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We describe the frequency, demographic and clinical features, and visual outcomes of ocular syphilis infections observed during 2012–2015 at a tertiary reference center in Paris, France. Twenty-one cases (29 eyes) were identified. The occurrence of ocular syphilis increased from 1 case in 2012 to 5 cases in 2013, 6 cases in 2014, and 9 cases in 2015 (2.22–25.21/1,000 individual patients/year for the period). Among case-patients, an annual 20%–33% were co-infected with HIV. Seventy-six percent of ocular syphilis infections occurred in men who have sex with men. Seventy-five percent of case-patients had a good final visual outcome (best-corrected visual acuity >0.3 logMAR score). Visual outcome was worse for HIV-positive patients than for HIV-negative patients (p = 0.0139). At follow-up, the best visual outcomes were observed in patients whose mean time from first ocular symptom to consultation was 15 days (SD ±19 days).

Syphilis is a sexually transmitted disease caused by the bacterium Treponema pallidum, which can infect almost any part of the body (1). Depending on the stage of the disease, acquired syphilis is classified into early (primary, secondary, and early latent) syphilis and late (or tertiary) syphilis (2,3). Although most cases of ocular syphilis occur in the context of tertiary syphilis, approximately one third of reported cases occur in the context of primary and secondary syphilis (4,5). Uveitis is observed in 0.6%–2.0% of patients with syphilis in any stage and up to 9% in patients co-infected with syphilis and HIV (HIV) (6–10). Syphilitic uveitis is considered a great mimicker because it can manifest as anterior uveitis, posterior uveitis, or panuveitis (11–13). Failure to recognize the ocular manifestations of syphilis or delayed treatment can lead to irreversible visual loss (14,15).

Syphilis has recently reemerged in the Western world in the context of the HIV crisis and massive sexual behavior changes among men who have sex with men (MSM). In 2015, nearly two thirds (62%) of the syphilis cases with information on transmission category were reported in MSM (16).

In this context, we set up a retrospective study to review ocular syphilis cases observed during 2012–2015 in a tertiary reference center in Paris, France. Our objectives were to analyze trends in ocular syphilis frequency, demographic characteristics, clinical severity, and patient outcome.

Methods
We reviewed the medical records of patients examined at the uveitis clinic (Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts, Paris, France) from January 2012 through December 2015. We retrospectively identified patients with a new diagnosis of ocular syphilis. During the study period, 1,493 new patients were examined for uveitis. Patients meeting all the following criteria...
were included: age ≥18 years, having intraocular inflammation specifically affecting the uvea, and having a syphilis infection confirmed by the Venereal Disease Research Laboratory (VDRL) test. We defined a positive serologic test result as a positive T. pallidum hemaggulination assay (TPHA) and a positive VDRL test result in accordance with the classification of syphilis (17,18). We used a standardized strategy to diagnose syphilis (19), including anti-Treponema antibody detection in all patients in whom uveitis was diagnosed, with additional tests guided by the clinical context and paraclinical findings.

We reviewed files to collect the following data: demographic information, including MSM status (in case of sexually transmitted disease, sexual orientation was recorded in the patient’s confidential file according to national ethics guidelines); duration of symptoms before diagnosis; and history of extraophthalmologic signs. Ophthalmologic examination included the measurement of the best-corrected visual acuity (BCVA) at initial presentation, at 15 days, and at the last follow-up. We converted BCVA to a visual acuity (VA) score on the basis of the logarithm of the minimum angle of resolution (logMAR).

We examined results from the slit lamp examination (in the anterior segment of the eye) and the fundus examination. We used the most informative imaging modality in patients with retinal inflammation, fundus fluorescein angiography, to report characteristic features of retinal vasculitis, optic nerve head inflammation, and macular edema. We used other fundus imaging studies such as indocyanine green angiography to assist in cases of choroidal inflammation, fundus autofluorescence imaging to assess retinal pigment epithelium integrity, and spectral-domain optical coherence tomography to evaluate retinal damage.

Applying the International Uveitis Study Group criteria, we classified uveitis into the following subtypes according to the site of inflammation: anterior uveitis, intermediate uveitis, posterior uveitis, or panuveitis (20,21). We considered placoid chorioretinitis to be the presence of ≥1 placoid, yellowish, outer retinal lesions. Fundus fluorescein angiography, indocyanine green angiography, and fundus autofluorescence indicate a typical fluorescent pattern in cases of placoid chorioretinitis (22,23).

Biological data included the results of treponemal and HIV serologic tests and the results of cerebrospinal fluid (CSF) analysis. The decision to perform lumbar puncture was left to the clinician’s discretion. We defined neurosyphilis as positive results in nontreponemal and treponemal serologic tests, combined with neurologic and CSF abnormalities, such as high leukocyte or protein concentrations (>0.5 g/L), and 1 CSF abnormality, such as positive VDRL or fluorescent treponemal antibody absorption test results (24–26) or a positive PCR test result for T. pallidum. Treatment and outcome data included the type, dose, duration, and route of antibiotic administration and corticosteroid treatment (local or systemic) as well as final VA.

