners along with 184 (2.3%) visits to other health professionals, 142 (1.8%) visits to emergency departments, 45 calls (0.6%) to 24-hour advice lines, and 39 (0.5%) stays in the hospital.

In 2012, among 10,379 episodes of fever and cough reported by Flutracking participants, participants reported 3,170 (30.5%) visits to general practitioners, 202 (1.9%) visits to other health professionals, 189 (1.8%) visits to emergency departments, 69 (0.7%) calls to 24-hour advice lines, and 37 (0.4%) stays in the hospital. In 2011, the proportion of participants with fever and cough, who also sought medical advice and had a positive laboratory test, was highest during September 11–October 16. During this period, 34.4% (573/1,665) of participants sought medical advice for their symptoms, and 4.5% (26/573) of participants who sought medical advice had a laboratory test for influenza, of whom 50.0% (13/26) reported having a positive influenza test result.

In 2012, the proportion of participants with fever and cough, who sought medical advice and had a positive laboratory test result, was highest during July 29–September 2. During this period, 34.5% (1,054/3,059) of participants sought medical advice for their symptoms, and 8.6% (91/1,054) of participants who sought medical advice had a laboratory test for influenza, of whom 50.0% (13/26) reported having a positive influenza test result.

### Table. Comparison of 6 weekly Flutracking surveillance pyramid results, Australia, 2011 and 2012

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive laboratory test result</td>
<td>9 (0.5)</td>
<td>21 (0.6)</td>
<td>15 (0.7)</td>
<td>32 (1.1)</td>
<td>13 (0.8)</td>
</tr>
<tr>
<td>Laboratory test for influenza</td>
<td>24 (1.2)</td>
<td>35 (1.1)</td>
<td>28 (1.2)</td>
<td>91 (3.0)</td>
<td>26 (1.6)</td>
</tr>
<tr>
<td>Sought medical advice (GP/ED/inpatient)</td>
<td>569 (28.9)</td>
<td>690 (32.4)</td>
<td>1,052 (32.0)</td>
<td>698 (31.1)</td>
<td>1,054 (34.5)</td>
</tr>
<tr>
<td>Reported fever and cough</td>
<td>1,967</td>
<td>3,289</td>
<td>3,289</td>
<td>2,246</td>
<td>3,059</td>
</tr>
<tr>
<td>No. surveys completed</td>
<td>64,869</td>
<td>67,812</td>
<td>81,365</td>
<td>67,006</td>
<td>81,385</td>
</tr>
<tr>
<td>Ratio of positive laboratory test results to fever and cough</td>
<td>1:218</td>
<td>1:157</td>
<td>1:150</td>
<td>1:96</td>
<td>1:128</td>
</tr>
</tbody>
</table>

*GP*, general practitioner; ED, emergency department;

### Conclusions

The addition of questions on health-seeking behavior and laboratory testing for influenza in the Flutracking surveillance system enabled rapid construction of a surveillance pyramid during 2011 and 2012 with progressive data available for each stratum of the pyramid on a weekly basis. Such analyses generally require integration of data from multiple and disparate surveillance systems.

Every Flutracking participant who reported laboratory-confirmed influenza represented 96 to 595 cases of cough and fever in the larger cohort. Although only a proportion of cough and fever cases would be true influenza, the proportion of true cases can be estimated (9).

The increased index of severity of illness among Flutracking participants in 2012 compared to 2011 is contemporaneous with a change in the circulating influenza strains from the predominant influenza A(H1N1)pdm09 strain to a subtype H3N2 influenza strain and the increased severity of illness reported by national and regional surveillance systems.

Although the Flutracking surveillance system relies on self-reports, its capacity to construct a surveillance pyramid from community ILI through to confirmed influenza and various strata of surveillance in near real-time is a unique attribute. Constructing such pyramids will facilitate the estimation of community level attack rates and severity of influenza, changes in health-seeking behavior, and influenza testing during seasonal and pandemic influenza periods.

### Acknowledgments

We thank John Fejsa and Stephen Clarke for their assistance with the online software and database development. We would also like to acknowledge the University of Newcastle for their continued support, and the Australian Government Department of Health and Ageing and the Hunter Medical Research Institute.
for their funding and support. In addition, we are grateful to the thousands of Flutracking participants who give their time freely each week to contribute to influenza surveillance.

Dr Dalton is a public health physician at Hunter New England Population Health, Newcastle, Australia, with extensive experience in foodborne disease and influenza surveillance. He is currently leading the Flutracking project.

References


Address for correspondence: Craig B. Dalton, Hunter New England Population Health, Locked Bag 10, Wallsend, New South Wales, 2287, Australia; email: craig.dalton@hnehealth.nsw.gov.au
Incidence of Influenza A(H1N1)pdm09 Infection, United Kingdom, 2009–2011

Saranya Sridhar, Shaima Begom, Alison Bermingham, Katja Hoschler, Walt Adamson, William Carman, Maria D. Van Kerkhove, and Ajit Lalvani

We conducted a longitudinal community cohort study of healthy adults in the UK. We found significantly higher incidence of influenza A(H1N1)pdm09 infection in 2010–11 than in 2009–10, a substantial proportion of subclinical infection, and higher risk for infection during 2010–11 among persons with lower preinfection antibody titers.

Case-based population-level surveillance and cross-sectional serologic surveys to estimate incidence and patterns of influenza infection are limited by the lack of accurate denominator data, inability to account for subclinical infections, difficulties in distinguishing between antibodies induced by natural infection and vaccination, and use of samples from high-risk groups. For these reasons, community-based longitudinal studies are ideal to estimate the incidence of infection and spectrum of illness. However, studies of this design describing the 2009 pandemic influenza A(H1N1)pdm09, reported only from Hong Kong, Singapore, and Vietnam, examine only the 2009–10 season (1–3).

The epidemiology of A(H1N1)pdm09 in the United Kingdom during 2009–2011 was characterized by 3 distinct waves: first wave, April–August 2009; second wave, September 2009–April 2010; and third wave, August 2010–April 2011. We report results from a community-based longitudinal cohort study that compared the epidemiology of influenza A(H1N1)pdm09 infection over the second and third waves. The North West London Research Ethics Committee approved this study (reference 09/H0724/27).

The Study

A total of 342 healthy adult staff and students of Imperial College London (London, UK) were recruited during September–November 2009 and followed for 2 consecutive influenza seasons: 2009–10 and 2010–11 (Figure 1). Participants’ median age was 28 years (interquartile range 20–36 years); 83% were <40 years of age. At each time point, collected serum samples were tested for antibodies to A(H1N1)pdm09 virus (A/England/195/09 strain) by the hemagglutination-inhibition (HI) assay (4). Participants were asked to record temperature, self-sample, and return nasal swabs when experiencing influenza-like symptoms. Swabs were tested for respiratory viruses with standardized real-time reverse transcription PCR. Influenza seroprevalence rates were defined as the proportion of persons with HI titers ≥32 (4).

Because our study began at the end of the first pandemic wave, cumulative incidence of A(H1N1)pdm09 infection over the first wave was estimated as the difference between age-specific seroprevalence rates at recruitment (T₀ in Figure 1) and published prepandemic (2008) seroprevalence rates for England (4). Incident infection was defined as antibody seroconversion (4-fold rise in HI titer) in paired serum samples collected at the start and end of a wave among unvaccinated persons (because HI assay cannot differentiate infection from vaccination) or detection of A(H1N1)pdm09 virus in nasal swabs. The incidence of infection was estimated for the second and third waves as the proportion of incident infections among unvaccinated participants.

Development of any symptoms was recorded on a Web-based questionnaire emailed to participants every 3 weeks. The average response rate was 75%. Illness episodes were categorized as acute respiratory infection (episode with any symptoms), influenza-like illness (ILI) episode with fever plus cough or sore throat), and fever (recorded temperature ≥38°C) alone. Visits to primary care or hospital during illness were also recorded. Data were analyzed using Stata version 9.0 (StataCorp, College Station, TX, USA) with the χ² test to compare proportions and t test to compare means after checking for normal distribution by assessing for kurtosis, skewness, and the Shapiro-Wilk test. Hosmer-Lemeshow test was used to estimate goodness-of-fit for each logistic regression.

At recruitment, after the first pandemic wave, A(H1N1)pdm09 seroprevalence was 26% (95% CI 21.4–31.2), with seroprevalence significantly higher in participants 18–25 years of age than in older age groups (Table 1). Participants with ILI in the preceding 3 months corresponding to the first wave had significantly higher (p<0.001) mean A(H1N1)pdm09 virus HI titers, which in conjunction with the age distribution, suggests first-wave infection rather than cross-reactive antibodies (5). Overall cumulative incidence
Incidence of Influenza A(H1N1)pdm09 Infection

during the first wave was 12.7% (95% CI 7.1%–18.4%) and 26.6% (95% CI 15.3%–37.8%) among participants 18–25 years of age with no increase in older age groups (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/ articlepdfs/19/11/13-0295-Techapp1.pdf).

The incidence of infection over the third pandemic wave was significantly higher (p = 0.02) than over the second wave (Figure 1). Among participants with prevaccine titers <8, the incidence of infection was significantly higher over the third wave than over the second wave (p<0.001); incidence did not differ for participants with prevaccine titers >8 (Table 2, Appendix, wwwnc.cdc.gov/EID/article/19/11/13-0293-T2.htm). Age-specific incidence was significantly higher (p = 0.01) over the third wave than the second wave among participants 26–40 years of age (third wave: 25.4% [95% CI 15.2–35.5]; second wave: 10.9% [95% CI 5.1–16.7]) but not the other age groups (Table 2, Appendix). For 11 infected participants with paired serum samples and virus detected in nasal swabs, 2 (18%) did not show antibody seroconversion (online Technical Appendix Table 2).

During an illness episode, 20% of infected participants reported fever or ILI, 17% visited their general practitioner, and none visited a hospital (Figure 2). Because predictions

Figure 1. Incidence of natural influenza A(H1N1)pdm09 infection in the study cohort during the 3 pandemic waves in context of the evolving pandemic, United Kingdom. Study outline is depicted in the upper panel in temporal context of the pandemic during the 2009–2011 influenza seasons. The bar chart shows UK influenza virologic surveillance data from WHO Flunet (www.who.int/influenza/gisrs_laboratory/flunet/en/) highlighting the periods of study recruitment and follow-up in relation to influenza A activity in the United Kingdom during 2009–2011. Light gray bars indicate influenza A of all subtypes; dark gray bars indicate the number of A(H1N1)pdm09 cases detected by virologic national surveillance. Healthy adults were recruited after the first pandemic wave (April–August 2009) had ended in the United Kingdom and were followed over 2 influenza seasons, with serum samples collected before and at the end of each influenza season. The median time between visits is shown. The second wave was defined as baseline (September–November 2009) to first follow-up (February–April 2010) and the third wave as the time between the second follow-up (August–November 2010) and the third follow-up (February–April 2011). The light gray bracket and numerals represent the estimated cumulative incidence of infection over the first pandemic wave by calculating the difference between and seroprevalence rates at baseline in the cohort and prepandemic (2008) published seroprevalence rates. Infection was defined as detection of A(H1N1)pdm09 virus in nasal swabs returned during the second or third wave or a 4-fold rise in A(H1N1)pdm09 virus HI titer in paired serum samples collected at the start and end of each wave. The number of infected persons with total persons at risk during each of the second and third waves with calculated incidence rate and 95% CIs are shown. WHO, World Health Organization; IQR, interquartile range; HI, hemagglutination-inhibition. *Infection rates in the first wave reflect cumulative incidence of infection, estimated by calculating the difference in proportion of persons with HI titer >32 between baseline (T₀) and published Health Protection Agency data before the pandemic in 2008.
of a small third pandemic wave were disproved (4), the reasons for this large wave remained unclear. Multivariate logistic regression was undertaken with infection as the dependent variable and age, sex, and prewave titers as independent variables. Each doubling increase in prewave HI titers, after adjustment for age and sex, was associated with significantly lower risk for infection (odds ratio 0.92, 95% CI 0.9–1.0, p = 0.04) during the third, but not the second, wave (Table 2, Appendix).

Conclusions

Incidence of A(H1N1)pdm09 infection was significantly higher among healthy adults during the third pandemic wave (2010–11) than during the second wave (2009–10). This study complements and corroborates clinical surveillance data and population-sampling serosurveys from the United Kingdom (4,6,7), United States (8) and elsewhere (9).

The reasons for this unexpectedly larger third wave in the postpandemic season remain unclear. We show an increased risk for A(H1N1)pdm09 infection associated with lower antibody levels at the start of the season, irrespective of age, during the third, but not the second, wave. Because no substantial viral genetic change occurred between the waves (7), our finding suggests that the third wave was driven by infection among susceptible persons remaining antibody-naïve at the end of the second wave. This thesis is supported by serosurveillance data showing lower infection rates over the third wave among age groups with the highest infection rates over previous pandemic waves (7,8). Our interpretation is further strengthened by a meta-analysis of serologic data from 19 countries that showed 20%–27% incidence of infection during the first pandemic year, suggestive of a large population susceptible to infection in subsequent seasons (10).

Incidence in our cohort was lower than that estimated for England by cross-sectional serosurveys (7,11). This finding may reflect our accounting for individual-level vaccination status and baseline antibody titers; data usually unobtainable with cross-sectional population-sample serosurveys. However, our study did not include children or elderly persons, which limits the generalizability of our findings. A major advantage of longitudinal cohort studies recording clinical data is identification of subclinical and asymptomatic infections. More than 80% of participants with influenza reported no symptoms. Cross-reactive cellular immune responses that are highly prevalent in the population (13) have recently been shown to be associated with protection against symptomatic illness (14).

Our analysis of pandemic influenza in a community cohort over successive seasons offers insight into
Figure 2. Proportion of influenza A(H1N1)pdm09–infected persons who had symptoms during their illness episode during the second wave (September 2009–April 2010), third wave (August 2010–April 2011), and entire study period, United Kingdom. Proportion of persons with reported symptoms over the study period is combined from the second and third waves. Symptoms were recorded by a Web-based symptom questionnaire emailed to participants every 3 weeks. Symptoms associated with illness episode were acute respiratory infection (ARI; illness episode with any symptoms), influenza-like illness (ILI; episode with fever plus cough or sore throat), fever (recorded temperature ≥38°C) alone, or visit to a general practitioner (GP). The graph depicts the average with 95% CIs calculated by using binomial distribution.

contributors of the unexpectedly larger third pandemic wave. Our analysis also highlights the necessity of using cohorts to complement routine case-based surveillance to estimate influenza burden.

Acknowledgments

We thank all willing participants of this study. We express our grateful appreciation to Marie Bautista, Sharleen Bowes, Bianca Fortunaso, and Sharna Lloyd-James for processing all the samples and our clinical research nurses.

A.L is a Wellcome Trust Senior Research Fellow in Clinical Science and NIHR Senior Clinical Investigator, S.S. is supported by the Imperial College Healthcare NHS Trust, and S.B. is supported by a Medical Research Council funded PhD studentship.

Dr Sridhar is a postdoctoral researcher at the Department of Respiratory Medicine, Imperial College London. His research interests encompass the immune epidemiology of influenza and tuberculosis and the development and evaluation of vaccination strategies against respiratory pathogens.

References


Address for correspondence: Saranya Sridhar, Department of Respiratory Medicine, Imperial College London, St. Mary’s Campus, Bldg 2, Norfolk Pl, London W2 1PG, UK; email: s.sridhar@imperial.ac.uk
Nontoxigenic Highly Pathogenic Clone of Corynebacterium diphtheriae, Poland, 2004–2012

Alekandra A. Zasada

Twenty-five cases of nontoxigenic Corynebacterium diphtheriae infection were recorded in Poland during 2004–2012, of which 18 were invasive. Alcoholism, homelessness, hepatic cirrhosis, and dental caries were predisposing factors for infection. However, for 17% of cases, no concomitant diseases or predisposing factors were found.

Corynebacterium diphtheriae is the causative agent of diphtheria. Its toxin is considered the major virulence factor. Since introduction of vaccine against the diphtheria toxin in the 1940s, infections caused by toxigenic Corynebacterium have been well controlled in industrialized countries that have high coverage rates of childhood vaccination with 3 doses of diphtheria-tetanus-pertussis vaccine (1). Nevertheless, emergence of nontoxigenic C. diphtheriae infections has been reported in some of these countries.

In line with other European countries, Poland routinely vaccinates against diphtheria (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/11-0297-Techapp1.pdf). According to data from the World Health Organization, >95% of children in Poland are fully vaccinated against diphtheria. The last diphtheria case was recorded in 2000 (www.who.int/immunization_monitoring/data/incidence_series.xls).

The absence of diphtheria during the past 13 years suggests that the high vaccination coverage rates in Poland protect against diphtheria. In 2004, the first case of sepsis caused by nontoxigenic C. diphtheriae was recorded (2). Other cases were recorded in 2006, and since 2007, several cases of C. diphtheriae invasive infections have been recorded every year (Table 1). In addition, local infections—usually wound infections—caused by nontoxigenic C. diphtheriae were recorded (Table 2). A total of 25 nontoxigenic C. diphtheriae infections were recorded in Poland in 2004–2012, of which 18 were invasive infections.