We tested data variable distributions for normality and, when appropriate, we performed t-tests or equivalent non-parametric tests. We used 1-way analysis of variance when ≥3 groups were present and defined statistical significance as p<0.05. We analyzed the data by using R 3.2.2 software (https://cran.rproject.org/bin/windows/base/old/3.2.2).

Results

We identified 21 cases of ocular syphilis (29 eyes) during January 2012–December 2015. Visits to outpatient clinics for confirmed ocular syphilis (the number of cases compared to the number of consultations for uveitis) increased from 1 in 2012 to 5 in 2013, 6 in 2014, and 9 in 2015 (2.22–25.21 visits/1,000 individual patients per year for the period) (Figure 1).

Demographic Data, Biologic Data, and Clinical Features

Twenty-one patients were affected, all men. The median age at presentation was 49 years (range 22–72 years). No difference in the mean age of patients was observed at diagnosis of ocular syphilis in 2013, 2014, and 2015. Median follow-up was 2 months (range 1–22 months).

At the time of uveitis diagnosis, 14 (67%) patients reported extraophthalmologic features, including rash, ulcer, mucocutaneous lesions, or neurologic signs. We noted the stages of syphilis and the presence of neurosyphilis in the 21 patients with ocular syphilis (Figure 2). Tertiary syphilis was diagnosed in only 1 (5%) patient, secondary syphilis in 11 (52%) patients, and an undetermined stage of syphilis in 9 (43%) patients. Of the 14 patients who had a lumbar puncture to assess CSF for evidence of neurosyphilis, 3 (21%) patients with ocular syphilis had neurosyphilis, and in 7 (50%) patients the CSF showed a lymphocytic reaction. We noted the frequency of different clinical presentations and anatomical types of ocular inflammation (Figure 3). The mean duration of ophthalmologic symptoms was
1 month before presentation at the uveitis clinic (range 1 day–4 months).

Isolated posterior uveitis was the most common type of uveitis observed within the cohort (69%), followed by panuveitis (14%). We summarized the frequency of clinical manifestations in patients with ocular syphilis (Table 1). Posterior placoid chorioretinitis was the most frequent finding in the posterior segment (58% of the total number of affected eyes); this condition accounted for 75% of cases of syphilis with eye involvement in 2013 and 81% in 2015. Patients with bilateral impairment had a mean BCVA of 0.9 logMAR (range 0–2.30), whereas patients with unilateral disease had a mean BCVA of 1.02 logMAR (p = 0.54). Worse final VA outcomes (BCVA ≥0.30 logMAR) were found in patients who waited a mean 61 days (SD ±53 days) before seeking medical care. Patients who had the best final VA outcome (BCVA <0.30 logMAR) had waited a mean 15 days (SD ±19 days) before seeking medical care.

Effect of HIV Status
The results of HIV serologic tests were available for all patients. Six of the 21 patients (29%) were HIV-positive, and ocular syphilis led to the eventual diagnosis of HIV infection in 2 of these 6 cases. All but 1 of the 6 HIV-positive patients were MSM. In patients with ocular syphilis, HIV co-infection remained stable, ranging 20%–33% during 2012–2015. Most of the 21 patients were MSM, including 16 patients in the whole cohort and 5 of the 6 HIV-infected patients. These percentages did not vary over the study period.

Subgroup analysis between HIV-negative and HIV-positive patients found no significant differences in the proportion of bilateral disease or initial VA. The clinical outcomes for affected eyes was significantly worse for HIV-positive patients (final mean BCVA 0.7 logMAR [range 0–2.3]) than for HIV-negative patients (0.09 logMAR [range 0–0.5]; p = 0.01) (Table 2). Affected eyes from HIV-positive patients had no significant difference in uveitis type compared with HIV-negative patients.

Treatment
Most of the patients (17/21) were treated with daily intravenous penicillin, 3 patients received intravenous ceftriaxone, and 1 received oral doxycycline for 2 weeks followed by

| Table 1. Clinical signs among patients with ocular syphilis seen at a uveitis clinic, Paris, France, 2012–2015 |
|----------------------------------|----------|----------|
| Clinical sign                         | No. (%)  | patients |
| Posterior placoid chorioretinitis     | 17 (58)  |          |
| Retinitis                            | 4 (14)   |          |
| Optic neuritis                       | 4 (14)   |          |
| Anterior uveitis (plus iris gumma)   | 1 (3.5)  |          |
| Neuroretinitis                       | 1 (3.5)  |          |
| Intermediate uveitis                 | 1 (3.5)  |          |
| Retinal vasculitis                   | 1 (3.5)  |          |
Table 2. Comparison of HIV-negative and HIV-positive patients with ocular syphilis seen at a uveitis clinic, by epidemiologic characteristics, clinical presentations, and outcomes, Paris, France, 2012–2015*