The Study

All patients were admitted to local hospitals and clinical samples for microbiological investigations were sent to the nearest laboratories. C. diphtheriae isolates were sent to National Institute of Public Health—National Institute of Hygiene for confirmation and toxigenicity testing, biotyping, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and ribotyping. Case classification and microbiological methods used are presented in the online Technical Appendix. Data collected for epidemiologic analysis included location; type of infection; year of presentation; and patient age, sex, concomitant diseases, socioeconomic status, and intravenous drug use (IVDU).

All isolates from local and invasive infections were identified as biotype gravis, except for the isolate from patient Loc-05, which was identified as biotype mitis. All 25 isolates were characterized by PFGE, and 20 isolates (18 from invasive and 2 from local infections) were characterized by MLST (3–5). All the isolates except the mitis isolate belonged to the same pulotype revealed by PFGE. All the isolates characterized by MLST belonged to genotype sequence type 8. Eight of the isolates (5 from invasive and 3 from local infections) also were genotyped by using ribotyping. All 8 isolates showed indistinguishable ribotype patterns (3).

All but 1 invasive infection were identified in male patients, whereas local infections affected male and female patients similarly. Age groups of patients most affected by invasive infections were 31–40 years, followed by 51–60 years; for local infections, persons 51–60 years of age were mostly affected (Figure). Patients’ ages ranged from 17 to 71 years. The cases occurred in various parts of Poland; no epidemiologic links were identified.

Epidemiologic data analysis revealed that predisposing factors of nontoxigenic C. diphtheriae invasive infections were related to conditions associated with low socioeconomic status, such as alcoholism, homelessness, and dental caries, as well as to hepatic cirrhosis. For 3 (17%) patients (Inv-08, Inv-09, Inv-13), no concomitant diseases or predisposing factors were identified. These were healthy men aged 17, 24, and 37 years of age, respectively. Predisposing factors for local infections were not analyzed. The sources of all infections described in the study were not identified. Despite IVDU being regarded as a risk factor for C. diphtheriae invasive infection, none of the patients were intravenous drug users.

Conclusions

Diphtheria is a rare disease in Europe. In 2006–2009 only 150 cases were reported in European Union and European Economic Area/European Free Trade Association countries. Most of the cases (114 cases) occurred in Latvia, where diphtheria is endemic. The other diphtheria cases

Author affiliation: National Institute of Public Health–National Institute of Hygiene, Warsaw, Poland

DOI: http://dx.doi.org/10.3201/eid1911.130297
C. diphtheriae a single clone of infections. This raises a valid question: is country over a 9-year period. This phenomenon has not clone despite isolation of the strains in different part of the, (5). All but 1 isolate from Poland represent a single, 10 8 5 10 8 5). Of isolates, respectively, originated from patients <15 years, and France, and biotype mitis dominated among the invasive isolates in Switzerland, and Italy (10), during the past few years. In Poland, persons most affected were 31–40 years and 51–60 years of age, whereas in other countries most patients were younger (up to 34 years of age). No C. diphtheriae infections among children were recorded in Poland, whereas in France, almost 20% of invasive infections were diagnosed in children. On the other hand, in Italy and the United Kingdom, 70% and 13% of isolates, respectively, originated from patients <15 years of age (5,8,10).

In Poland, all but 1 strain isolated from local and invasive infections belonged to biotype gravis, whereas biotype mitis dominated among the invasive isolates in Switzerland and France, and biotype gravis dominated among isolates from local infections in Italy and the United Kingdom (5,8–10). All but 1 isolate from Poland represent a single clone despite isolation of the strains in different part of the country over a 9-year period. This phenomenon has not been documented in any countries reporting nontoxigenic C. diphtheriae infections. This raises a valid question: is a single clone of C. diphtheriae circulating in Poland or does the identified clone have increased pathogenic properties? This question remains unanswered because the carrier state of C. diphtheriae has not been examined in the Polish population.

Taking these data and the literature review into consideration, C. diphtheriae infections frequently are associated with endocarditis. Muttaiyah et al. (11) and Mishra et al. (12) demonstrated that most patients with C. diphtheriae endocarditis have underlying cardiac disease, prosthetic valves, or a history of IVDU. This finding, however, was not observed among patients in Poland.

The portal of entry for invasive nontoxigenic C. diphtheriae infection has not been fully elucidated. However, some authors shown that skin lesions are the most likely sources (9,13,14). In the cases presented here, skin ulceration was uncommon (1 case), but dental caries were found in >22% of cases. Dental caries could be a portal of entry.

The main limitation of this work is lack of complete data. Nevertheless, nontoxigenic C. diphtheriae can be concluded to be an emerging pathogen in Poland and has

Table 1. Cases of bloodstream infections caused by nontoxigenic Corynebacterium diphtheriae, Poland, 2004–2012

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y/sex</th>
<th>Concomitant disease</th>
<th>Location</th>
<th>Year</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inv-01</td>
<td>38/M</td>
<td>Dental caries</td>
<td>Warsaw</td>
<td>2004</td>
<td>Endocarditis diagnosed</td>
</tr>
<tr>
<td>Inv-02</td>
<td>ND/M</td>
<td>ND</td>
<td>Bydgoszcz</td>
<td>2006</td>
<td>Homeless</td>
</tr>
<tr>
<td>Inv-03</td>
<td>51/M</td>
<td>HIV suspected</td>
<td>Gdynia</td>
<td>2007</td>
<td></td>
</tr>
<tr>
<td>Inv-04</td>
<td>37/M</td>
<td>Dental caries</td>
<td>Gdynia</td>
<td>2007</td>
<td></td>
</tr>
<tr>
<td>Inv-05</td>
<td>53/M</td>
<td>Alcoholism, hepatic cirrhosis</td>
<td>Rzeszów</td>
<td>2008</td>
<td></td>
</tr>
<tr>
<td>Inv-06</td>
<td>50/F</td>
<td>Portal and posthepatitic C cirrhosis, dental caries</td>
<td>Bydgoszcz</td>
<td>2008</td>
<td></td>
</tr>
<tr>
<td>Inv-07</td>
<td>32/M</td>
<td>Alcoholism, abscess of the liver</td>
<td>Gdynia</td>
<td>2008</td>
<td></td>
</tr>
<tr>
<td>Inv-08</td>
<td>24/M</td>
<td>Not identified</td>
<td>Gdynia</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Inv-09</td>
<td>17/M</td>
<td>Not identified</td>
<td>Kraków</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Inv-10</td>
<td>60/M</td>
<td>Alcoholism</td>
<td>Sosnowiec</td>
<td>2009</td>
<td></td>
</tr>
<tr>
<td>Inv-11</td>
<td>60/M</td>
<td>Dental caries, frostbite of feet</td>
<td>Bydgoszcz</td>
<td>2010</td>
<td>Homeless</td>
</tr>
<tr>
<td>Inv-12</td>
<td>36/M</td>
<td>Alcoholism, delirium</td>
<td>Gdynia</td>
<td>2010</td>
<td></td>
</tr>
<tr>
<td>Inv-13</td>
<td>37/M</td>
<td>Not identified</td>
<td>Legnica</td>
<td>2010</td>
<td>Endocarditis diagnosed</td>
</tr>
<tr>
<td>Inv-14</td>
<td>ND/M</td>
<td>ND</td>
<td>Sosnowiec</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td>Inv-15</td>
<td>50/M</td>
<td>Skull trauma, skin ulceration</td>
<td>Radom</td>
<td>2011</td>
<td>Homeless</td>
</tr>
<tr>
<td>Inv-16</td>
<td>71/M</td>
<td>Stroke</td>
<td>Kraków</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td>Inv-17</td>
<td>65/M</td>
<td>Hepatic cirrhosis, encephalopathy, diabetes mellitus</td>
<td>Gdańsk</td>
<td>2012</td>
<td></td>
</tr>
<tr>
<td>Inv-18</td>
<td>ND/M</td>
<td>Stroke</td>
<td>Poznań</td>
<td>2012</td>
<td>Endocarditis diagnosed</td>
</tr>
</tbody>
</table>

ND, no data.

Table 2. Local infections caused by nontoxigenic Corynebacterium diphtheriae, Poland, 2004–2012

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y/sex</th>
<th>Location</th>
<th>Year</th>
<th>Site of C. diphtheriae isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loc-01</td>
<td>ND/M</td>
<td>Bydgoszcz</td>
<td>2007</td>
<td>Wound</td>
</tr>
<tr>
<td>Loc-02</td>
<td>29/F</td>
<td>Warszawa</td>
<td>2007</td>
<td>Fistula</td>
</tr>
<tr>
<td>Loc-03</td>
<td>ND/M</td>
<td>Bydgoszcz</td>
<td>2007</td>
<td>Wound</td>
</tr>
<tr>
<td>Loc-04</td>
<td>51/M</td>
<td>Warszawa</td>
<td>2008</td>
<td>Wound</td>
</tr>
<tr>
<td>Loc-05</td>
<td>61/F</td>
<td>Bydgoszcz</td>
<td>2010</td>
<td>Shank cyst</td>
</tr>
<tr>
<td>Loc-06</td>
<td>56/F</td>
<td>Gdynia</td>
<td>2010</td>
<td>Wound</td>
</tr>
<tr>
<td>Loc-07</td>
<td>59/M</td>
<td>Warszawa</td>
<td>2012</td>
<td>Wound</td>
</tr>
</tbody>
</table>

ND, no data.
the potential to cause serious infections. The number of non-toxigenic *C. diphtheriae* infections might be higher because reporting of only toxigenic *C. diphtheriae* infections is mandatory in Poland. Moreover, in clinical settings, detection of coryneform bacteria in blood cultures is often dismissed as contamination, and in severe cases of the disease, *C. diphtheriae* might never be identified as the etiologic agent of bloodstream infection.

Homelessness, alcohol abuse, IVDU, and diabetes mellitus were mentioned in the literature as risk factors for *C. diphtheriae* invasive infections. In the cases presented here, 31% of patients were homeless, and 22% reported alcohol dependency but only 1 patient had diabetes mellitus. No patients reported IVDU. In 17% of cases, hepatic cirrhosis was ascertained, which suggests that it also may be another predisposing factor to infection. Moreover, dental caries is a highly probable portal of entry of *C. diphtheriae* invasive infection and has not been documented by other authors. However, such infections also might occur in persons with no identified predisposing factors.

**Acknowledgment**

The author thanks Anna Zielicka-Hardy for editing the manuscript.

Dr Zasada is a microbiologist at the National Institute of Public Health–National Institute of Hygiene working in the Laboratory of Highly Pathogenic Bacteria of the Department of Bacteriology. Her research interests include *C. diphtheriae* infection and *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* infections.

**References**


Address for correspondence: Aleksandra A. Zasada, National Institute of Public Health–National Institute of Hygiene, Department of Bacteriology, Chocimska 24, 00-791 Warsaw, Poland; email: azasada@pzh.gov.pl
Tula Hantavirus Infection in Immuno-compromised Host, Czech Republic

Hana Zelená,1 Jakub Mrázek,1 and Tomáš Kuhn1

We report molecular evidence of Tula hantavirus as an etiologic agent of pulmonary-renal syndrome in an immunocompromised patient. Acute hantavirus infection was confirmed by using serologic and molecular methods. Sequencing revealed Tula virus genome RNA in the patient’s blood. This case shows that Tula virus can cause serious disease in humans.

Hantaviruses are enveloped RNA viruses carried by rodents and insectivore species. At least 5 hantavirus species are known to circulate in Europe: Dobrava-Belgrade virus, Puumala virus (PUUV), Seoul virus, Saaremaa virus, and Tula virus (TULV). The first 3 are well-characterized human pathogens; however, little is known about TULV human pathogenicity.

The species Tulavirus was first described by Plyusnin et al. (1) in voles (Microtus arvalis and M. levis) caught in Tula, Russia, in 1987. The presence of TULV was also documented in other vole species in several European countries including Germany, Switzerland, Slovenia, Czech Republic, Slovakia, Austria, Poland, and Serbia (2). In Central Europe, M. arvalis is the main reservoir of TULV. The TULV antigen was found in 10% of the population of common voles in southern Moravia in the Czech Republic (3). The pathogenic potential of Tula virus in humans is considered to be low.

The causative agents of hemorrhagic fever with renal syndrome in Central Europe are Dobrava-Belgrade virus and PUUV (4). These viruses seem to circulate in geographic areas that overlap with the areas where TULV circulates. Despite the massive population of common voles in the Czech Republic and a high prevalence of TULV in its rodent reservoir, human TULV infection has not been reported.

The Patient

A 14-year-old boy from a rural region in the northeast part of the Czech Republic (Opava region) has received treatment for acute lymphoblastic leukemia since July 2011. Because of the biologic properties of the malignity, the boy was classified into the high-risk group of the treatment protocol. The intensive part of the treatment was finished in August 2012, and the patient has continued maintenance therapy since then.

During his first week of maintenance therapy, the patient experienced a respiratory infection with temperatures of ≈38°C, mild dyspnea, and a cough. These symptoms spontaneously disappeared. One week later, the patient had temperatures up to 38.5°C. He reported a headache, lack of appetite, and vomiting but no cough or respiratory distress. Upon the patient’s admission to the hospital, at the end of September 2012, his conditions deteriorated. He was febrile at 39.3°C and moderately dehydrated. Dyspnea with desaturation developed, so he was transferred to the intensive care unit to receive oxygenotherapy. The antileukemic maintenance therapy therefore had to be interrupted. The x-ray and high-resolution computed tomographic scan revealed severe bilateral bronchopneumonia with a major fluidothorax and bilateral dyspneumia. He was then given amoxicillin/clavulanate, amikacin, and antimycotic drugs. Oliguria also developed, with a minimum of 0.3 mL/kg/h, and it was managed by diuretic medication. Hemodialysis was not needed. He had transiently increased blood pressure followed by hypotension.

Laboratory results revealed eosinophilia in the patient’s differential leukocyte count at a maximum of 59.3% (reference range 0%–5%), anemia with a minimal value of hemoglobin of 60.0 g/L (reference range 135–175 g/L), thrombocytopenia at 12 × 10^9/L (reference range 150–440 × 10^9/L), and C-reactive protein 70 mg/L (reference range 0–10 mg/L). Elevated values were detected for serum urea measured at 8.40 mmol/L (reference range 1.8–6.4 mmol/L), creatinine at 103 µmol/L (reference range 27–88 µmol/L), and D-dimers at 3.53 µg/mL (reference range 0–0.5 µg/mL). Other coagulation parameters were not affected. Moreover, erythrocyturia and hyaline cylinders were observed in urine samples. The serum amylase and liver enzyme levels were within reference ranges. The relapse of acute lymphoblastic leukemia was excluded by the bone marrow examination. Because of the patient’s severe thrombocytopenia, thromboconcentrate was administered.

During the course of the patient’s hospitalization, his clinical condition, computed tomographic scan, and chest radiographic findings, and laboratory parameters improved. His renal failure gradually subsided with a transient polyuric phase. After 3 weeks of hospitalization, the patient resumed maintenance antileukemic therapy, and he was discharged from the hospital in good condition.
Serum samples taken on days 11, 12, 20, and 39 were tested for IgG and IgM antibodies to hantaviruses by using ELISA (Anti-Hanta Virus Pool 1 “Eurasia”; Euroimmun, Lübeck, Germany). The serum sample taken on day 12 was further tested for IgG and IgM antibodies by using Immunoblot (Anti-Hanta Profile 1; Euroimmun). ELISA results are considered positive when the index value (optical density divided by the cutoff value) is >1.1. Serology results suggested that the causative agent was a hantavirus antigenically closer to PUUV (Table 1).

RNA was extracted from an EDTA plasma sample taken on day 11. Hantavirus RNA was detected by nested reverse transcription PCR performed with pan-hantaviral large (L) segment specific primers (5) (Table 2). Direct sequencing was performed with each separate nested primer and BigDye Terminator v1.1 Cycle Sequencing Kit (LifeTechnologies, Grand Island, NY, USA) on ABI 3130 platform.

TULV RNA detection was confirmed by another PCR and sequencing experiment with small (S) segment Tula virus-specific primers previously published (6,7) for the first and second PCR step respectively (Table 2). The sequences were aligned to consensus sequence by using Seqscape software (Life Technologies) and compared with sequences available at BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees using neighbor-joining analysis with maximum composite likelihood method and bootstrap values were constructed by using MEGA 5.2 software (www.megasoftware.net).

The EDTA plasma sample collected during the acute phase was positive for hantavirus RNA. Sequencing analysis of both L- and S-segments confirmed that the causative agent was TULV. The phylogenetic trees for partial L- and S-segments (Figures 1 and 2, respectively) indicated that the identified Tula virus strain belongs to the lineage representing strains from middle Europe (Czech Republic, west Slovakia, Austria, and Slovenia). Partial L- and S-segment sequences of the TULV isolated RNA have been deposited in GenBank under accession numbers KC522413 and KC494908, respectively.