<table>
<thead>
<tr>
<th>HIV status</th>
<th>HIV-negative patients, n = 15 patients, 22 eyes</th>
<th>HIV-positive patients, n = 6 patients, 7 eyes</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range), y</td>
<td>48 (32–72)</td>
<td>38.5 (22–61)</td>
<td>0.28</td>
</tr>
<tr>
<td>Median duration from onset of uveitis to presentation (range), d</td>
<td>7 (1–90)</td>
<td>30 (1–120)</td>
<td>0.98</td>
</tr>
<tr>
<td>Uveitis type, no. (% of 29 eyes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placoid chorioretinitis</td>
<td>15 (51)</td>
<td>2 (7)</td>
<td></td>
</tr>
<tr>
<td>Retinitis</td>
<td>2 (7)</td>
<td>2 (7)</td>
<td></td>
</tr>
<tr>
<td>Optic neuritis</td>
<td>3 (10.5)</td>
<td>1 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Anterior uveitis (plus iris gumma)</td>
<td>0</td>
<td>1 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Neuroretinitis</td>
<td>1 (3.5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Intermediate uveitis</td>
<td>1 (3.5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Retinal vasculitis</td>
<td>1 (3.5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bilateral disease, no. (%)</td>
<td>7 (46)</td>
<td>1 (16.5)</td>
<td></td>
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<tr>
<td>Neurosyphilis, no. (%)</td>
<td>4 (26)</td>
<td>6 (50)</td>
<td></td>
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<tr>
<td>Mean initial visual acuity (range), logMAR score</td>
<td>0.9 (0–2.3)</td>
<td>1 (0–2)</td>
<td>0.61</td>
</tr>
<tr>
<td>BCVA (range) 2 weeks after starting treatment, logMAR score</td>
<td>0.5 (0–2.3)</td>
<td>1.2 (0.4–2)</td>
<td>0.0030</td>
</tr>
<tr>
<td>BCVA (range) at final follow-up, logMAR score</td>
<td>0.09 (0–0.5)</td>
<td>0.7 (0–2.3)</td>
<td>0.0139</td>
</tr>
</tbody>
</table>

*BCVA, best-corrected visual acuity; logMAR, logarithm of the minimum angle of resolution.

ceftiraxone for 2 weeks. The duration of treatment ranged from 2 to 3 weeks, although 2 patients received a total of 4 weeks of treatment (1 who was treated first with doxycycline and 1 who was diagnosed with tertiary syphilis). The penicillin dosage was 24 million IU/day for 13 patients, whereas 20 million IU/day was used in 4 patients (including 1 who was diagnosed with neurosyphilis). Five of the 7 patients with neurosyphilis were treated for 2 weeks.

In addition to receiving antibiotic therapy, 11 patients also received corticosteroid treatment orally (9 patients), periocular treatment (4 patients), or both (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/2/17-1167-Techapp1.xlsx). Corticosteroids were administered in 4 cases to treat an ocular Jarisch-Herxheimer reaction (JHR) (sudden onset of vitritis, papilledema, or retinal vasculitis) despite appropriate antibiotic therapy. In 1 patient (patient 20), systemic corticosteroids were used to prevent systemic JHR (online Technical Appendix Table 2).

Outcomes

Before antimicrobial drug therapy at initial presentation, 62% of patients had BCVA <0.3 logMAR (20/40 Snellen); after antimicrobial drug therapy at final follow-up, 75% had BCVA ≥0.3 logMAR (20/40 Snellen) (Figure 4). Of the 5 patients with a final BCVA ≥0.3 logMAR (20/40 Snellen), only 2 had a treatment delay (≤12 weeks); therefore, we cannot correlate duration of treatment and visual prognosis because of the small number of patients with a poor outcome.

Most patients had better final VA than at presentation, with a mean initial BCVA of 0.9 logMAR (range 0–2.3) to a mean final BCVA of 0.2 logMAR (range −0.2 to 2.3). BCVA at presentation did not predict BCVA posttreatment (r = 0.5) (Figure 4). All patients had a good outcome in terms of quiescence of uveitis and final BCVA regardless of whether or not they had been treated with systemic or subconjunctival corticosteroids. No statistically significant difference in final BCVA after treatment was found between patients on the basis of whether or not they received systemic or subconjunctival corticosteroids: 0.40 logMAR (range 0–2.3) versus 0.15 logMAR (range −0.2 to 0.5), respectively (p = 0.208). Initial BCVA did not significantly differ between patients who had and those who had not received systemic or subconjunctival corticosteroids (p = 0.627).

In all patients who had lumbar puncture, no statistical difference was observed in final visual outcome between eyes in which a CSF lymphocytic reaction was diagnosed (final BCVA 0.46 [range 0–0.5] logMAR) and eyes with no evidence of CSF abnormalities (0.23 [range −0.2 to 2.3] logMAR). We also studied differences in syphilis trends regarding visual outcomes in 2013, 2014, and 2015. We observed no significant differences in visual recovery over time (p = 0.5783). In 13 patients for whom ≥3 months of follow-up was available for laboratory testing, quantitative VDRL screening was reported to decrease, except in 1 patient who had recurrence of uveitic syphilis because of an interruption in antiretroviral therapy (online Technical Appendix Table 2).