Conclusions

Although the presence of TULV in the common vole population in the Czech Republic has been documented, no evidence of its pathogenicity in humans has been shown. Specific antibodies against TULV have been identified in a healthy blood donor in the Czech Republic (8) and in German forestry workers (9), suggesting that TULV can be transmitted to humans. A case of a serologically detected symptomatic TULV infection that followed a rodent bite has been reported in Switzerland (10). However, because of the late occurrence of specific antibodies and because the symptoms were atypical for hantavirus infection, the evidence for the Tula virus as an etiologic agent in this case is questionable (11). Renal and pulmonary syndrome with biphasic course associated with TULV was documented in northern Germany. The diagnosis was made on the basis of the highest neutralizing titer against TULV and detection of TULV RNA in common voles in the region where the patient lived (12).

We provide the molecular evidence of human symptomatic TULV infection. The clinical symptoms included both renal and pulmonary involvement with dominating respiratory failure corresponding to the hantavirus

<table>
<thead>
<tr>
<th>Primer</th>
<th>Step</th>
<th>Target segment</th>
<th>Sequence (5′→3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAN-L-F1</td>
<td>1st PCR</td>
<td>Large</td>
<td>ATGTAAYTBAGTGCGWGATGC</td>
<td>(5)</td>
</tr>
<tr>
<td>HAN-L-R1</td>
<td>1st PCR</td>
<td>Large</td>
<td>AACADTCGWGTCCRTCATC</td>
<td>(5)</td>
</tr>
<tr>
<td>HAN-L-F2</td>
<td>2nd PCR, sequencing</td>
<td>Large</td>
<td>TGCGWGATGCACIAARTGGTC</td>
<td>(5)</td>
</tr>
<tr>
<td>HAN-L-R2</td>
<td>2nd PCR, sequencing</td>
<td>Large</td>
<td>GCRTCRTCWGARTGRTGDGCA</td>
<td>(5)</td>
</tr>
<tr>
<td>S1</td>
<td>1st PCR</td>
<td>Small</td>
<td>GGMCAACACAGAGTGG</td>
<td>(6)</td>
</tr>
<tr>
<td>S2</td>
<td>1st PCR</td>
<td>Small</td>
<td>AGCTCAGGATCCATRTCATC</td>
<td>(6)</td>
</tr>
<tr>
<td>MaS4F</td>
<td>2nd PCR, sequencing</td>
<td>Small</td>
<td>CATCACAGGATCCATRTGGATGC</td>
<td>(7)</td>
</tr>
<tr>
<td>MaS5C</td>
<td>2nd PCR, sequencing</td>
<td>Small</td>
<td>TCCTGAGGCTGCAAGGTT</td>
<td>(7)</td>
</tr>
</tbody>
</table>
pulmonary syndrome. The course of the disease was severe, and the delayed occurrence of TULV IgG was most likely caused by the patient’s immunodeficiency. The laboratory findings were typical for hantavirus infection, with strongly decreased platelet count but only moderately elevated serum creatinine and urea. Furthermore, during the acute stage, viral RNA was detected in the patient’s serum, which strongly suggests that TULV is a causative agent of the critical stage. This case illustrates that TULV can cause life-threatening disease in an immunocompromised patient, although under normal circumstances it is a nonpathogenic virus (8).

Acknowledgment

We thank Zuzana Dostálová for language correction.

Dr. Zelená is a head of the Department of Virology (Institute of Public Health Ostrava) and of the National Reference Laboratory for Arboviruses of the Czech Republic. Her main research interests include arboviruses and vector-borne viruses, imported and emerging viruses, and diagnostic electron microscopy.

References


Human Bocavirus in Children with Acute Gastroenteritis, Chile, 1985–2010

Jorge Levican, Esteban Navas, Joaquín Orizola, Luis Fidel Avendaño, and Aldo Gaggero

We detected human bocavirus in 89 (19.3%) of 462 fecal samples collected during 3 periods from 1985 through 2010 from children <5 years of age in Chile who were hospitalized with acute gastroenteritis. Our findings confirm the long-term circulation of human bocavirus in Chile.

Human bocavirus (HBoV) was discovered in 2005 on the basis of large-scale molecular virus screening of respiratory samples (1). More recently, HBoV was detected in fecal samples of children who had gastroenteritis with or without symptoms of respiratory infection and in samples from healthy controls (2–4). Although HBoV is assumed to have coexisted with humans for a long time, there is little evidence to confirm long-term circulation.

The Study

We analyzed 462 fecal specimens from hospitalized children 0–60 months of age (median 13.8 months) with acute gastroenteritis in Chile. The samples belonged to a collection obtained from 1985 through 2010. Three periods were analyzed: 1985–1986 (period A, 86 samples), 1997–2004 (period B, 261 samples), and 2009–2010 (period C, 115 samples) (Table). The patients did not show respiratory symptoms during their clinical evaluation. Analysis for rotavirus, calicivirus, enteric adenovirus, and astrovirus was conducted (data not shown), and only samples negative for these viruses were selected. The samples were maintained at −80°C until analysis.

DNA from fecal samples was extracted by using a High Pure Nucleic Acid Viral Kit (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer’s instructions. Using PCR with specific primers as described, we performed the HBoV detection (3–5). Positive and negative controls were included in each amplification round. PCR products were purified, and nucleotide sequences were determined by Macrogen Inc. (Seoul, South Korea) and submitted to GenBank (accession nos. KC757418–KC757460).

Phylogenetic relationships between isolates from Chile and GenBank reference strains were studied by using MEGA5 software (6). We inferred the evolutionary history using the neighbor-joining method. Bootstrap (1,000 replications) was used to assess the reliability of individual nodes in each phylogenetic tree. Evolutionary distances were computed by using the Kimura 2-parameter method (6). Variance analysis of the bocavirus detection frequency was conducted by using the Kruskal-Wallis test (α = 0.05) using Statdisk 12.0.1 software (Marc Triola and Pearson Education, Inc., New York, NY, USA). The Ethics Committee of the Faculty of Medicine, University of Chile approved the study.

The 89 (19.3%) samples positive for HBoV were distributed throughout the study period; 22.1%, 21.1%, and 13.0% for periods A, B, and C, respectively (Table). HBoV1 was the most frequently detected species, with 65 (14.1%) cases, followed by HBoV2 and HBoV3, with 18 (3.9%) and 6 (1.3%), cases, respectively (Table). HBoV4 was not detected.

Twenty-two (of 65 HBoV1) nonstructural (NS) 1 partial coding sequences were obtained, and consistent with previous reports, phylogenetic analysis showed that HBoV1 constitutes a genetically homogeneous entity (4,7). The nucleotide divergence average for the HBoV1 Chile isolates was 0.7% (range 0%–1.7%). In the phylogenetic tree analysis, 15 of 22 isolates clustered with prototype strain st1 (GenBank accession no. DQ000495), and the remaining 5 clustered with prototype strain st2 (GenBank accession no. DQ000496) (Figure, panel A). There was no temporal clustering of the HBoV1 isolates. Similarly, the phylogenetic analysis of 391 nt from the nucleocapsid 1 region of 5 HBoV3 isolates from Chile revealed an average divergence of 1.2% (range 0%–1.6%), and the phylogenetic tree analysis showed that the isolates grouped into 2 clusters with no evident temporal clustering (Figure, panel B).

In contrast, HBoV2 was a genetically heterogeneous group. Analysis of the nucleotide sequence of the NS1 partial region of 16 of 18 isolates yielded an average of 3.0% nt divergence (range 0%–6.0%). CH23–85, the most divergent isolate, showed 5.0%–6.0% nt divergence with other HBoV2 isolates from Chile. Phylogenetic tree analysis demonstrated that this isolate was closely related to the Pakistan strain PK5510, with which it formed a separate cluster (98% nt identity). This same analysis demonstrated 2 additional clusters among HBoV2 isolates from Chile whose intragroup average nucleotide identity reached 99.6% and 99.5%, respectively.

Kapoor et al. reported a similar clustering pattern by phylogenetic analysis of NS1 and nucleocapsid 1 of HBoV2. They recognized 3 clusters, which enabled them to categorize HBoV2 into 3 genotypes: genotype 1,
represented by prototype strain PK5510 (GenBank accession no. FJ170278); genotype 2, with prototype strain PK2255 (GenBank accession no. FJ170279); and genotype 3, with prototype strain UK648 (GenBank accession no. FJ170280) (4).

Following this scheme, we determined that 1 of 13 isolates belongs to genotype 1 (CH23–85), 6 to genotype 2 (CH27–99, CH28–01, CH30–02, CH31–04, CH32–04, CH34–10), and 9 to genotype 3 (CH24–85, CH25–97, CH26–98, CH29–02, CH33–09, CH35–10, CH41–04, CH42–09, CH43–09) (Figure, panel C). Unlike HBoV1, which was present in all periods analyzed showing a downward trend in the last period, the different genotypes HBoV2 show a marked dynamism. Thus, period A (1985–1986) revealed only 2 isolates (genotypes 1 and 3).

We found no other isolates of genotype 1 during the remaining study time (Figure, panel C). Genotype 3 became the only genotype prevalent during 1997 and 1998 and remained in the other years studied. Genotype 2 appeared in 1999 and persisted until the last year analyzed; it probably led to increased HBoV2 detection in 2009–10 (Figure, panel C, Table). This prompted speculation that HBoV2 infection is a dynamic phenomenon that can manifest with the emergence of different variants of the agent at different times. This possibility also is rooted in the observation that HBoV2 is highly prone to recombination, and as a consequence presents a high degree of diversity. This diversity is proposed to have given rise to HBoV1, a species with completely different biologic characteristics (7). However, because of the lack of continuous chronologic follow-up of the phenomena found in this study, we cannot ensure such assumptions, and a larger longitudinal study is required to confirm this hypothesis.

**Conclusions**

Although HBoV1 was originally detected in respiratory secretions of patients with respiratory infection, numerous studies have demonstrated its presence in 1.5%–19% of fecal samples (1–3,5,8,9). However, after primary respiratory infection this agent can persist with asymptomatic shedding for several months (10,11). Thus, HBoV1 in fecal samples could be due mainly to passive transfer from the respiratory tract (8,12).

Unlike HBoV1, HBoV2–4 have enteric tropism, and their role in gastroenteritis remains unclear (9). HBoV2 has been detected in feces from children with gastroenteritis in a broad range of percentages (1%–21%) alone or in co-infection with other enteropathogens. In contrast to our study, it has been frequently reported as the main HBoV species detected in feces (4,5,9,13). Moreover, in accordance with previous reports that found HBoV3 in low percentages (0%–2%), we detected HBoV3 only in periods A and B (2.3% and 1.5%, respectively) (Table) and did not detect HBoV4 (5,7,9,14). Although samples were maintained at −80°C until tested, we cannot exclude the possibility that molecular detection of HBoV may be reduced by long-term storage. This fact, along with the co-infection exclusion, may explain the low detection rates of the strains with enteric tropism.

Although the frequency of detection of HBoV species varied among the periods studied, variance analysis indicated no significant differences (p = 0.099) (Table). We cannot confirm circulation or variation in detection in periods during which surveillance was not conducted.

This study confirms long-term circulation of HBoV in Chile and demonstrates the heterogeneity of HBoV2. These findings justify prospective studies to better understand the role of these viruses in childhood gastroenteritis.
HBoV in Children, Chile

Acknowledgments

We acknowledge the excellent technical assistance of Rosa Corvalán. We thank Dona Benadof for supplying several samples for the study.

This study was supported in part by a project from the Fundación Estudios Biomédicos Avanzados, Facultad de Medicina, Universidad de Chile.

Dr. Levican is a doctoral student and a scientist affiliated with the Virology Program, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile. His main research interest is the detection and characterization of enteric human viruses.

References


Address for correspondence: Aldo Gaggero, Programa de Virología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile. Independencia 1027, Santiago 8380453, Chile; email: agaggero@med.uchile.cl
An epidemic caused by influenza A (H7N9) virus was recently reported in China. Deep sequencing revealed the full genome of the virus obtained directly from a patient’s sputum without virus culture. The full genome showed substantial sequence heterogeneity and large differences compared with that from embryonated chicken eggs.

Recently, a novel influenza A (H7N9) virus infected humans in China (1,2), leading to great concerns about its threat to public health (3). However, almost all the current genomes of the novel subtype H7N9 virus have been sequenced after culture in embryonated chicken eggs or mammalian cells. Switching the evolutionary selection pressure from in vivo human respiratory tract to embryonated chicken eggs might introduce mutations into the final genome sequences during culture (4). We report determination of the full genome of the influenza A (H7N9) virus derived directly by deep sequencing, without virus culture, from a sputum specimen of an infected human. Deep sequencing provides a direct way to evaluate the genome characteristics and potential virulence and transmissibility of the novel influenza A (H7N9) virus.

The Study

We collected a sputum specimen from a 54-year-old woman with fever, cough, sputum production, and pneumonia. Influenza A (H7N9) virus was detected in the specimen by specific real-time reverse transcription PCR (RT-PCR). The specimen was then processed with a viral particle–protected nucleic acid purification method (5). Total RNA was extracted and amplified by sequence-independent PCR (5) and then sequenced with an Illumina/Solexa GAII sequencer (Illumina, San Diego, CA, USA). Reads generated by the Illumina/Solexa GAII with lengths of 80 bases were directly aligned to those nucleotide sequences of influenza A viruses in the National Center for Biotechnology Information nonredundant nucleotide database by the blastn program in the BLAST (6) software package, version 2.2.22 (www.ncbi.nlm.nih.gov/blast) with parameters –e 1e-5 -F T (–e 1e-5 for selection of highly similar reads and -F T for masking the low-complexity reads) after filtering of the sequence adapters and RT-PCR primers. No assembly was performed before alignment. We obtained 19,177 reads aligned to influenza A viruses.

We then conducted a reference-guided assembly based on the 19,177 reads by the Seqman program in the DNAStar software package version 7.1 (www.dnastar.com). The novel influenza A (H7N9) virus A/Anhui/1/2013 was selected as the reference. With 80% minimum sequence similarity tolerance and 12 bp minimum match size, those 19,177 reads were assembled into 439 contigs. The top 8 contigs covered by the most reads corresponded to the 8 genome segments of the novel influenza A (H7N9) virus. The other contigs did not align to the reference virus, which might have resulted from sequencing or assembling errors. Calculating the consensus sequence, we obtained the genome of the influenza A (H7N9) virus directly from the sputum specimen of this patient. Further RT-PCR and Sanger sequencing confirmed the quality of the assembled subtype H7N9 virus genome. Sequences were deposited in GenBank under accession nos. KF226105–KF226120 and KF278742–KF278749.

The influenza A (H7N9) genome that we report varies from that obtained by Sanger sequencing after passage in the allantoic sac and amniotic cavity of 9–11-day-old specific pathogen–free embryonated chicken eggs for 48–72 hours at 35°C (Table 1). In the nucleocapsid protein (NP) segment, 15 point mutations were found; 13 were synonymous and 2 induced amino acid changes S321N and M371I. In the nonstructural (NS) protein segment, 15 point mutations were found; all caused amino acid changes R59H, P107L, and V111Q. In the polymerase basic (PB1) protein segment, 3 point mutations were found, 1 of which caused amino acid change V707F. In the polymerase acidic (PA) protein segment, 3 point mutations were found, 1 of which caused amino acid change V707F. In the polymerase basic 1 (PB1) protein segment, 2 point mutations were found, both of which were synonymous. In the PB2 segment, 2 point mutations were found, 1 of which caused amino acid change S534F.

The influenza A (H7N9) genome also demonstrates significant intraspecimen heterogeneity. Deep sequencing revealed that the average coverage (ratio of the total number of nucleotides of all reads to the length of the reference gene) of the 8 genes was quite inhomogeneous.
Average coverage (± SD) was highest for neuraminidase (NA) (131.94 ± 30.25) and second highest for NP (130.41 ± 27.01). The average coverages of PB2, PB1, PA, matrix protein, and hemagglutinin were 99.89 (± 22.49), 95.35 (± 21.34), 43.35 (± 14.13), 53.73 (± 17.67), and 69.82 (± 19.02), respectively. Average coverage was lowest for NS (27.73 ± 11.31).