Discussion

Our study examined the trends in ocular syphilis in patients attending the uveitis clinic in a tertiary uveitis center in France. During the 4-year study period, consistent 10-fold increase in ocular syphilis was observed. This finding is in agreement with an annual epidemiologic report of the European Centre for Disease Prevention and Control (ECDC), which observed that in some countries in Europe, including France, the United Kingdom, and
Germany, a >50% increase in syphilis cases occurred during 2010–2014 (16). In France during 2012–2015, the number of early syphilis cases increased from nearly 750 to 1,450 reported cases. This trend was dramatically sharp among MSM (27). Indeed, a 15-fold increase was also found in the incidence of ocular syphilis in the United Kingdom during 1998–2003 (15).

A predominantly male cohort of patients has been reported in all studies, including ours (7,14,22,23,28). In our study, 76% of ocular syphilitic cases occurred in MSM. The proportion of HIIV-positive MSM remained stable during the period among ocular syphilis case-patients. A recent report has shown that MSM account for the greatest increases in syphilis cases (29). In our study, most case-patients had syphilis diagnosed during the second stage of the disease.

Syphilis is known to display diverse ophthalmologic manifestations, which our study affirmed. The most common finding was posterior uveitis manifesting as placoid chorioretinitis (58%), which has been noted in previous reports (14,23,29–31). Posterior placoid chorioretinitis is an extremely specific clinical sign for the diagnosis of syphilis. Therefore, ≈60% of the patients had clinical features consistent with the diagnosis of syphilis on retinal imaging even before the diagnosis of systemic syphilis was established. Syphilis can be highly suspected by the ophthalmologist and requires confirmation with blood testing. Moreover, to detect other ocular presentations, blood testing for syphilis should be included in routine laboratory testing for all patients with ocular inflammation. However, clinical presentations of other conditions also were identified (anterior uveitis, intermediate uveitis, retinitis, and optic nerve edema). Notably, only 20% of patients had an inflamed anterior segment.

We found disparities in ocular syphilis trends by clinical phenotype and evidence of neurosyphilis on CSF analysis during the 4-year study period. By clinical feature, the greatest number of cases of posterior placoid chorioretinitis occurred in 2013 (4 patients) and 2015 (7 patients) and the least in 2014 (1 patient). Meanwhile, among the patients who underwent a lumbar puncture, 75% of those with ocular syphilis had a CSF lymphocytic reaction in 2013 (3/4 patients), which decreased to 33% in 2014 (2/6 patients) and showed an increase again to 50% in 2015 (2/4 patients). These differences in ocular syphilitic forms could be related to several treponemal strains originating from distinct regions and diverse isolation dates (32). Five different strains in 14 patients have been identified previously (33).

According to current guidelines, the recommended regimen for neurosyphilis and ocular syphilis is benzyl penicillin, 18–24 million units/day by intravenous injection for 10–14 days (2,3). In our study, this regimen proved to be effective. The duration of treatment was similar whether or not lumbar puncture confirmed neurosyphilis. Current guidelines recommend CSF examination only if patients have clinical evidence of neurologic involvement or opthalmic symptoms.

In our study, of 14 case-patients who had undergone CSF analysis, 7 (50%) had a lymphocytic reaction and 3 (21%) had neurosyphilis based on a positive reactive treponemal or nontreponemal test or PCR test on CSF samples. These numbers are consistent with a previous report of 26% of 31 ocular syphilis case-patients having neurosyphilis diagnosed on CSF examination. In that study, neurosyphilis was diagnosed if CSF fluorescent treponemal antibody absorption test was reactive or if a lymphocytic pleocytosis of >20 cells/μL was detected (34). In contrast, the US Centers for Disease Control and Prevention’s estimate of 50%–75% of ocular syphilis patients with evidence of neurosyphilis based on a positive CSF VDRL result (35) is higher than in our study.

In our study, CSF results did not influence the duration of treatment or the outcome. Furthermore, the sensitivity of PCR tests on CSF could be questioned because sensitivity was low even in intraocular samples. Indeed, because of the variable sensitivity of CSF tests (26), no CSF test result can definitively exclude a diagnosis of neurosyphilis (33). Because experience with PCR tests on intraocular fluids is limited (36,37), we suggest not relying on CSF results in such complex cases, preferring to examine CSF only when an alternate or concomitant infection is suspected (e.g., Herpesviridae and Mycobacterium tuberculosis infections), because syphilis is known to mimic many other causes of uveitis. Recently, PCR in...
CSF has been shown to be highly specific in the diagnosis of neurosyphilis (26), offering guidance in case of treatment failure or in supporting the clinical diagnosis of ocular syphilis or neurosyphilis when several co-infections other than syphilis are also detected. Therefore, ocular syphilis should be considered in any patient with positive syphilis serologic tests and ophthalmologic findings consistent with ocular syphilis and should be treated with a regimen of penicillin appropriate for neurosyphilis regardless of CSF results.

This study has shown that VA on initial examination did not predict the final visual outcome because no correlation existed between BCVA at presentation and posttreatment. However, a longer delay in consulting for visual symptoms was associated with worse final VA outcomes. We found the best final outcomes (BCVA <0.30 logMAR; >6/12 Snellen) in patients whose mean time from the first visual symptom to consultation was 15 days (SD ±19) and, conversely, the worst final BCVA (≥0.30 logMAR; ≤6/12 Snellen) in patients whose mean time from onset to consultation for visual symptoms was 2 months (SD ±53 days). Similarly, a longer duration between the first presentation of uveitis and treatment for syphilis has been associated previously with a significantly higher logMAR VA (e.g., 12 weeks in a study by Bollemeijer et al. [31]).

The use of corticosteroids to control the degree of inflammation remains undefined in the current treatment guidelines. Oral and topical corticosteroids have been used in the past in association with antibiotic treatment (4,23,38–40). Although topical corticosteroids can be used freely to help control anterior segment inflammation (40), intravitreal injections of triamcinolone appear to be harmful (39,41). Previous studies highlight the usefulness of systemic corticosteroids (intravenous and oral) and periorcular injections to treat macular edema (4), papillitis (23), and posterior placoid chorioretinopathy (40). The results of our study suggest that the use of perioral or oral steroids associated with antibiotic treatment could be useful as adjunctive therapy for ocular syphilis in the treatment of ocular JHR (vitritis), papillitis, and retinal vasculitis. Recently, Bollemeijer et al. found no difference in visual outcome whether or not patients had received any adjunctive systemic or local steroids; however, they indicate that adjunct corticosteroid treatment might have been preferable in the most severe cases (31).

If cases of persisting inflammation despite appropriate antibiotic therapy or an ocular JHR, corticosteroids seemed to be effective. In our study, improvements in VA after the use of periorcular injection or systemic corticosteroids were observed in all 11 patients, independent of the route of corticosteroid administration (intravenous, oral, or subconjunctival). No difference in final VA after treatment was found between patients who had or had not received any adjunctive treatment with systemic or periocular corticosteroids.

Discrepancies exist in previous reports of HIV co-infection and visual outcome in syphilitic uveitis. In fact, in previous studies, HIV positivity has been associated with a worse visual outcome in cases of syphilitic uveitis. Other recent studies have not supported this conclusion (1,7,30,42–45). Bollemeijer et al. hypothesized that favorable outcomes might be attributable to the immune status of HIV-positive patients receiving highly active antiretroviral therapy (31). In our study, 3 of the HIV-positive patients had CD4+ cell counts <350 cells/µL, which might explain the worse visual outcomes associated with HIV co-infection. We report worse posttreatment VA in HIV-positive patients compared with HIV-negative patients (Table 2).

The main limitations of this study stem from its intrinsic retrospective nature, leading to a heterogeneous follow-up schedule for ophthalmic examination. We highlight the same limitation as noted in previous case series (the study was conducted in 1 uveitis center from referrals, so this population might not represent the total spectrum of syphilitic uveitis) (31). Nonetheless, we strongly believe that the study provides a thorough description of the cases of ocular syphilis diagnosed at a reference national eye center. This report is strengthened by the fact that we have epidemiologic information on case-patients, including the sex of their sex partners and HIV status, as well as ophthalmologic examination and visual outcome information.

Syphilis can involve visual function, and therefore clinicians dealing with patients at risk for syphilis should consider that uveitis is one of its potential indications. Despite the historic stigma of syphilis and the generally low prevalence in the population at large, *T. pallidum* screening of patients with uveitis should be conducted (45). The nonspecific symptoms of uveitis include severe redness, pain, floaters, photophobia, and blurred vision. All patients with ocular syphilis should undergo HIV testing and comprehensive counseling on the prevention of sexually transmitted diseases. A minimal decline has been observed in the number of HIV diagnoses per 100,000 population over the past decade. However, the trend by transmission mode shows that the number of HIV diagnoses among MSM has continued to increase in countries in Europe (46). These trends in HIV infection are consistent with the increasing incidence of syphilis infection among MSM. At this juncture, the availability of oral HIV preexposure prophylaxis and persons’ decisions to rely on it rather than condoms might require future evaluation of an increased risk for ocular impairment attributable to increasing numbers of syphilis infection.
About the Author
Ms. Pratas formerly was a medical engineer at Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts. Currently, she is completing a PhD at the University of East Anglia. Her research interests include biomedical research in immunology and epidemiology of human infectious diseases.

References

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Address for correspondence: Marie-Hélène Errera, Centre Hospitalier National d’Ophtalmologie des Quinze-Vingts, 28 rue de Charenton, 75012 Paris, France; email: errera.mhelene@gmail.com
Human adenovirus type 4 (HAdV-4) is most commonly isolated in military settings. We conducted detailed molecular characterization on 36 HAdV-4 isolates recovered from civilian adults with acute respiratory disease (ARD) in the northeastern United States during 2011–2015. Specimens came from college students, residents of long-term care facilities or nursing homes, a cancer patient, and young adults without co-morbidities. HAdV-4 genome types 4a1 and 4a2, the variants most frequently detected among US military recruits in basic training before the restoration of vaccination protocols, were isolated in most cases. Two novel a-like variants were recovered from students enrolled at a college in Tompkins County, New York, USA, and a prototype-like variant distinguishable from the vaccine strain was isolated from an 18-year-old woman visiting a physician’s office in Ulster County, New York, USA, with symptoms of influenza-like illness. Our data suggest that HAdV-4 might be an underestimated causative agent of ARD among civilian adults.