Besides the gene abundance, the genome sequence of influenza A (H7N9) virus also demonstrated heterogeneity (the heterozygous peak threshold 80%). In total, 22 positions were confirmed by PCR and Sanger sequencing to be heterogeneous (Table 2). In the NP segment, 4 positions demonstrated heterogeneity; 3 were synonymous and 1 induced amino acid change E421K. In the NS segment, 3 positions demonstrated heterogeneity; 2 were synonymous and 1 induced amino acid change R140W. In the hemagglutinin segment, 7 positions demonstrated heterogeneity; 6 were synonymous and 1 induced amino acid change H242Y. In NA, 3 positions demonstrated heterogeneity; 2 induced amino acid changes (S92L and S108L) and 1 was synonymous. In the PA segment, 2 positions demonstrated heterogeneity; both were synonymous. In the PB2 segment, 3 positions demonstrated heterogeneity; all were nonsynonymous (S532L, S533L, and S534F). All these heterogeneous sites were confirmed by PCR and Sanger sequencing; only 1 site overlapped with the mutation sites after passage in embryonated chicken eggs.

Compared with the reference influenza A (H7N9) virus strain A/Anhui/1/2013, the influenza A (H7N9) virus demonstrated prominent sequence differences (Table 2). In particular, the amino acid at the 627 position of PB2 of A/Anhui/1/2013 is K, whereas the corresponding amino acid in the subtype H7N9 genome is E. The amino acid at the 368 position of PB1 of A/Anhui/1/2013 is V, whereas the corresponding amino acid in the subtype H7N9 genome is I. The E627K mutation in PB2 and the I368V mutation in PB1 are closely associated with the virulence and transmissibility of avian influenza A virus in mammals (1). E627K in PB2 was observed in A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013 viruses (1). A/Zhejiang/DTID-ZJU01/2013 virus does not have this mutation but has a complementary mutation D701N in PB2 (2). I368V in PB1 was observed in A/Shanghai/2/2013 and A/Anhui/1/2013 viruses, but A/Shanghai/1/2013 virus does not have this mutation (1).

MEGA5.0 (www.megasoftware.net) was used to construct the phylogenetic trees on the basis of the nucleotide sequences of all influenza A (H7N9) viruses in the Global Initiative on Sharing All Influenza Data (GISAID) database (7). We conducted 2 rounds of phylogenetic analysis. First, to examine whether this subtype H7N9 virus is clustered with the available subtype H7N9 strains, we included all influenza A (H7N9) viruses in the GISAID database. To construct the multiple sequence alignment, we used the MUSCLE package with default
parameters (www.megasoftware.net/); then, to construct
the phylogenetic trees with 1,000 bootstrap replicates, we
used the minimum-evolution method. Results suggested
that all 8 genome segments are closely related to the
available influenza A (H7N9) virus strains.

We next included all influenza A (H7N9) viruses
isolated in China in 2013 to closely investigate the rela-
tionships between this virus and available subtype H7N9
genomes isolated during epidemics. However, the phylo-
genetic topologies based on different gene segments were
not consistent (Figures 1, 2; online Technical Appendix
Figures 1–6, wwwnc.cdc.gov/EID/article/19/11/13-0664-
Techapp1.pdf), suggesting that the influenza A (H7N9) vi-
rus may have persistently evolved for a while (8).

Conclusion

Using deep sequencing technologies, we derived the full-
length genome of the novel influenza A (H7N9) virus direct-
ly from the sputum specimen of a patient, without conducting
virus culture. The full genome revealed substantial sequence
heterogeneity within the specimen, obvious sequence varia-
tions from that obtained from embryonated chicken eggs, and

<table>
<thead>
<tr>
<th>Protein</th>
<th>Heterogeneity, nucleotide position in gene sequence:</th>
<th>Amino acid position in protein sequence:</th>
<th>Direct sequencing</th>
<th>Culture</th>
<th>Consensus of isolate from humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA 174: C&gt;T</td>
<td>58</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>PA 1305: C&gt;T</td>
<td>435</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>PA 616</td>
<td>616</td>
<td>K</td>
<td>K</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>PA 707</td>
<td>707</td>
<td>V</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>PB1 200</td>
<td>200</td>
<td>I</td>
<td>I</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>PB1 368</td>
<td>368</td>
<td>I</td>
<td>I</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>PB1 454</td>
<td>454</td>
<td>L</td>
<td>L</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>PB1 637</td>
<td>637</td>
<td>V</td>
<td>V</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>PB1-F2 42</td>
<td>42</td>
<td>C</td>
<td>C</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>PB1-F2 51</td>
<td>51</td>
<td>T</td>
<td>T</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>PB1-F2 70</td>
<td>70</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>PB1-F2 77</td>
<td>77</td>
<td>L</td>
<td>L</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>PB2 534</td>
<td>534</td>
<td>S</td>
<td>F</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>PB2 591</td>
<td>591</td>
<td>K</td>
<td>K</td>
<td>Q</td>
<td>Q</td>
</tr>
<tr>
<td>PB2 627</td>
<td>627</td>
<td>E</td>
<td>E</td>
<td>K</td>
<td>K</td>
</tr>
</tbody>
</table>

*HA, hemagglutinin; M, matrix; NA, neuraminidase; NP, nucleocapsid protein; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic.
†Position of first nucleotide = 1.
‡Position of first amino acid = 1.
§Types of amino acids.
¶Virus cultured in chicken eggs.
#Thirteen influenza A (H7N9) viruses isolated from humans; data from Global Initiative on Sharing All Influenza Data.
prominent differences from the available influenza A (H7N9) strains, most of which were sequenced after culture.

Acknowledgment

We acknowledge those who contributed to the generation of the genome sequences of influenza A (H7N9) viruses in GISAID, on which this research is based.

This work was supported by the National S&T Major Project, “China Mega-Project for Infectious Disease” (grant No. 2013ZX10004101).

Dr Ren is an assistant professor at the Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. His research focuses on the bioinformatics and computational biological questions of pathogens.

References


Address for correspondence: Qi Jin, No. 6 St Rongjing East, BDA, Beijing, 100176, People’s Republic of China; email: zdsys@vip.sina.com
Mild Illness in Avian Influenza A(H7N9) Virus–Infected Poultry Worker, Huzhou, China, April 2013

Huakun Lv,1 Jiankang Han,1 Peng Zhang, Ye Lu, Dong Wen, Jian Cai, Shelan Liu, Jimin Sun, Zhao Yu, Heng Zhang, Zhenyu Gong, Enfu Chen, and Zhiping Chen

During April 2013 in China, mild respiratory symptoms developed in 1/61 workers who had culled influenza A(H7N9) virus–infected poultry. Laboratory testing confirmed A(H7N9) infection in the worker and showed that the virus persisted longer in sputum than pharyngeal swab samples. Pharyngeal swab samples from the other workers were negative for A(H7N9) virus.

During March–May 2013, a respiratory disease caused by avian influenza A(H7N9) virus was identified among humans in China (1–6). Most infected persons were >60 years of age, and most cases were severe and involved serious complications, including death (1). Few children and adults have been reported with mild illness caused by influenza A(H7N9) virus infection (7,8). After an epidemiologic link was reported between exposure to poultry and confirmed influenza A(H7N9) cases (9–11), local governments closed contaminated wholesale wet markets (large markets where live chickens were sold to vendors) and assigned government office workers to assist in a temporary poultry culling campaign.

The largest number of confirmed cases was reported in Zhejiang Province, where 46 cases and 11 deaths occurred (data from the Chinese Disease Surveillance Information Report and Management System; as of July 20, 2013). Of the 46 cases, 12 were reported from Huzhou city, where the environment of a wholesale wet market was contaminated by influenza A(H7N9) virus (9).

Approximately 25,000 live chickens were processed daily at this market, and on April 8, 2013, the Huzhou city government launched their campaign to close the market and slaughter the remaining poultry.

Sixty-one government workers participated for 3 hours in the culling campaign. The workers wore personal protective equipment, including protective clothing, ordinary disposable masks, and latex gloves; neither goggles nor face shields were worn (Figure 1). During the culling process, workers disarticulated chickens’ necks and placed the dead birds in individual sacks.

Avian influenza A(H7N9) infection was subsequently confirmed in 1 of the 61 workers. We conducted an epidemiologic investigation and clinical review of the confirmed case. In addition, we administered questionnaires to the 60 co-workers and obtained pharyngeal swab samples from them to test for influenza A(H7N9) virus.

Case Report

The male patient was a 41-year-old administrative manager in a sub-district government office in Huzhou city. The patient had been a chronic smoker, but discontinued smoking 4 years earlier because of chronic pharyngolaryngitis. He did not report any other underlying medical conditions, including hypertension and diabetes.

The patient’s only contact with poultry during the 10 days before symptom onset occurred on April 8, when he participated in the campaign to cull poultry. Five days later, on April 13, the patient’s eyes were swollen, but there was no tearing or discharge. Midday on April 14, the patient experienced low-grade fever (self-reported axillary temperature 37.8°C), dry throat, cough with a small amount of white sputum, weakness, and muscle soreness. Later that afternoon, he visited the outpatient clinic of Huzhou First People’s Hospital. Clinical records were not available, however, at that visit, the patient was told his temperature was normal and that he probably had a cold, and he was sent home without medications.

The next morning, April 15, the patient returned to the clinic for medical evaluation and was found to have an oral temperature of 37.5°C, normal auscultation of the heart and lungs, leukocyte count of 5.3 × 10^9 cells/L (reference range 4.0–10.0 × 10^9 cells/L), neutrophil count of 3.25 × 10^9 cells/L (reference range 2.0–7.0 × 10^9 cells/L), and C-reactive protein level of 2.29 mg/L (reference range 0–4.0 mg/L). The patient was sent home without treatment, but later that afternoon, he returned to the clinic. At that third visit, a pharyngeal swab sample was collected and submitted to the Huzhou Municipal Center for Disease Prevention and Control for testing by real-time reverse transcription PCR (rRT-PCR) (12); the sample was found to be positive

DOI: http://dx.doi.org/10.3201/eid1911.130717

1These authors contributed equally to this article.
for influenza A(H7N9) virus. That same evening, leftover sample was confirmed positive for influenza A(H7N9) virus by a provincial reference laboratory.

During the early morning hours of April 16, immediately after laboratory confirmation of influenza A(H7N9) infection, the patient was transferred to a hospital designated for the care of persons infected with the virus. The patient’s oral temperature was recorded twice a day: his temperature was 37.6°C on the first morning of hospitalization and <37.5°C thereafter. Routine blood tests showed that the patient’s maximum C-reactive protein level (4.19 mg/L on day 4 of hospitalization) was slightly elevated, his leukocyte and lymphocyte counts were normal, his lactate dehydrogenase level was normal, and his neutrophil count ranged from normal to slightly below normal (Table 1). A cardiac ultrasound examination revealed no abnormalities in the heart, and results of an abdominal ultrasound of the liver, kidneys, and spleen was also unremarkable. Computer tomographic scans of the chests on April 16 and 18, showed an old lesion in the lung that seemed unrelated to the acute infection.

The patient was administered oseltamivir (75 mg 2×/day) on hospitalization days 1–6; noninvasive ventilation and symptomatic and supportive treatment were also administered daily. Each day while the patient was hospitalized, pharyngeal swab samples and sputum samples were collected and tested for the presence of influenza A(H7N9) virus by rRT-PCR. All pharyngeal swab samples, except the 1 obtained the day before hospitalization, were negative for the virus. Sputum samples were influenza A(H7N9) virus–positive on hospitalization days 1–3 and converted to virus-negative on hospitalization day 4.

As of April 22, 2013, the patient had made a good recovery and was discharged from the hospital. Figure 2 shows the timeline of events, from exposure to hospital discharge, for the patient.

On April 16, 2013, we administered a questionnaire to, recorded oral temperatures for, and obtained pharyngeal swab samples from the patient’s 60 co-workers (Table 2). None of the workers had fever or other signs or symptoms of infection at the time of screening, but 13 of the 60 reported transient symptoms during April 9–16. All pharyngeal swab samples from these workers were negative for influenza A(H7N9) virus by rRT-PCR.

Conclusions

Our epidemiologic investigation and clinical review showed that mild upper respiratory symptoms developed in a man 6 days after he had contact with influenza A(H7N9) virus–infected poultry. We found that sputum samples from this patient remained positive for A(H7N9) virus longer than pharyngeal swab samples. This finding is in agreement with those of Chen et al. (10) and Lo et al. (13). Thus, it is a limitation of our screening of the patient’s 60 coworkers that we did not collect sputum specimens.

Table 1. Blood test results on hospitalization days 1–4 for a man infected with avian influenza A(H7N9) virus, Huzhou city, Zhejiang Province, China, 2013

<table>
<thead>
<tr>
<th>Index</th>
<th>April 16</th>
<th>April 17</th>
<th>April 18</th>
<th>April 19</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morning</td>
<td>Afternoon</td>
<td>Morning</td>
<td>Afternoon</td>
<td>Morning</td>
</tr>
<tr>
<td>Leukocyte count (10⁹ cells/L)</td>
<td>7.0</td>
<td>5.9</td>
<td>4.9</td>
<td>4.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Neutrophil count (10⁹ cells/L)</td>
<td>5.6</td>
<td>3.9</td>
<td>3.0</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Lymphocyte count (10⁹ cells/L)</td>
<td>0.6</td>
<td>1.5</td>
<td>1.6</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Lactate dehydrogenase (IU/L)</td>
<td>183.0</td>
<td>ND</td>
<td>156.0</td>
<td>161.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not determined.
Because of this, we may have missed identifying other mild infections among the workers who culled poultry.

The patient in this report is 1 of only a few adults with mild respiratory symptoms who have been confirmed to be infected with avian influenza A(H7N9) virus (8). Our investigation strongly suggests that he became infected with the virus after working for 3 hours as poultry culler in a contaminated wet market. Future investigations of persons exposed to influenza A(H7N9)–infected poultry may consider testing for the virus in sputum samples rather than throat swab samples.

This work was supported by a grant from Zhejiang Provincial Public Technology Applied Research Projects (No. 2012C33063).

Mr. Lv is a chief physician at the Zhejiang Provincial Center for Disease Prevention and Control, Hangzhou, China. His research interests focus on epidemiology and acute communicable disease prevention and control.

References


Address for correspondence: Zhiping Chen, Zhejiang Provincial Center for Disease Prevention and Control, 630 Xincheng Rd, Binjiang District, Hangzhou 310051, China; email: zhpchen@cdc.zj.cn
Pulmonary nontuberculous mycobacterial (pNTM) disease is clinically challenging. Therapy entails complex antimycobacterial drug combinations, typically for 18 months (1), often with poor tolerability (2) and limited success (3). pNTM disease is increasingly common in Canada (4) and the United States (5–7), but its prevalence is not well understood. Determining the epidemiology of pNTM disease is difficult for several reasons. It is generally not reportable, so population-level data are not routinely compiled. The diagnosis requires clinical and radiologic information in addition to microbiological examination (≥2 positive sputum cultures or 1 bronchoscopic or biopsy culture) (1). Finally, the chronic nature of pNTM disease dictates longitudinal study, illustrated by considering that only a minority with pNTM disease appear to be treated (18% in 1 study) (6), treatment succeeds in only 56% (3), and disease recurs in >30% of patients (2,8). These data indicate that most pNTM cases are expected to be chronic. Cases detected by isolation of nontuberculous Mycobacterium spp. in 1 year, generally remain prevalent over several subsequent years, regardless of the reliable appearance of subsequent isolates, with a disease duration that may depend primarily on patient survival.

The traditional method of identifying cases for NTM disease epidemiology studies by using mycobacterial laboratory databases and measuring annual prevalence is not ideal. Such studies assume that, in patients with pNTM disease, the organism is isolated during every year of disease, an invalid assumption (6). Recent investigators have focused on prevalence within a defined period (period prevalence) as an improved estimate of pNTM disease, including a 2-year study in Oregon (5), 3-year sampling of 4 US health care delivery systems (6), and ≤11-year US-wide sample of Medicare beneficiaries (7). Important limitations of these studies included the patient populations and geographic regions selected and the limited data about temporal prevalence changes. Expanding on methods of previous studies to overcome some prior limitations, we performed a population-based study of pNTM disease in Ontario, Canada, using 5-year periods for prevalence calculations and compared prevalence from 1998–2002 to 2006–2010.

The Study

We performed a retrospective cohort study of all Ontario residents who had pulmonary nontuberculous Mycobacterium spp. isolated during 1998–2010, identified from the records of the Public Health Ontario Laboratory, capturing ≥95% of NTM disease in Ontario. Culture was performed by using Bectec 460 TB system until 2000 and thereafter with BACTEC MGIT 960 (Becton Dickinson, Baltimore, MD, USA). Before 2008, speciation was performed by using a combination of DNA probes (AccuProbe, Gen-Probe, San Diego, CA, USA) for Mycobacterium avium complex (MAC) and M. gordonae and high-performance liquid chromatography for other species and thereafter solely by DNA probes (AccuProbe, Gen-Probe) or line-probe assays (GenoType, Hain Lifescience, Germany). Because MAC was not identified to individual species for most of our study, we present data only for MAC.