Human adenovirus type 4 (HAdV-4), the only human adenovirus classified within species E, was first identified in the early 1950s in association with military outbreaks of febrile respiratory illness and is well-recognized worldwide as a prevalent causative agent of acute respiratory disease (ARD) and ocular disease (1–6). Surveillance studies conducted in the United States and other countries have demonstrated a leading role for this particular adenovirus type in the etiology of outbreaks of febrile respiratory illness in military recruit training facilities (7–11), where crowding and environmental contamination appear to facilitate transmission among nonvaccinated trainees (12,13). By using restriction enzyme analyses of viral DNA, several studies have reported extensive intratypic genetic variability for HAdV-4 (14–16). Two major clusters of genetic homology have been identified among circulating genomic variants: prototype (p)–like viruses, which are closely related to prototype strain RI-67, and a-like viruses, which exhibit, among other characteristics, distinct BamHI restriction profiles (15), a distinct inverted terminal repeat (17,18), and a different genetic make-up in the E3 region (A.E. Kajon, unpub. data).

HAdV-4 respiratory infections are preventable by vaccination with the live oral formulation of the nonattenuated p-like strain exclusively licensed for military use (19,20). After 15 years of discontinuation of HAdV-4 vaccination protocols with the consequent resurgence of continuous outbreaks of HAdV-4–associated illness in US recruit training facilities nationwide, US Department of Defense reinstated the vaccine in November 2011, dramatically reducing the number of cases of HAdV infection in basic training camps (21,22).

The absence of a sentinel system for HAdV surveillance outside of the military has made assessing the burden of disease attributable to HAdV-4 infection among civilians difficult. The limited epidemiologic data available in the published literature suggest that respiratory disease associated with HAdV-4 infection is detected at a significantly lower frequency than disease associated with species C or B HAdV types among children and that HAdV-4 infection occurs rarely among civilian adults (23–27).

Consequently, the apparent increased frequency of detection of cases and case clusters of HAdV-4 respiratory infection in the northeastern United States, documented...
by the New York State Department of Health (NYSDOH) or the Centers for Disease Control and Prevention (CDC) (data not shown), caught our attention. In this article, we report the molecular characterization of 36 select HAdV-4 isolates from a selection of retrospectively evaluated cases among civilians.

Methods

Source of Specimens
We obtained HAdV-4–positive specimens from patients with ARD/influenza-like illness (ILI) characterized by fever >37.8°C and cough, sore throat, or other respiratory symptoms. We selected cases that were originally identified by the NYSDOH as part of its activities for the US Sentinel PhysicianILI Surveillance Network (https://www.cdc.gov/flu/weekly/pdf/flu-surveillance-overview) or that represented special HAdV cases referred to the CDC for investigation because of their clinical disease severity or occurrence during an outbreak. Institutional review board review was not required for the processing of clinical samples or for the evaluation of patient information, which were obtained during routine diagnostic workups at the Clinical Virology Laboratory, Yale–New Haven Hospital (New Haven, Connecticut, USA), and the University of Rochester Medical Center (Rochester, New York, USA). Review board approval was also not required for the typing protocol used on deidentified HAdV isolates at the Lovelace Respiratory Research Institute (Albuquerque, New Mexico, USA).

Virus Isolation and Initial Identification
Virus isolation from respiratory specimens and typing was performed at CDC (Atlanta, Georgia, USA) or the Virology Laboratory, Wadsworth Center NYSDOH (Albany, New York, USA). The virus isolates were initially identified as HAdV-4 by molecular procedures as previously described (28,29).

Genome Typing by Restriction Enzyme Analysis
Cultured isolates were shipped to Lovelace Respiratory Research Institute for propagation and extraction of intracellular viral DNA from infected A549 cell monolayers as previously reported (30). We digested viral DNA samples with a panel of 6 endonucleases to identify genomic variants following the initial guidelines of Li and Wadell (15) and as previously applied to the genomic characterization of US military strains (16) to facilitate comparisons. In brief, we digested 1 μg of purified viral DNA with BamHI, Drai, EcoRI, EcoRV, SmaI, and XhoI (New England Bio labs, Ipswich, MA, USA) in a 20-μL reaction following the manufacturer’s recommended conditions. For the isolates selected for complete genomic sequencing, we performed genome typing by in silico digestion of their viral DNA with the same panel of enzymes using Geneious Pro 9 (Biomatters Ltd, Auckland, New Zealand) (31).

Genome Sequencing and Analysis
We then carried out next-generation sequencing reactions at the Wadsworth Center’s Applied Genomics Technology Core with the purified viral genomic DNA prepared at Lovelace Respiratory Research Institute. The 12 isolates that were selected for next-generation sequencing were representative of the set of identified variants on the basis of restriction enzyme analysis, location, and date of detection. We prepared libraries with the NexteraXT kit (Illumina, San Diego, CA, USA) and performed paired-end sequencing on the Illumina MiSeq using the NextSeq 500 cycle v2 kit (Illumina). We assembled all genomic sequences de novo using the Illumina BaseSpace cloud application for SPAdes 3.5 (http://spades.bioinf.spbau.ru/release3.5.0/manual.html); we then remapped the sample sequences to the consensus sequence using Geneious Pro 9 (https://www.geneious.com/). Confirmation of sequence accuracy for specific regions of the genome was carried out by Sanger sequencing of PCR amplicons as needed. We annotated the HAdV-4 genomes using curated reference sequences available for the prototype RI-67 strain (GenBank accession no. AY594253), the vaccine strain CL68578 (GenBank accession no. AY487947), and several military isolates representing previously described genomic variants (GenBank accession nos. EF371058, AY599837, and AY599835).

For phylogenetic analysis, we aligned the genomic sequences generated in this study with reference strains (Table 1) using MAFFT in Geneious Pro 9. We constructed a maximum-likelihood tree on the basis of the Kimura 2-parameter model (32) with 500 bootstrap replicates using MEGA6 (33).

Results

Case Descriptions
The HAdV-4–positive ARD/ILI cases we evaluated occurred in otherwise healthy teenagers, young adults in college, and older adult patients who were residing in long-term care facilities, with 1 older adult patient hospitalized in a cancer center. Some cases required prolonged hospitalization or had fatal outcomes.

Nosocomial Outbreak of HAdV-4 Respiratory Infection in Long-Term Care Facility for Elderly—Boston, Massachusetts, April–May 2006
A detailed description of this outbreak was published by Kandel et al. in 2010 (34). In brief, the outbreak occurred in a unit with 40 residents of mean age 88 (range 66–99) years. During April–May 2006, fifteen residents had symptoms...
Table 1. Clinical isolates and reference strains used in the phylogenetic analysis of HAdV-4 strains recovered from cases of acute respiratory infection detected in northeastern United States, 2011–2015

<table>
<thead>
<tr>
<th>Genome type</th>
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</tr>
<tr>
<td>4a1</td>
<td>KY996450</td>
</tr>
<tr>
<td>4a1 Smal v</td>
<td>KY996449</td>
</tr>
<tr>
<td>4a2</td>
<td>KY996448</td>
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</tr>
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of ARD. HAdV-4 infection was confirmed for 4 residents who had positive virus culture results through PCR amplification and sequencing of the hexon hypervariable regions 1–6 as described by Lu and Erdman (28). The nasopharyngeal aspirates from 3 symptomatic residents gave negative virus culture results, and the remaining 8 residents were not sampled. Three of the 4 patients with confirmed HAdV-4 infections died of complications from ARD. Isolates from 2 of the confirmed cases of HAdV-4–associated pneumonia identified during this outbreak were processed for viral DNA extraction and detailed characterization. The 2 respiratory isolates were genome typed as variant 4a1 by restriction enzyme analysis at Lovelace Respiratory Research Institute.

Adult Case of Severe Pneumonia—Connecticut, July 2011

One author (M.L.L.) was involved in testing, consulting, and advising on this case as the clinical laboratory director and as an infectious disease specialist. A 26-year-old man with an unremarkable medical history sought treatment at an emergency department for a 3-day illness involving severe headache, photophobia, nausea, vomiting, and chills. He had seen his doctor 2 days earlier and was treated with azithromycin without improvement. His lumbar puncture results were normal, but a chest radiograph showed a left upper lobe infiltrate. Blood work showed a normal white blood cell count with 27% band cells, anemia, thrombocytopenia, and elevated creatinine. The patient was admitted and treated with ceftriaxone and azithromycin. On the following day, he experienced severe respiratory distress, so he was intubated and transferred to intensive care. His nasopharyngeal swab tested positive by panadenovirus PCR at the Clinical Virology Laboratory, Yale New Haven Hospital. On the 5th day after hospital admission, the patient remained febrile, and his chest radiograph showed diffuse bilateral infiltrates; dialysis was initiated for a creatinine of 4.9 mg/dL (or 430 µmol/L, reference range 53–106 µmol/L). By using the panadenovirus PCR, the plasma viral load was determined to be $1.16 \times 10^4$ copies/mL. Molecular typing conducted at CDC confirmed the presence of HAdV-4 in both the nasopharyngeal swab and plasma specimens. By using a HAdV-4–specific PCR (35), the plasma viral load was determined to be $5.00 \times 10^4$ copies/mL. The patient eventually recovered with supportive therapy. The HAdV-4 isolate obtained from the nasopharyngeal swab was genome typed at Lovelace Respiratory Research Institute as variant 4a2 by restriction enzyme analysis.

Cases of ILI among College Students—New York, 2011–2015

During December 2011–October 2015, several HAdV-4–positive cases were identified at the Wadsworth Center Virology Laboratory among students enrolled at 7 colleges in 6 New York counties (Table 2; Figure 1). Persons arrived at their corresponding student health clinics with symptoms of ILI but tested negative for influenza by the CDC human influenza virus real-time reverse transcriptase PCR diagnostic panel. Four different HAdV-4 genomic variants were isolated from this group of patients 18–25 years of age (Table 2).