Full criteria for pNTM disease include the presence of all clinical (symptoms and radiology) and microbiological components (1). We defined surrogate criteria as microbiological criteria only (1), (≥2 positive sputum cultures or 1 bronchoscopic or lung biopsy culture), which has a positive predictive value of 70%–100% (5,6,9,10). Period prevalence of disease was calculated as the number of persons who fulfilled the disease criteria during a 5-year period (1998–2002 or 2006–2010), divided by the Ontario population at the period midpoint. We left a 3-year gap (2003–2005) between periods to minimize patient overlap. We excluded M. gordonae from period prevalence because it is rarely pathogenic (1). We selected a conservative 5-year period on the assumption that the median survival with pNTM disease is 5–10 years (10,11), using the low end of the survival range based on assumptions that a small
proportion of the cohort would not have true disease (misclassified by surrogate definition) and disease of an additional small proportion would be cured.

Annual isolation prevalence (number of persons in a calendar year with ≥1 pulmonary Mycobacterium spp. isolate divided by the contemporary population) and annual disease prevalence (number of persons in a calendar year whose illnesses fulfilled criteria for disease divided by the contemporary population) are presented for illustrative purposes. A generalized linear model with negative binomial distribution was used to assess annual rate changes, and a simple model binomial approach was used to compare 5-year period prevalence rates by using SAS 9.2 (SAS Institute, Cary, NC, USA). This study was approved by the University of Toronto Research Ethics Board with the requirement for informed consent waived.

Ontario’s population increased from 11.3 million to 13.2 million during 1998–2010. Total annual isolations of pulmonary Mycobacterium spp. rose from 11.4 to 22.2 per 100,000 persons (p = 0.0025, mean annual increase 6.3%) (Table 1; Figure). The relative frequency of different nontuberculous Mycobacterium isolates remained constant. The most common pulmonary nontuberculous Mycobacterium isolates in 2010 were MAC (12.2 isolations/100,000 persons), M. xenopi (3.9/100,000), M. gordonae (3.0/100,000), M. fortuitum (0.8/100,000), and M. abscessus (0.6/100,000). Among patients with different Mycobacterium spp. isolates in 2010, the following proportions were judged to have disease: MAC, 52%; M. abscessus, 50%; M. xenopi, 38%; and other non-M. gordonae species, 38%. Annual prevalence for all NTM disease combined rose from 4.9 cases to 9.8 cases per 100,000 persons (p<0.0001, mean annual increase 6.5%) (Table 1; Figure). Five-year prevalence of pNTM disease (M. gordonae excluded) increased from 29.3 cases per 100,000 persons in 1998–2002 to 41.3 per 100,000 in 2006–2010 (p<0.0001) (Table 2).

### Conclusions

The 5-year prevalence of pNTM disease was substantial and increased significantly during our population-based assessment in Ontario, Canada. Our measurements of period prevalence (29.3 and 41.3 cases/100,000 persons) were substantially higher than observed in Oregon (8.6/100,000), probably partially because of the shorter period (2 years) and more stringent definition for disease (medical records review) used in the Oregon study (5). Other studies did not present period prevalence for the entire study populations, only by age strata, and used durations of 3 years (6,7) or ≤11 years (7). We selected a 5-year period assuming it would provide the most accurate estimate of disease prevalence based on the chronic nature of pNTM disease. Prior studies provided age-stratified data, with high period prevalence in older patients (20.4/100,000 to >200/100,000, depending on period length and specific age range) (5–7), as expected, because pNTM disease is a disease of the elderly (1,4,6,12). Although age data were unavailable for our study, annual disease prevalence of pulmonary MAC in Ontario has a strong age association, with an average increase of 14/100,000 per decade increase during 50–80 years (4).

Changes in microbiological methods and the number of samples submitted annually did not account for the increases in pulmonary nontuberculous Mycobacterium isolation (13). The attenuation in the rate of increase in isolation prevalence around the middle of the study corresponded with a previously reported plateau in the annual number of specimens submitted (13). However, the annual

### Table 1. Annual prevalence of all pulmonary nontuberculous mycobacterial disease, Ontario, Canada, 1998–2010*

<table>
<thead>
<tr>
<th>Year</th>
<th>Isolation prevalence†</th>
<th>Disease prevalence‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>11.4</td>
<td>4.9</td>
</tr>
<tr>
<td>1999</td>
<td>14.3</td>
<td>6.3</td>
</tr>
<tr>
<td>2000</td>
<td>15.1</td>
<td>6.1</td>
</tr>
<tr>
<td>2001</td>
<td>18.7</td>
<td>7.6</td>
</tr>
<tr>
<td>2002</td>
<td>21.0</td>
<td>8.1</td>
</tr>
<tr>
<td>2003</td>
<td>18.9</td>
<td>7.3</td>
</tr>
<tr>
<td>2004</td>
<td>22.8</td>
<td>8.6</td>
</tr>
<tr>
<td>2005</td>
<td>22.6</td>
<td>9.1</td>
</tr>
<tr>
<td>2006</td>
<td>23.4</td>
<td>9.7</td>
</tr>
<tr>
<td>2007</td>
<td>24.0</td>
<td>10.3</td>
</tr>
<tr>
<td>2008</td>
<td>24.5</td>
<td>10.4</td>
</tr>
<tr>
<td>2009</td>
<td>24.9</td>
<td>10.7</td>
</tr>
<tr>
<td>2010</td>
<td>22.2</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Annual (1-year) prevalence, per 100,000 population, in a calendar year.
†Prevalence of ≥1 pulmonary nontuberculous Mycobacterium isolate. Mean annual increase: 6.3% (p = 0.025).
‡Prevalence of ≥2 sputum nontuberculous Mycobacterium isolates or 1 bronchoscopic or biopsy nontuberculous Mycobacterium isolate. Mean annual increase: 6.5% (p<0.0001).

![Annual isolation prevalence and disease prevalence per 100,000 persons of pulmonary nontuberculous mycobacteria, Ontario, Canada, 1998–2010.](image)
isolation prevalence continued to rise, and the annual disease prevalence rose steadily throughout the study period. We suspect a multifactorial explanation for the increase in pNTM disease: an increase in susceptible hosts (aging, chronic lung disease) contributes (4); decades-old increases in water aerosol exposure could cause recent increases in pNTM disease, given the potential latency of pNTM disease; more computed tomographic scanning probably leads to sampling patients with previously unidentified abnormalities; and reduced tuberculosis, with an associated reduction in cross-immunity, may play a role. The latter is supported by observations of increased extrapulmonary NTM infection in children not vaccinated with _M. bovis_ BCG (14, 15). pNTM disease in Ontario is substantial and increased greatly from early (1998–2002) to recent (2006–2010) periods.

Dr Marras is an attending staff physician in the Division of Respiratory, Department of Medicine, University Health Network and Mount Sinai Hospital, Toronto, and assistant professor of medicine, University of Toronto. His primary research interest is nontuberculous mycobacterial disease.

References


Address for correspondence: Theodore K. Marras, Toronto Western Hospital, 7E-452, 399 Bathurst St, Toronto, ON M5T 2S8, Canada; email: ted.marras@uhn.on.ca

Search past issues of EID at wwwnc.cdc.gov/eid
Severe Fever with Thrombocytopenia Syndrome, South Korea, 2012

Kye-Hyung Kim, Jongyoun Yi, Gayeon Kim, Su Jin Choi, Kang Il Jun, Nak-Hyun Kim, Pyoeng Gyun Choe, Nam-Joong Kim, Jong-Koo Lee, and Myoung-don Oh

We report a retrospectively identified fatal case of severe fever with thrombocytopenia syndrome (SFTS) in South Korea from 2012. SFTS virus was isolated from the stored blood of the patient. Phylogenetic analysis revealed this isolate was closely related to SFTS virus strains from China and Japan.

Severe fever with thrombocytopenia syndrome (SFTS) causes signs and symptoms including high fever, vomiting, diarrhea, thrombocytopenia, leukopenia, and multiple organ failure and has a 6%–30% case-fatality rate (1–4). Caused by a novel bunyavirus, SFTS virus (SFTSV), SFTS was initially reported in China in 2011 (1). SFTSV has been detected in Haemaphysalis longicornis ticks, which have been implicated as a vector of the virus (1). H. longicornis ticks widely inhabit the Korean Peninsula (5,6), and the Korea Centers for Disease Control and Prevention reported that SFTSV was detected in samples from H. longicornis ticks collected during 2011–2012 in South Korea (7). Seroconversion and viremia of SFTSV have been demonstrated in domesticated animals such as goats, sheep, cattle, pigs, and dogs; these animals have been implicated as intermediate hosts in SFTSV-endemic areas (8,9). SFTSV was also detected in Japan in February 2013 (10). We report a retrospectively identified case of SFTS in South Korea from 2012 and the characterization of the SFTSV isolated from the patient.

The Study

On August 3, 2012, fever developed in a previously healthy 63-year-old woman who lived in Chuncheon-si, Gangwon Province, South Korea; the same day, she noticed a lump on the left side of her neck. She visited a local clinic, and ciprofloxacin and ceftriaxone were started on the first day of illness. The patient reported that, 2 weeks before her fever started, she noticed an insect bite on her neck while she was working on a crop farm in Hwacheongun, Gangwon Province (in the northernmost part of South Korea). She did not recall having contact with any domestic animals on the farm and had no history of travel outside South Korea in the month before illness onset.

On the third day of her illness, she began having watery diarrhea, 6 times per day. On the fourth day of the illness, thrombocytopenia and leukopenia were recorded at the local clinic (Table). Because of worsening thrombocytopenia, she was transferred to another hospital. Ciprofloxacin was changed to doxycycline, and ceftriaxone was continued. A computed tomography scan of the neck showed an enlarged (1.6 cm), necrotic lymph node. Multiple lymph nodes on the left cervical and left axillary areas were also swollen. On the sixth day, the patient was transferred to Seoul National University Hospital.

At admission to the hospital, the patient was febrile but alert. Her temperature was 38.7°C, blood pressure 126/70 mm Hg, heart rate 86 beats per minute, and oxygen saturation 92% on room air. Her face was puffy, with a sunburned appearance, and both conjunctivae were congested. The insect bite site on her posterior neck was swollen and erythematous, and the draining cervical lymph node was enlarged. Petechiae were observed on her shoulders and lower extremities.

Laboratory test results showed pancytopenia and elevated serum aminotransferase levels; prothrombin and activated partial thromboplastin times were normal, but fibrinogen level was decreased (Table). A urine dipstick test showed albuminuria (+++), and microscopic examination of the urine revealed >100 erythrocytes per high-power field. Test results for antibodies against Orientia tsutsugamushi, Hantaan virus, and leptospira were negative. A chest radiograph showed bilateral increased vascular markings, and the plasma level of B-type natriuretic peptide increased to 134 pg/mL (reference range <100 pg/mL); these findings suggested cardiac dysfunction.

On the eighth day of her illness, the patient spoke incoherently and was unable to communicate. Cerebrospinal fluid analysis showed no erythrocytes or leukocytes and a normal chemistry profile. A computed tomography scan of the brain showed no evidence of hemorrhage or infarction and no other abnormalities. She was transferred to the intensive care unit. On the ninth day, she was intubated and placed on continuous renal replacement therapy. On the tenth day of illness (August 12, 2012), the patient died of multiple organ failure. Ceftriaxone and doxycycline were continued until the patient’s death. Antiviral drugs, corticosteroids, immunosuppressive agents, or intravenous immunoglobulin were not given.

Author affiliations: Seoul National University College of Medicine, Seoul, South Korea (K.-H. Kim, G. Kim, S.J. Choi, K.I. Jun, N.-H. Kim, P.G. Choe, N.-J. Kim, J.-K. Lee, M.-D. Oh); and Pusan National University School of Medicine, Busan, South Korea (J. Yi)

DOI: http://dx.doi.org/10.3201/eid1911.130792

These authors contributed equally to this article.
Because viral infection was suspected but no virus could be identified, an anticoagulated blood sample was obtained from the patient on the eighth day of illness and stored at −70°C. When testing for SFTSV became available 7 months later, we inoculated monolayers of Vero cells with the patient’s blood sample and cultured the cells at 37°C in a 5% carbon dioxide atmosphere. A culture supernatant obtained 13 days after the inoculation was used for genetic analysis. The culture supernatant was also used to inoculate DH82 cells when the cell line became available; 5 days after the inoculation, we observed a cytopathic effect of SFTSV in DH82 cells. The SFTSV-infected Vero cell monolayer was fixed according to described methods (11) and cut on ultramicrotome (RMC MT-XL) at 65 nm. Ultrathin sections were stained with saturated 4% uranyl acetate and 4% lead citrate before examination with a transmission electron microscope (HITACHI-7100; Hitachi High-Technologies, Ibaraki, Japan) at 75 kV (Figure 1).

RNA was extracted from the stored blood and from virus-infected Vero cells by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription PCR (RT-PCR) was performed to amplify the partial large (L) segment of the viral RNA from the stored blood to confirm SFTSV, as described (12). RT-PCR results were positive, and direct sequencing was done. A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed no sequences from organisms other than SFTSV.

Using the culture supernatant, full lengths of all 3 genome segments (L, medium [M], and small [S]) were sequenced by RT-PCR and direct sequencing was performed by using primers designed from previously published SFTSV sequences. After polyadenylation of 3’ ends of the genomic and complementary RNAs, the sequences of the segment ends were obtained by rapid amplification of cDNA ends. The complete sequences of the L, M, and S segments were deposited in GenBank (accession nos. KF358691–KF358693). Sequences that had homology to our isolate were identified by BLAST search. The L, M, and S segments of the isolate showed 95.8%–99.8%, 94.1%–99.9%, and 94.8%–99.7% identity, respectively, to previously reported SFTSV sequences. We also constructed a phylogenetic tree by the neighbor-joining method using RNA-dependent RNA polymerase gene nucleic acid sequences to compare the isolate we obtained to representative SFTSV strains from China and Japan; the isolate and the other strains were closely related (95.9%–99.9% sequence relatedness) but not identical (Figure 2).

<table>
<thead>
<tr>
<th>Laboratory test (reference range)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, % (36–48)</td>
<td>NA</td>
<td>NA</td>
<td>30</td>
<td>30</td>
<td>38</td>
<td>32</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Hemoglobin, g/dL (12–16)</td>
<td>12.9</td>
<td>13.6</td>
<td>12.4</td>
<td>12.4</td>
<td>12.9</td>
<td>12.8</td>
<td>9.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Leukocytes, cells/μL (4,500–10,000)</td>
<td>1,800</td>
<td>1,300</td>
<td>1,600</td>
<td>1,600</td>
<td>2,100</td>
<td>3,150</td>
<td>4,300</td>
<td>4,700</td>
</tr>
<tr>
<td>Neutrophils, % (50–75)</td>
<td>47</td>
<td>60</td>
<td>56</td>
<td>51</td>
<td>76</td>
<td>35</td>
<td>32</td>
<td>33</td>
</tr>
<tr>
<td>Lymphocytes, % (20–44)</td>
<td>36</td>
<td>36</td>
<td>39</td>
<td>41</td>
<td>18</td>
<td>58</td>
<td>57</td>
<td>62</td>
</tr>
<tr>
<td>Atypical lymphocytes, % (0)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8.0</td>
</tr>
<tr>
<td>Platelets, /μL (130,000–400,000)</td>
<td>136,000</td>
<td>98,000</td>
<td>50,000</td>
<td>25,000</td>
<td>32,000</td>
<td>70,000</td>
<td>159,000</td>
<td>116,000</td>
</tr>
<tr>
<td>AST, IU/L (0–40)</td>
<td>56</td>
<td>NA</td>
<td>180</td>
<td>383</td>
<td>537</td>
<td>1059</td>
<td>2279</td>
<td>NA</td>
</tr>
<tr>
<td>ALT, IU/L (0–40)</td>
<td>32</td>
<td>NA</td>
<td>66</td>
<td>115</td>
<td>137</td>
<td>199</td>
<td>403</td>
<td>NA</td>
</tr>
<tr>
<td>Creatine kinase, IU/L (20–270)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5,127</td>
<td>6,966</td>
<td>7,830</td>
<td>15,224</td>
<td>NA</td>
</tr>
<tr>
<td>LDH, IU/L (100–225)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5270</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Creatinine, mg/dL (0.7–1.4)</td>
<td>NA</td>
<td>0.60</td>
<td>0.70</td>
<td>NA</td>
<td>0.59</td>
<td>0.99</td>
<td>2.17</td>
<td>3.01</td>
</tr>
<tr>
<td>aPTT, sec (26–35.3)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>44.1</td>
<td>45.4</td>
<td>75.6</td>
<td>71.6</td>
<td>400</td>
</tr>
<tr>
<td>Prothrombin time, INR (0.8–1.2)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.98</td>
<td>0.99</td>
<td>1.08</td>
<td>1.06</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL (230–380)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>172</td>
<td>151</td>
<td>136</td>
<td>124</td>
<td>57</td>
</tr>
</tbody>
</table>

Figure 1. Transmission electron microscopy image of Vero cells infected with severe fever with thrombocytopenia syndrome virus (arrows). Scale bar indicates 500 nm.
Conclusions

We confirmed a case of SFTS in South Korea in 2012 by isolation of SFTSV from a stored blood sample collected shortly before the patient’s death. The patient had a history of an insect bite while working on a crop farm in Hwacheon-gun, Gangwon Province, the northernmost part of South Korea. Phylogenetic analysis of the RNA-dependent RNA polymerase gene showed that our virus isolate was closely related to SFTSV strains reported from China and Japan.

As of July 5, 2013, the Korea Centers for Disease Control and Prevention had confirmed 13 cases of SFTS by RT-PCR; of these patients, 8 were dead and 5 alive (13).

Except for our patient, who died in 2012, all cases occurred during 2013.

Dr Kye-Hyung Kim is an infectious disease physician and a senior researcher at Seoul National University College of Medicine, Seoul, South Korea. Her main research interests are medical virology and infectious disease epidemiology.

References


Address for correspondence: Myoung-don Oh, Department of Internal Medicine, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea; email: mdohmd@snu.ac.kr
Seoul Virus in Rats (Rattus norvegicus), Hyesan, North Korea, 2009–2011

To the Editor: Seoul virus (SEOV), a member of the family Bunyaviridae, genus Hantavirus, is primarily carried by Rattus norvegicus rats. Because members of Rattus species are widely distributed, SEOV has the potential to cause human disease worldwide. It has been reported that SEOV causes a milder form of hemorrhagic fever with renal syndrome than Hantaan virus and Dobrava-Belgrade virus and is responsible for 25% of cases of hemorrhagic fever with renal syndrome in Asia (1). Although it is well known that SEOV is endemic to China (2) and South Korea (3), little is known about its distribution in North Korea (4).

In September 2009, June and September 2010, and September 2011, a total of 89 R. norvegicus rats were trapped in the city of Hyesan (128°30′E, 41°30′N) during the operation of a cooperative rodent surveillance program of China and North Korea. The captured rodents were euthanized with barbiturate (100 mg/kg), weighed, measured, classified by sex, and then autopsied. Lung samples were probed for the large segment of SEOV by reverse transcription PCR by using the RT primer P14 (5), the primary PCR primers HAN-L-F1 and HAN-L-R1, and the nested PCR primers HAN-L-F2 and HAN-L-R2 (6). PCR products were sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

A high rate of SEOV infection was detected in R. norvegicus rats; 15 (16.8%) of 90 rodent samples tested positive for SEOV by reverse transcription PCR. Infection rates at each surveillance time were 26.7% (4/15) in September 2009, 7.5% (3/40) in June 2010, 28.6% (6/21) in September 2010, and 15.4% (2/13) in September 2011. All infected R. norvegicus rats were adults; 9 were male and 6 were female. The rate of nucleotide substitution in these 15 SEOV amplicons (330 bp; GenBank accession nos. KC576788–KC576802, JX853574) was calculated by Bayesian Markov chain Monte Carlo analysis using BEAST 1.74 (7). The mean substitution rate, calculated by using the uncorrelated lognormal distribution relaxed molecular clock model and a Bayesian skyline model for the large segment of SEOV, was 8.27 × 10^{-3} substitutions/site/year, with a 95% high posterior density interval that ranged from 1.02 × 10^{-4} to 1.79 × 10^{-3}. This substitution rate is about 3 times greater than that for middle and small segments (2).

Phylogenetic relationships were assessed by using the uncorrelated lognormal distribution relaxed molecular clock model with the SRD06 substitution model (8) in BEAST 1.74. The Hantaan virus strain AA57 (GenBank accession no. AB620033) sequence was used as the outgroup. The resulting phylogenetic tree (Figure) showed that SEOV strains in the city of Hyesan shared >97.3% identity and were all clustered in their own lineages, subdivided into 2 co-existing sublineages. Although the geographic distance from Hyesan to northeastern China (e.g., Liaoning Province) is much less than that between northeastern and southeastern China (e.g., Zhejiang Province) or central China (e.g., Hubei Province), the phylogenetic distance between SEOV strains in North Korea and those in each location in China in clade A, calculated by using MEGA5.1 (9), was 0.03, but was only 0.01–0.02 between locations in China.

One possible explanation for this discrepancy in phylogenetic and geographic distances between SEOV strains in China and those in North Korea may be differences in the extent of human contact. Although human interactions among different regions of China are extensive, by comparison, those between China and North Korea are considerably reduced for political reasons. In addition, combining with small segment (GenBank accession no. HQ992815) sequence analysis (data not shown), the fact that SEOV strain L0199 from Laos were not clustered in clade A–D(2) showed that Laos was another possible area of origin for SEOV.

Our work contributes to the known epidemiology of exposure to the SEOV pathogen in Hyesan. Hyesan adjoins Changbai County in Jilin Province of China. However, SEOV was not detected in Changbai County during the surveillance program (data not shown), which was consistent with previous research (10). This study further highlights the need for long-term surveillance.

This work was supported partly by the fund for International Science and Technology Cooperation Program of China (grant no.2012DFA30540), the Science and Technology Planned Project of General Administration of Quality Supervision (grant no. 2012IK251), and Infectious Diseases Special Project, Ministry of Health of China (2011ZX10004-001).

Lisi Yao,1 Zhehao Kang,1 Yongxian Liu,1 Fenglin Song1, Xiaolong Zhang, Xiaomei Cao, Yunshu Zhang, Yu Yang, Xiaohong Sun, Jing Wang, Kongxin Hu, Licheng Liu, Weijun Chen, Lijun Shao, Baoliang Xu, and Baolin Wang

Author affiliations: Chinese Academy of Inspection and Quarantine, Beijing, China (L. Yao, X. Zhang, X. Cao, Y. Yang, X. Sun, J. Wang, K. Hu, B. Xu, B. Wang); Ryanggang-do Institution of Commodity Entry-Exit Quarantine, Hyesan, North Korea (Z. Kang); Jilin Entry-Exit Inspection and Quarantine Bureau, Changchun, China (Y. Liu, Y. Zhang, L. Shao); Liaoqing Entry-Exit Inspection and Quarantine Bureau, Dalian, China (F. Song); and Beijing Institute of Genomics, Beijing (L. Liu, W. Chen).

DOI: http://dx.doi.org/10.3202/eid1911.130207

1These authors contributed equally to this article.
LETTERS

References


Schmallenberg Virus Infection in Dogs, France, 2012

To the Editor: In 2011, Schmallenberg virus (SBV) emerged in Europe (1); the virus spread into France in January 2012 (2). During January–March 2012, a total of >1,000 cases were reported in France, mainly in stillborn and newborn lambs with congenital malformations.

In March 2012, neurologic disorders were detected in five 15-day-old puppies (Belgian shepherd) from a dog breeding kennel in northwestern France (Orne). We report data suggesting that these puppies were infected with SBV.

In June 2012, the kennel veterinarian contacted a veterinary school (Unité de Médecine de l’Élevage et du Sport Breeding and Sport Medicine Unit, Maisons-Alfort, France) after neurologic signs of ataxia, exotropia, a
head tilt, and stunted growth were observed in a litter of 5 puppies. Four of the puppies had died at 5–6 weeks of age. The veterinarian collected blood samples from the surviving puppy at 3 months of age, and the puppy was euthanized for necropsy. Severe torticollis was observed during the necropsy, but no other macroscopic signs were detected. The brain, including the cerebellum; a part of the spine; and cerebrospinal fluid (CSF) were collected for further investigation. Specific PCR analyses for canine coronavirus, *Neospora caninum*, *Toxoplasma gondii*, and canine minute virus were performed on CSF; all test results were negative. The brain tissue was fixed in formalin and processed for histologic examination. Features of degenerative encephalopathy, including neuronal vacuolation, neuropil vacuolation, and minimal gliosis, were observed.

Because some clinical signs were evocative of SBV infection and the puppy was born in an area where the virus was circulating actively in cattle and sheep, veterinarians decided to investigate SBV as a possible etiology. Serum samples from the 3-month-old puppy and the dam were tested by virus neutralization test (VNT), according to the protocol used for ruminant assays. The results were negative for the puppy but positive (titer 128) for the mother. Specific competitive SBV ELISA (IDVet, Montpellier, France) against the SBV N protein showed similar results.

Real-time reverse transcription PCR (RT-PCR) was performed (3) to detect the presence of the SBV genome in the cerebellum. Because the sample was paraffin-embedded, RNA was extracted from 5-µm sections, as described (4). All of the extracted cerebellum sections had positive test results (cycle threshold range 33-36); the extraction and PCR controls all showed negative results. To confirm these positive results, conventional RT-PCR was used to amplify a 573-nt sequence of the SBV S segment. The amplification product was sequenced, and a BLAST analysis was performed (www.ncbi.nlm.nih.gov/BLAST). An identity of 100% was obtained with the SBV small gene segment from a ruminant (GenBank accession no. KC108860). An immunohistochemical assay was also performed; the result was negative.

The remaining 7 female dogs in the breeding kennel were tested for SBV in October 2012; 1 showed positive results by VNT (titer 256), which confirmed that SBV was circulating in the kennel. This positive dam had a litter of puppies in December 2012, but no signs developed, and the puppies were not tested. In March 2013, repeat testing was done on serum samples from the 2 dogs that had shown positive results. Results for both animals were positive by VNT (titers 32 for the dam and 128 for the other dog) and ELISA.

Taken together, specific SBV antibodies in the mother and the SBV genome in her puppy suggest that these dogs experienced SBV infection. The absence of detectable SBV antibodies in the puppy in this investigation suggests that transplacental infection occurred before the onset of fetal immune competence. Maternal infection probably occurred in January or February 2012; entomologic monitoring conducted in France showed the presence of *Culicoides* spp. midges, a vector of SBV, during this period in northwestern France. In addition, because the puppies were born in March 2012 and SBV antibodies were still detectable in the mother in March 2013, the duration of SBV antibodies in dogs appears to be ≥1 year. In cattle and sheep, the SBV genome persists in an infected fetus and is detectable after birth by real-time RT-PCR, despite gestation length (5,6).

Few reports on orthobunyavirus infections in dogs are available. Two serologic studies from the United States (7) and Mexico (8) found antibodies against La Crosse virus, South River virus, and Jamestown Canyon virus in dogs. Two other reports described cases in which La Crosse virus was detected in canine littermates who had clinical encephalitis (9) or neurologic disorders (10).

It is unclear if the apparent SBV infection we detected in these dogs was an isolated event or if other cases occurred elsewhere but were not detected because they were not investigated. Further serologic and clinical surveys are needed to estimate SBV prevalence in dogs and the virus’ involvement in the occurrence of neurologic signs in puppies.

**Acknowledgments**

We thank Francine Larangot for collecting biological samples and Muriel Coulpier and Julie Valloire-Lucot for contributing to this study.

This project was funded by ANSES, Royal Canin, and Unité de Médecine de l’Elevage.

Corinne Sailleau, Cassandra Boogaerts, Anne Meyrueix, Eve Laloy, Emmanuel Bréard, Cyril Viarouge, Alexandra Desprat, Damien Vitour, Virginie Doceul, Catherine Boucher, Stéphan Zientara, Alexandra Nicolier, and Dominique Grandjean

Author affiliations: ANSES, Maisons-Alfort, France (C. Sailleau, E. Bréard, C. Viarouge, A. Desprat, D. Vitour, V. Doceul, S. Zientara); Ecole Nationale Vétérinaire d’Alfort, Maisons-Alfort (C. Boogaerts, A. Meyrueix, E. Laloy, D. Grandjean); Royal Canin, Aimargues, France (C. Boucher); and Vet Diagnostics, Lyon, France (A. Nicolier)

DOI: http://dx.doi.org/10.3201/eid1911.130464

**References**

To the Editor: Human infection with a novel low pathogenicity influenza A(H7N9) virus in eastern China has recently raised global public health concerns (1). The geographic sources of infection have yet to be fully clarified, and confirmed human cases from 1 province have not been linked to those from other provinces. While some studies have identified epidemiologic characteristics of subtype H7N9 cases and clinical differences between these cases and cases of highly pathogenic influenza A(H5N1), another avian influenza affecting parts of China (2–4), the spatial epidemiology of human infection with influenza subtypes H7N9 and H5N1 in China has yet to be elucidated. To test the hypothesis of co-distribution of high-risk clusters of both types of infection, we used all available data on human cases in mainland China and investigated the geospatial epidemiologic features.

Data on individual confirmed human cases of influenza (H7N9) during February 19, 2013, through May 17, 2013, and of influenza (H5N1) from October 14, 2005, through May 17, 2013, were included in the analysis. The definitions of these cases have been described (3,5). A total of 129 confirmed cases of influenza (H7N9) (male:female ratio 2.39:1) and 40 confirmed cases of influenza (H5N1) (male:female ratio 0.90:1) were included in the analysis. The median age of persons with influenza (H7N9) was 27 years; z = −7.73; p<0.01). Most (75.0%) persons with influenza (H5N1) had direct contact (e.g., occupational contact) with poultry (including dead and live birds) or their excrement and urine, whereas most (64.3%) persons with influenza (H7N9) had only indirect exposure to live poultry, mainly during visits to live poultry markets.

Reported cases of influenza (H5N1) were distributed over 40 townships in 16 provinces, whereas cases of influenza (H7N9) were relatively more concentrated, in 108 townships but only 10 provinces (Figure). To identify a spatial overlap between the primary cluster of influenza (H7N9) cases, detected in April 2013 (relative risk [RR] 78.40; p<0.01), and the earliest space-time cluster of influenza (H5N1) cases, detected during November 2005–February 2006 (RR 65.27; p<0.01), we used spatio-temporal scan statistics with a maximum spatial cluster size of 5% of the population at risk in the spatial window and a maximum temporal cluster size of 25% of the study period in the temporal window (6) (Figure). The results suggest that the overlap is not perfect and is concentrated around an area southeast of Taihu Lake (south of Jiangsu Province), bordering the provinces of Anhui and Zhejiang. Smaller clusters of influenza (H7N9) cases were identified in the boundary of Jiangsu and Anhui Province (8 cases; RR 64.86; p<0.01) and Jiangxi Province (Nanchang County and Qing-shanhu District) (4 cases; RR 105.67; p<0.01). A small cluster of influenza (H5N1) cases was detected during 2012–2013 along the boundaries of Guanshanhu, Yunyan, and Nanning Counties in Guizhou Province (3 cases; RR 496.60; p<0.01).

In addition, family clustering, defined as ≥2 family members with laboratory-confirmed cases, was found for influenza (H7N9) cases during March–April 2013 in Shanghai and Jiangsu Provinces and for influenza (H5N1) cases during December 2007 in Jiangsu Province.
Family clustering may indicate person-to-person viral transmission or may reflect common exposure to infected poultry or their excrement in the household or in a contaminated environment (7). No evidence supports person-to-person viral transmission as the means of transmission in family clusters.

In conclusion, we found compelling evidence that the high-risk areas for human infection with subtype H7N9 and H5N1 viruses are co-distributed in an area bordering the provinces of Anhui and Zhejiang, which suggests that this area might be a common ground for the transmission of emerging avian influenza viruses in China. We also found that visits to live poultry markets or exposure to contaminated environments are a pathway to infection with influenza (H7N9) virus, whereas infection with influenza (H5N1) is more tied to occupational hazards. These differences may reflect the differences in the pathogenicity of the viruses in poultry, which influences disease progression and identification of clinical signs further down the poultry market chain. Further empirical investigation into our findings could identify risk factors that might be involved in disease transmission to humans in high-risk areas and could help public health authorities develop targeted control and surveillance strategies to prevent disease transmission.

This work was partly supported by grants from National Natural Science Foundation of China (81102169) and National Basic Research Program of China (2012CB955500-955504).

Figure. Geographic and temporal distribution of human cases of infection with avian influenza subtypes H7N9 (circles) and H5N1 (triangles), China. A) Distribution and space-time clusters of human influenza (H7N9) and influenza (H5N1) cases, calculated by using Kulldorff’s scan statistics in SaTScan version 9.1.1 (6). B) Spatial overlap between influenza (H7N9) and influenza (H5N1) case clusters in an area bordering the provinces of Anhui and Zhejiang. C) Primary cluster of influenza (H5N1) cases in Guizhou Province (relative risk [RR] 496.60). D) Secondary cluster of influenza (H5N1) cases in Anhui and Zhejiang Provinces (RR 65.27). E) Primary (RR 78.40) and secondary clusters of influenza (H7N9) cases on the boundary of Jiangsu and Anhui Provinces (RR 64.86) and in Jiangxi Province (RR 105.67).

Liya Wang,1 Wenyi Zhang,1 Ricardo J. Soares Magalhaes,1 Archie C.A. Clements,1 Wenbiao Hu,1 Fan Ding,1 Hailong Sun, Shenlong Li, Qiyong Liu, Zeliang Chen, Yansong Sun, Liuyu Huang, and Cheng-Yi Li

Author affiliations: Institute of Disease Control and Prevention of People’s Liberation Army, Beijing, China (L. Wang, W. Zhang, H. Sun, S. Li, Z. Chen, Y. Sun, L. Huang, C.-Y. Li); University of Queensland, Brisbane, Queensland, Australia (R.J. Soares Magalhaes, A.C.A. Clements, W. Hu); and Chinese Center for Disease Control and Prevention, Beijing (F. Ding, Q. Liu)

DOI: http://dx.doi.org/10.3201/eid1911.130815

1These authors contributed equally to this article.
NewVariantofRabbitHemorrhagicDiseaseVirus,Portugal,2012–2013

To the Editor: During November 2012–February 2013, rabbit hemorrhagic disease virus (RHDV) strains belonging to the new variant RHDV were isolated in Portugal from wild European rabbits (Oryctolagus cuniculus subsp. aligerus). The major capsid protein, VP60, of these strains was partially characterized. RHDV had been previously detected in Portugal in 1989 (1). Before 2011, RHDV outbreaks in wild European rabbit (O. cuniculus) populations in the Iberian Peninsula were exclusively caused by strains belonging to genogroup 1 (2,3).

In the Iberian Peninsula, 2 subspecies of European rabbit are found, O. cuniculus subsp. aligerus and O. cuniculus subsp. cuniculus. These subspecies are equally susceptible to RHDV (3). In 2011, a new variant was isolated in young rabbits belonging to O. cuniculus subsp. cuniculus from a rabitry in the province of Navarra, Spain (4). The topology of the phylogenetic tree that included this variant and the susceptibility of kits <2 months old suggest that this strain is similar to that described in France in 2010 (5).

Before the new variant of RHDV emerged and, on the basis of phylolgetic relationships, RHDV strains had been divided into 6 genogroups (G1–G6) (1), with strains of G6, or RHD-Va, having a distinct antigenic profile (6). All of these strains replicate in the liver and are responsible for causing death in rabbits >2 months of age. Nonpathogenic and weakly pathogenic RHDV-related strains have also been described. The nonpathogenic and weakly pathogenic strains are phylogenetically distinct from the G1–G6 strains with ≈20% of nucleotide divergence (7); they typically replicate in the intestines (8,9). New variant RHDV causes death in kits as young as 30 days old and affects vaccinated and unvaccinated animals (4). Phylogenetically, this new variant falls between the nonpathogenic groups (4,5). During November 2012–February 2013, our laboratory, CIBIO, Universidade do Porto, Portugal, received liver samples from wild adult rabbits and kits, belonging to O. cuniculus subsp. aligerus, from 3 areas of Portugal, Valpaços, Barrancos, and Algarve. The rabbits had appeared dead and had clinical signs suggesting rabbit hemorrhagic disease (RHD). We analyzed the samples for RHDV by reverse transcription PCR. For this process, total RNA was extracted by using the RNasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Reverse transcription was performed by using oligo(dT) as primer (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcription (Invitrogen) as recommended by the manufacturer. Screening of the samples consisted of PCR with a pair of primers as described by Dalton et al. (4). This pair amplifies a 738-bp fragment of the gene encoding the capsid protein, VP60 (PCR conditions are available on request). After purification, PCR products were sequenced on an automated sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the same pair of primers.

The virus was detected in 15 samples, 5 from each locality. The obtained sequences were aligned with those available from public databases. Retrieved sequences represent the RHDV groups G1–G6, the nonpathogenic groups, and the new variant (GenBank accession nos. KF442960–KF442964). A phylogenetic tree was inferred in MEGA5 (10) by using a maximum-likelihood (ML) approach. Reliability of the nodes was assessed with a bootstrap resampling procedure consisting of 500 replicates of the ML trees. The best-fit nucleotide substitution model was determined by using MEGA5.


References

Address for correspondence: Cheng-Yi Li, Institute of Disease Control and Prevention, 20 Dong-Da St, Fengtai District, Beijing 100071, People’s Republic of China; email: licy_60@163.com
Our sequences exhibit the highest nucleotide sequence identity with the RHDV N11 strain from Spain (99%; GenBank accession no. JX133161.1), which corresponds to the new RHDV variant. Thirteen nucleotide substitutions were detected in comparison to the Spanish sequence, 3 of which were nonsynonymous. The inferred ML phylogenetic tree is in agreement with those published (1,3,9). G1–G6 (pathogenic) RHDV strains and nonpathogenic and weakly pathogenic RHDV-related strains (generally referred to as RCV) form 2 groups (Figure). The nonpathogenic strain from Australia (RCV-A1_Australia_MIC-07) does not cluster with other nonpathogenic groups and European brown hare syndrome virus (EBHSV_France) appears in a basal position in the tree. As described, the new variant (N11_Spain) appears between RCV and the nonpathogenic Australian strain (4,5). The strains isolated from rabbits in Portugal cluster with the new variant and form a highly supported group (bootstrap value 1.00). These results support the conclusion that the virus recovered in Portugal belongs to the new variant RHDV described in Spain and France.

This confirms the presence of the virus in wild rabbits on the Iberian Peninsula. We also confirm that both European rabbit subspecies are susceptible to the new variant. The appearance and rapid spread of the new variant RHDV into the Iberian wild rabbit populations raise concern for the survival of these populations in this region. These conservation concerns are particular highlighted for the O. cuniculus subsp. algirus, because it only occurs in the southwestern part of the Iberian Peninsula, and it is a key prey species for several carnivores, namely, for the most endangered feline, the Iberian Lynx (Lynx pardinus). Therefore, monitoring the spread and evolution of this new variant is crucial in determining the most appropriate conservation measures.

Acknowledgments

The samples from Valpaços and Algarve were provided by Jorge Pires and Vitor Palmilha, respectively.

The Portuguese Foundation for Science and Technology supported the doctoral fellowship of A.M.L. (SFRH/BD/78738/2011) and the postdoctoral fellowship of J.A. (SFRH/BPD/73512/2010). The Portuguese Foundation for Science and Technology Projects PTDC/CVT/108490/2008 and FCT-ANR/ BIA-BIC/0043/2012 supported this work. Also, project “Genomics Applied to Genetic Resources,” cofinanced by North Portugal Regional Operational Programme 2007/2013 (ON.2-O Novo Norte), under the National Strategic Reference Framework, through the European Regional Development Fund, supported this work. F.P. and K.P.D. gratefully acknowledge the financial support of Organização Interprofesional Cunicola.
Mycobacterium yongonense in Pulmonary Disease, Italy

To the Editor: Mycobacterium yongonense is a recently described species (1) that belongs to the M. avium complex (MAC) and is associated with pulmonary infection. The strain on which the description of species was based was isolated in South Korea from the sputum of a patient with unspecified pulmonary disease. We describe 2 M. yongonense strains isolated from patients in Italy.

Patient 1 was a 74-year-old woman who had experienced fatigue, diarrhea, and weight loss. Her medical history included liver cirrhosis resulting from hepatitis C virus infection and surgery for colon cancer; the patient also reported tuberculosis in childhood. Chest radiograph revealed a cavitary lesion, a finding confirmed by computed tomography scan (Figure). Cultures in liquid and solid media grew a nonchromogenic mycobacterium from sputum and stool samples; results were negative for urine samples.

The patient was treated with clarithromycin, rifabutin, and ethambutol and showed some improvement. A bronchoscopic investigation was performed, and microscopic examination of bronchoalveolar lavage samples revealed the presence of acid-fast bacilli that subsequently were grown in culture. The patient began improving markedly starting with the second month of treatment, which will be continued for a total of 18 months.

Patient 2 was a 74-year-old woman, living in a community of nuns, who reported cough and dyspnea. Her medical history included renal failure and surgery for breast cancer. A bronchoalveolar lavage was performed; samples yielded in culture Pseudomonas aeruginosa and a nonchromogenic mycobacterium.

### References


### Address for correspondence: Pedro J. Esteves, CIBIO-UP, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, InBIO, Laboratório Associado, Campus Agrário de Vairão, Rua Padre Armando Quintas, Vairão, Portugal; email: pjesteves@cibio.up.pt

---

**Letters**

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article’s publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.
The patient was treated with cefepime, to which *P. aeruginosa* was susceptible in vitro, and rapidly improved. The isolation of the nontuberculous mycobacterium was considered irrelevant, and no specific treatment was undertaken.

To determine the specific mycobacteria species isolated from these patients, we conducted a commercial line-probe assay (GenoType Mycobacterium CM; Hain Lifesciences, Nehren, Germany). Both strains were identified as *M. intracellulare*. However, the known cross-reaction of *M. intracellulare* probe with most MAC species (2) led us to determine the complete sequence of the 16S rRNA gene. Both strains showed 100% similarity with *M. yongonense* and *M. marseillense* (3) strains.

To confirm this unusual finding, we investigated other genetic regions. We detected 100% identity with *M. yongonense* in the internal transcribed spacer 1 region and in a 1,384-bp region of the *hsp65* gene and found 2 mismatches in a 420-bp fragment of the *sodA* gene (99.5% similarity). In contrast, *M. marseillense* showed 6 mismatches (98.6% similarity) in the internal transcribed spacer 1 region and 24 (98.3% similarity) in *hsp65*; no *sodA* sequence is available in GenBank for this species. Partial sequencing of other genetic targets not available in GenBank for *M. yongonense* enabled us to confirm the close relatedness of the strains to *M. intracellulare* (100% similarity in *dnaK* gene; 99.3% identity in *gyrB* and *gyrC* genes).

The finding of the same novel *Mycobacterium* species in these 2 unrelated patients reflects variability in the significance of nontuberculous mycobacteria isolated from clinical specimens. *M. yongonense* was probably a contaminant in the second case, but in the first, its involvement as causative agent of disease seems incontrovertible. The specific criteria of the American Thoracic Society (4) were fulfilled: radiographic imaging clearly documented the presence of a cavitary pulmonary lesion, no other pathogen possibly responsible of disease was detected by bronchoscopic investigation, and the same mycobacterium was isolated repeatedly from sputum (its presence in stool probably results from swallowed sputum) and bronchoalveolar lavage samples. Confirmation is further provided by the response to the specific therapy, according to international guidelines (4,5), for MAC pulmonary disease (MICs were 2, 1, and 8 µg/mL for clarithromycin, rifabutin, and ethambutol, respectively).

The initial description of *M. yongonense* noted that it has a distinct *rpoB* sequence (1), identical to that of a distantly related scotochromogenic species, *M. parascrofulaceum*. In a more recent article (6), the same authors investigated 2 more strains of *M. yongonense* with similar characteristics and suggested that the recent acquisition of the *rpoB* gene resulted from a lateral gene transfer event from *M. parascrofulaceum*. The *rpoB* genes of the strains we investigated, however, were substantially different from that of *M. scrofulaceum* and were instead related to that of *M. intracellulare* (99.4% similarity) and, less closely, to that of other species belonging to the MAC, including *M. marseillense* (97.4%). Discrepancy in the *rpoB* sequence means some uncertainty remains that our strains are *M. yongonense*, but the 100% identity in major phylogenetically relevant regions strongly supports this hypothesis and suggests the possibility of a variant of the species preceding the acquisition of the *rpoB* gene from *M. parascrofulaceum*. Less evidence exists for identifying the strains as *M. marseillense* because of the clear divergence in the genes investigated, other than 16S rRNA.

The complete epidemiology of *M. youngonense* is unknown, in part because few strains have been identified. However, as in the cases we describe, use of suboptimal identification methods may mean that some isolates have been misidentified as other mycobacteria species.
GenBank accession numbers for the *M. yongonense* strains identified in this study (FI-13004 and FI-13005) are KF224989–KF224999.

Enrico Tortoli,
Alessandro Mariottini,
Piera Pierotti,
Tullia M. Simonetti,
and Gian Maria Rossolini

Author affiliations: San Raffaele Scientific Institute, Milan, Italy (E. Tortoli); Careggi University Hospital, Florence, Italy (A. Mariottini, M.T. Simonetti, G.M. Rossolini); SS. Maria Annunziata Hospital, Florence (P. Pierotti); University of Florence, Florence (G.M. Rossolini); and University of Siena, Siena, Italy (G.M. Rossolini)

DOI: http://dx.doi.org/10.3201/eid1911.130911

References


Address for correspondence: Enrico Tortoli, Emerging Bacterial Pathogens Unit, San Gabriele Bldg, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy; email: tortoli.enrico@hsr.it

---

**Subcutaneous Infection with Dirofilaria spp. Nematode in Human, France**

To the Editor: The article by Foissac et al. titled Subcutaneous infection with *Dirofilaria immitis* nematode in human, France (1) presents an interesting and challenging diagnostic dilemma. The paper described, but did not illustrate, the worm as having a strongly ridged external surface of the cuticle—a feature known not to exist on *Dirofilaria immitis*, the dog heartworm. However, molecular sequencing of the specimen demonstrated much closer similarity to *D. immitis* than to *D. repens*, the most common cause of zoonotic subcutaneous dirofilariasis infection in Europe.

Well-described morphologic features of parasites, including in tissue sections, have long been the standard for diagnosis. More recently, molecular diagnostics have helped in many of these difficult cases. However, in some cases, the morphology and molecular diagnosis are discordant. On the basis of the data in the article, the worm does not seem to represent *D. repens*. A more likely possibility is some other species for which no sequences are yet available for comparison. In such a worm, the regions sequenced must be similar to *D. immitis*, and distinct from *D. repens*, to achieve the observed results.

When one encounters a case such as this, where well-validated morphologic features (Figure) are contradictory to the molecular analysis, one must exercise caution in arriving at a final diagnosis. One disadvantage

---

Figure. Cross-section of the filarial nematode seen in the subcutaneous nodule on the thigh of a woman in France. The features, as described in the original report (1), include prominent, longitudinal ridging of the cuticle (arrows), 2 reproductive tubes, and the intestine (asterisk). Scale bar indicates 50 µm. Image courtesy of Jean-Philippe Dales.
of morphologic and molecular diagnostics is an absence of information on poorly described and characterized pathogens or new pathogens that have yet to be identified. No good algorithm exists to resolve these conflicts other than to explore all possibilities. The diagnosis in the described case is probably best left as a *Dirofilaria* species of the *Dirofilaria* (*Nochtilla*) type, members of which exhibit marked cuticular ridging, and not *D. (Dirofilaria) immitis* type, members of which have as a feature an absence of cuticular ridging.

Mark L. Eberhard
Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: http://dx.doi.org/10.3201/eid1911.130606

Reference


In Response: We agree with Eberhard (1) that it is difficult to make a species identification when data derived from morphologic examinations do not correlate with those of molecular diagnostics. Errors may be the result of poor indexing of sequences deposited in sequence databases or inaccurate estimation of the degree of genomic polymorphisms within a species and between closely related species. On the other hand, a morphologic difference between 2 organisms, if it is associated with only 1 characteristic, should not be considered sufficient to classify them as 2 distinct species. Such a phenotypic variation may be the result of a single mutation or deletion. Consequently, the absence of a certain character does not exclude the categorization of an organism as a given species.

Molecular identification of the *Dirofilaria* spp. worm in our clinical case was made on the basis of 2 distinct sequences, each of which exhibited marked differences between *D. immitis* and *D. repens* (2). The first sequence targeted internal transcribed spacer regions of ribosomal genes and revealed up to 100% homology with *D. immitis* sequences from GenBank, whereas a maximum homology of 80% was observed with *D. repens* sequences from GenBank. The second sequence targeted the cytochrome oxidase 1 gene and showed 100% homology with *D. immitis*, whereas >90% homology was observed for *D. repens*. For both analyzed targets, GenBank contained several sequences for *D. immitis* and *D. repens* that were deposited by various investigators, and all sequences yielded consistent results. Therefore, there is no basis to suggest that the sequences deposited in GenBank were incorrect.

Nevertheless, we agree that an alternate hypothesis is possible. The worm reported in our article could conceivably belong to a species that differs slightly from both *D. immitis* and *D. repens*, displaying morphologic similarities with *D. repens* but being more closely associated with *D. immitis* at the genomic level.

Charles Mary, Maud Foissac, Matthieu Million, Philippe Parola, and Renaud Piarroux
Author affiliations: Assistance Publique–Hôpitaux de Marseille, Marseille, France (C. Mary, M. Foissac, M. Million, P. Parola, R. Piarroux); and Aix-Marseille Université, Marseille (C. Mary, M. Foissac, M. Million, P. Parola)

DOI: http://dx.doi.org/10.3201/eid1911.131176


Address for correspondence: Charles Mary, Laboratoire de Parasitologie, Hôpital de la Timone, 264 Rue Saint Pierre, 13385 Marseille, France; email: cmary@ap-hm.fr
**Cytomegaloviruses: From Molecular Pathogenesis to Intervention**

Matthias J. Reddehase, editor
(with the assistance of Niels A.W. Lemmermann)
Caister Academic Press, London, United Kingdom, 2013
Pages: 1,046; Price: US $600

Many health professionals rely on journal articles to keep up with advances in their field because textbooks are often 1–2 years out of date by the time they come to press, and are far more expensive than the occasional PDF downloaded from a university library Web site. This 2-volume text on cytomegalovirus (CMV) is costly and cites data from before 2012, but provides a solid foundation on which to apply new findings. Volume I is mainly focused on the basic science of and related animal experiments on CMV; volume II is aimed at the clinical reader, again with chapters on relevant animal model studies. Each chapter reads as a short review article, and is easily digestible. Many well-recognized experts in this field contributed content, which should be reassuring to the reader. The text and referencing style are easy to read and the figures and tables are illustrative and helpful. The volumes come in a compact size, making them convenient to carry, and also are downloadable as eBooks.

Volume I gives in-depth overviews of primate and murine CMVs, CMV metabolomics, miRNAs, and proteomics. Most of the chapters are dedicated to viral gene expression and function and virus interaction with human host cells, describing immune response, aspects of viral tropism, entry, pathogenesis, and latency. The terminology used in Volume I is specialized and may be difficult for readers who do not work in these fields.

Volume II covers essential clinical background: the epidemiology of CMV infections in pregnancy, CMV infections in solid and bone marrow transplants, CMV therapy and drug resistance, diagnostic methods, and vaccine development. Additional chapters describe the host immune response to CMV infection, and mechanisms of infection in specific targets, such as the placenta.

Chapters in both volumes are detailed and clearly written; the editors are to be commended on maintaining this standard. However, additional detail and discussion (i.e., pros and cons) about alternative targets for CMV PCR monitoring of transplant patients, such as pp65 antigen and pp67 mRNA versus DNA, would have been helpful. Also, an in-depth chapter on the characteristics of reinfec ting or superinfecting strains of CMV in various clinical situations would be useful; such strains are frequently referred to without description throughout the clinical text. Perhaps one surprising omission on the clinical side is a chapter comparing and contrasting the various clinical guidelines for the treatment of CMV infections in transplant patients; this would highlight differences in how published data are interpreted.

Chapter II.23, Putative Disease Associations with Cytomegalovirus: a Critical Survey, explores the possible role of CMV as an etiologic agent for specific clinical syndromes, including glioblastomamultiforme, cardiovascular disease, and the role that CMV may play in immunosenescence. This makes fascinating and educational reading, especially in how the authors tease out the relevant (and irrelevant) evidence for and against these potential etiologic roles.

The price tag is considerable, although the 2 volumes can be purchased separately (http://www.horizonpress.com/hsp/supplementary/cmv2/vol1-vol2.html). As a medical–clinical virologist, I find Volume II to be a useful reference text. Those working on the basic virology of CMV may consider Volume I a useful addition to their libraries. These volumes give comprehensive, yet succinct overviews of the current state of knowledge of many aspects of CMV and are detailed enough to satisfy most readers.

**Julian Tang**

Author affiliation: Alberta Provincial Laboratory for Public Health, Edmonton, Alberta, Canada.

DOI: http://dx.doi.org/10.3201/eid1911.131226

Address for correspondence: Julian Tang, Alberta Provincial Laboratory for Public Health–Virology, Rm 2B1.03; 8440 112 St, Walter McKenzie Health Sciences Bldg, University of Alberta Hospital, Edmonton, Alberta T6G 2J2, Canada; email: julian.tang@albertahealthservices.ca

---

**CME**

Sign up to receive email announcements when a new article is available.

Get an online subscription at wwwnc.cdc.gov/eid/subscribe.htm
Keep those journals coming to your mailbox...

Renew your subscription today!

EMERGING INFECTIOUS DISEASES®

☑ YES, I would like to receive Emerging Infectious Diseases. Please add me to your mailing list.

Return:
Email: eideditor@cdc.gov
Fax: 404 639-1954
Mail to:
EID Editor
CDC/NCID/MS D61
1600 Clifton Rd, NE
Atlanta, GA 30333

Number on mailing label (required)
Name:
Full mailing address: (BLOCK LETTERS)

Full text free online at www.cdc.gov/eid
Sweet wine, unblended, served Odysseus well in the escape with his companions from the Cyclops’ cave during his epic return to Ithaca. The wine, a gift from Maron, grandson of Dionysus, was exceptional. “When he drank it he mixed twenty parts of water to one of wine, and yet the fragrance from the mixing bowl was so exquisite that it was impossible to refrain from drinking.” Odysseus carried a large skin with this wine, in case on the way he had to deal with some unknown savage of great strength who “would respect neither right nor law.”

Mixing wine with water before drinking was a mark of civilized behavior in ancient Athens and an essential feature of the symposium, a gathering in which drinking together was intertwined with conducting business. The practice spawned a line of equipment for transporting, mixing, and consuming wine. One such implement was the krater, a vessel in which wine was diluted to the right consistency for drinking. Kraters, often too large to be used for serving, were positioned in the center of a room and sometimes were decorated with images of symposium proceedings.

Athenian pottery was common in the Mediterranean region as far back as 2,800 years ago. Clay vessels of different sizes, shapes, and uses were widely traded. And while little painting of that period has survived, even on stone, intact and fragmented painted vases from various locations abound because they were exceptionally durable, more so than metal. As a result, they became a repository, not only of painting but of religious and social norms and daily activities, from raising children to burying the dead. Historical and mythologic scenes, also used heavily, provided the opportunity to inject life scenes with life lessons.

Pottery was difficult work half art, half magic. Clay pots were created in workshops, usually led by master potters, who knew how to manipulate kiln temperature by letting air in and out at different times, never knowing for certain how the final product would turn out. Many pots were clearly signed. Others are recognizable as products of a certain workshop or area known for a distinctive style. The potter was not necessarily the painter, although many times they were one and the same. The painter who created the krater on this month’s cover was named after Sappho, the famous poet of antiquity, a popular subject for artistic representation. His name, “the Sappho painter,” came from a vase, now in a museum in Warsaw, showing perhaps the oldest portrait of Sappho, a woman playing a long-armed lyre. The name “Sappho” is incised next to the figure.

The black-figure technique, featured on the krater on this month’s cover, evolved from earlier geometric designs in Corinth and then Athens. The surface of the pot was covered with a black pigment on which details were incised that would turn red in the final stage of firing. Certain conventions prevailed in the painted scenes. Figures were flat, although not entirely without perspective. Faces were shown in profile, young men generally beardless,
older women heavyset. Inscriptions floated conspicuously in-between figures.

Odysseus Escaping from the Cave of Polyphemos is the image on a wide mouth black-figure krater with two columnlike handles. The theme was a popular one: a famous hero set against Polyphemos—“A horrid creature, not like a human being at all, but resembling rather some crag that stands out boldly against the sky on the top of a high mountain.” The tired Odysseus and his crew found the monster’s cave. “His cheese-racks were loaded with cheeses, and he had more lambs and kids than his pens could hold.” But what he had in prosperity, Polyphemos lacked in hospitality. “The cruel wretch … gripped up two of my men at once and dashed them down upon the ground as though they had been puppies. Their brains were shed upon the ground, and the earth was wet with their blood. Then he tore them limb to limb and supped upon them.”

Homer’s hero realized that extraordinary measures would be needed for him and his companions to get out of the monster’s cave alive. “Look here, Cyclops … you’ve been eating a great deal of man’s flesh, so take this and drink some wine.” The undiluted potion had the anticipated effect. “This drinks like nectar and ambrosia all in one,” the Cyclops exclaimed. “Be so kind … as to give me some more and tell me your name at once.” Odysseus obliged. “My name is Nobody … This is what my father and mother and my friends have always called me.”

Drunk and sick, the monster fell “backwards and a deep sleep took hold upon him.” Odysseus and his men then thrust a burning beam of wood into the monster’s eye “till the boiling blood bubbled all over it as we worked it round and round.” The Cyclops cried and shouted “in a frenzy of rage and pain,” alerting his friends that Nobody was killing him, “by fraud or by force.” For Odysseus and his friends, the problem now was how to get out of the cave when Polyphemos moved the huge bolder to let the sheep out in the morning.

“The male sheep were well grown and carried a heavy black fleece, so I bound them noiselessly in threes together … there was to be a man under the middle sheep, and the two on either side.” And, “There was a ram finer than any of the others, so I caught hold of him by the back, ensconced myself in the thick wool under his belly, and flung on patiently to his fleece, face upwards, keeping a firm hold on it all the time.”

On the krater depicting the story, the faces betray no emotion. Polyphemos has just lost his vision. “Nobody,” tied with rope under the ram and clinging for dear life, awaits the outcome of his daring escapade. The ram receives a tender stroke from his master. “My good ram, what is it that makes you the last to leave my cave this morning?”

Outside the cave at last, his men safely on the ship, Odysseus cannot contain himself. “Like a craftsman, I had to leave my name on my handiwork,” he shouts. “Cyclops … If anyone asks you who it was that put your eye out and spoiled your beauty, say it was the valiant warrior Odysseus, son of Laertes, who lives in Ithaca.”

In the times of Homer, monsters lived in caves, the mountains, and the sea. They symbolized humans’ worst fears—being eaten was one of them. “He gobbled them up like a lion in the wilderness, flesh, bones, marrow, and entrails, without leaving anything uneaten.” There was a clear dichotomy. Cyclopes were bad, sheep good. Cyclopes ate humans. Humans ate sheep and shared food and wine with others.

In our times, the landscape of monsters has expanded to include the dark side within us and even within sheep. In the absence of the mythical Cyclopes, humans can still be eaten, now by disease, which sometimes devours the brain, disabling and finally destroying the body. Unbeknown to Odysseus, his escape vessel, Polyphemos’ prized sheep, could destroy him if its self-replicating prions could be transmitted to humans. For BSE, a prion disease of domestic cattle, good evidence exists from natural exposure and laboratory studies that this could happen. For scrapie, a prion disease of sheep, the evidence is not there, although the risk remains unknown.

Like pottery making, models of disease are fraught with uncertainties. They can be influenced by subtle differences in prion strains from different species of animals. The difficulty lies in demonstrating human susceptibilities in animal models. However, animals are not humans, humans are not identical to each other, and small risk is difficult to demonstrate statistically. We cannot tell if atypical scrapie prions cause human disease because, despite the names we ascribe to atypical proteins, we do not know who they are or what they are capable of until, like Odysseus, they finally announce themselves.

Bibliography


Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: pmpl1@cdc.gov
Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association’s Physician’s Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title
Tropheryma whipplei Endocarditis

CME Questions

1. Which of the following statements regarding epidemiologic factors among patients with Tropheryma whipplei endocarditis in the current study is most accurate?
   A. All patients were male
   B. Most patients acquired the infection in Africa or Asia
   C. Nearly all patients were older than 70 years
   D. Most patients had previous cardiac valve disease

2. What was the most common presenting symptom of T. whipplei endocarditis in the current study?
   A. Fever
   B. Neurologic complications of stroke
   C. Arthralgias
   D. Weight loss

3. Which of the following statements regarding laboratory findings among patients with T. whipplei endocarditis is most accurate?
   A. C-reactive protein levels were normal in most patients
   B. Nearly all patients had positive findings on heart valve analysis for T. whipplei
   C. Nocardia spp. were frequently found as co-infections with T. whipplei
   D. Nearly all saliva and fecal samples were positive for T. whipplei

4. What other findings were present among patients with T. whipplei endocarditis in the current study?
   A. Valves infected with T. whipplei demonstrated rare signs of inflammation
   B. Valvular infiltrates consisted primarily of neutrophils
   C. Most patients had a positive serological profile for classic Whipple disease
   D. There was wide variability in the genotypes represented

Activity Evaluation

| 1. The activity supported the learning objectives. | Strongly Disagree | 1 | 2 | 3 | 4 | 5 | Strongly Agree |
| 2. The material was organized clearly for learning to occur. | Strongly Disagree | 1 | 2 | 3 | 4 | 5 | Strongly Agree |
| 3. The content learned from this activity will impact my practice. | Strongly Disagree | 1 | 2 | 3 | 4 | 5 | Strongly Agree |
| 4. The activity was presented objectively and free of commercial bias. | Strongly Disagree | 1 | 2 | 3 | 4 | 5 | Strongly Agree |
Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association’s Physician’s Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title
Possible Association between Obesity and Clostridium difficile Infection

CME Questions

1. You are seeing a 40-year-old woman with diarrhea and fever for the past 3 days. You are concerned regarding the possibility of Clostridium difficile infection (CDI). The patient also reports that she has had increasing abdominal pain for the past year, and you consider whether she has inflammatory bowel disease (IBD). Which of the following statements is most accurate regarding the relationship between IBD and CDI?
   A. IBD is associated with increased morbidity and mortality associated with CDI
   B. Most patients with IBD acquire CDI in inpatient settings
   C. CDI generally develops more slowly after hospital admission among patients with IBD compared with patients without IBD
   D. IBD does not affect the risk of antibiotic exposure

2. Which of the following factors was significantly more common in the healthcare-onset (HO) vs community-onset (CO) groups in the current study?
   A. Obesity
   B. Antibiotic use
   C. Diabetes
   D. End-stage renal disease

3. How did the CO cohort most differ from the CO–healthcare facility–associated (HCFA) cohort in the current study?
   A. The CO cohort was older
   B. The CO cohort was more likely to use acid suppressive medications
   C. The CO cohort was more likely to be obese
   D. The CO cohort was more likely to be male

4. Which group of patients had the highest rate of IBD in the current study?
   A. CO
   B. CO-HCFA
   C. HO
   D. CO-HCFA and HO

Activity Evaluation

1. The activity supported the learning objectives.
   Strongly Disagree  2  3  4  Strongly Agree
   1  2  3  4  5

2. The material was organized clearly for learning to occur.
   Strongly Disagree
   1  2  3  4  Strongly Agree
   1  2  3  4  5

3. The content learned from this activity will impact my practice.
   Strongly Disagree
   1  2  3  4  Strongly Agree
   1  2  3  4  5

4. The activity was presented objectively and free of commercial bias.
   Strongly Disagree
   1  2  3  4  Strongly Agree
   1  2  3  4  5
NEWS & NOTES

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Review of Institute of Medicine and National Research Council Recommendations for One Health Initiative
Potential Role of Deer Tick Virus in Powassan Encephalitis Cases in Lyme Disease–endemic Areas of New York, USA
Twenty-Year Summary of Surveillance for Human Hantavirus Infections, United States
Spontaneous Generation of Infectious Prion Disease in Transgenic Mice
Antiviral Susceptibility of Highly Pathogenic Avian Influenza A(H5N1) Viruses Isolated from Poultry, Vietnam, 2009–2011
Powassan Virus in Mammals, Alaska and New Mexico, USA, and Russia, 2004–2007
Rift Valley Fever in Namibia, 2010
Reemergence of Vaccinia Virus during Zoonotic Outbreak, Pará State, Brazil
Outbreak of Human Infection with Sarcocystis nesbitti, Malaysia, 2012
Distinct Lineage of Vesiculovirus from Big Brown Bats, Maryland, USA
Acute Toxoplasma gondii Infection among Family Members, United States
Surveillance for Avian Influenza A(H7N9), Beijing, China, 2013
Historic Prevalence and Distribution of Avian Influenza Virus A(H7N9) among Wild Birds
Novel Variants of Clade 2.3.4 Highly Pathogenic Avian Influenza Viruses, China
Lack of MERS Coronavirus Neutralizing Antibodies in Humans, Eastern Province, Saudi Arabia
Novel Orthoreovirus from Mink, China, 2011
Novel Cause of Tuberculosis in Meerkats, South Africa
Cerebellar Cysticercosis Caused by Larval Taenia crassiceps Tapeworm in Immunocompetent Woman, Germany
Novel Hepatitis E Virus in Farmed Mink, Denmark
Novel Reassortant Influenza A(H1N2) Virus Derived from A(H1N1)pdm09 Virus Isolated from Swine, Japan
Myocarditis after Trimethoprim-Sulfamethoxazole Treatment for Ehrlichiosis

Complete list of articles in the December issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 2–6, 2013
APHA
American Public Health Association’s 141st Annual Meeting and Exposition
Boston, MA, USA
http://www.apha.org

November 4–7, 2013
3rd ASM-ESCMID
Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications
Copenhagen, Denmark
http://www.asm.org/conferences

November 5–7, 2013
ESCAIDE 2013
European Scientific Conference on Applied Infectious Disease Epidemiology
Stockholm, Sweden
http://www.escaide.eu

November 13-17, 2013
ASTMH–American Society of Tropical Medicine and Hygiene
https://www.astmh.org

November 30–December 4, 2013
ASLM
African Society for Laboratory Medicine
Innovation and Integration of Laboratory and Clinical Systems
Cape Town, South Africa
http://www.ASLM2014.org

April 2-5, 2014
16th International Congress on Infectious Diseases
Cape Town, South Africa
http://www.isid.org/icid/

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

1912 Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 19, No. 11, November 2013
Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination. The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

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

Keywords. 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) t if 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. A short abstract (150 words), 1-sentence summary, and biographical sketch. 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. 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. 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. 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 short abstract (150 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 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.

Etymology. Etymology (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.