During 2011–2015, ILI surveillance efforts identified 5 additional cases of acute HAdV-4 respiratory infection of variable severity (Table 2; Figure 1). These cases included infections in 2 adult patients (NY3 and NY25) sampled in
Table 2. Basic demographics, clinical characteristics, and virology findings of 33 cases of HAdV-4 acute respiratory infection detected by New York State Department of Health surveillance, New York, USA, 2011–2015*

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Specimen collection date</th>
<th>Specimen</th>
<th>Patient age, y/sex</th>
<th>Setting</th>
<th>County</th>
<th>Diagnosis</th>
<th>Genome type†</th>
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<tr>
<td>NY2</td>
<td>2011 Dec</td>
<td>NSW</td>
<td>18/M</td>
<td>College 2</td>
<td>Tompkins</td>
<td>ILI</td>
<td>4a2</td>
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<td>4a2</td>
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<td>4a2</td>
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<td>4a Smal vz</td>
</tr>
<tr>
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<td>20/F</td>
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<td>NY13</td>
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*HAdV-4, human adenovirus type 4; ICU, intensive care unit; ID, identification; ILI, influenza-like illness; NPS, nasopharyngeal swab; OPS, oropharyngeal swab; TA, tracheal aspirate; TS, throat swab; v, variant exhibiting unpublished profiles for the specified endonucleases.
†Determined by restriction enzyme analysis of viral genomic DNA performed in vitro, in silico, or both with BamHI, DraI, EcoRI, EcoRV, Smal, and XhoI and designated according to Li and Waddell (15).
‡Restriction enzyme analysis by in silico digestion.

physicians’ offices, a case of pneumonia reported in a nursing home (NY9), a fatal case involving respiratory complications in a patient at a cancer center (NY10), and a case of ARD detected in a teenager at a pediatric clinic (NY23).

**Adult Case of Severe Pneumonia—Ontario County, New York, August 2012**

One author (M.M.) was involved in testing, consulting, and advising on this case as the director of the Virology Laboratory at the Strong Memorial Hospital, University of Rochester Medical Center. A 43-year-old woman with cough and an unremarkable medical history sought treatment at the emergency department of University of Rochester Medical Center, Monroe County (NY8; Table 2; Figure 1). She was prescribed levofloxacin for presumed community-acquired pneumonia and sent home. Four days later, she was admitted with worsening cough, shortness of breath, and rigors and was found to have bilateral infiltrates on chest radiograph, anemia, and leukocytosis. She subsequently required intubation for declining respiratory status. Despite treatment with multiple broad-spectrum antimicrobial drugs, she experienced severe hypoxic hypercarbic respiratory failure requiring venous-venous extracorporeal mechanical oxygenation. Her nasopharyngeal swab was positive for HAdV by FilmArray (BioFire Diagnostics, bioMérieux, Marcy l’Etoile, France). She was treated with 3 doses of cidofovir. Her hospital course was complicated by severe acute kidney injury, acute tubular necrosis, and anuria requiring continuous veno-venous hemofiltration. Additional complications included cerebral edema, intracranial hemorrhage, and persistent hypertension. She was weaned from the ventilator after 40 days and was discharged to in-patient rehabilitation on day 53 of hospitalization. A year after discharge, she continued to experience bronchiectasis and dyspnea on exertion but had otherwise returned to her previous level of function. HAdV was isolated from cultures of the patient’s tracheal aspirate and nasopharyngeal swab, and a PCR of her peripheral
Adenovirus Type 4 Infections, United States

blood demonstrated a virus load of $3.47 \times 10^5$ copies/mL. Molecular typing at the Wadsworth Center identified the virus as HAdV-4. The HAdV-4 isolate was genome typed as variant 4a1 by both in vitro and in silico restriction enzyme analyses (Table 2).

### Virology Findings

Among these 36 select cases (some associated with outbreaks and some epidemiologically unlinked ARD cases) that occurred during 2009–2015 in the northeastern United States, 5 different genomic variants of HAdV-4 were identified by gel-based or in silico restriction enzyme analysis (Figure 2). Isolate NY25 (GenBank accession no. KY996447) was identified as 4p-like (Figure 3) and was indistinguishable from the prototype strain RI-67 or the vaccine strain CL68578 by restriction enzyme analysis (Figure 2). Of the 35 a-like isolates, 18 were classified as genome type 4a1, 12 as genome type 4a2, 4 as genome type 4a Smal v (having a 4a-like genome with a novel Smal profile), and 1 as genome type 4a Smal/XhoI v (having a 4a-like genome with novel Smal and XhoI profiles) (Table 2).

To investigate a possible transmission event of the vaccine strain administered orally to US military recruits since October 2011 (22), we further characterized the 4p-like strain isolated from case NY25 by whole-genome sequencing. We identified 10 point mutations scattered throughout the genome (6 nonsynonymous and a 3-nt [CAG] in-frame insertion at position 23402 within the L4 coding region and open reading frame of the 100-kDa protein), distinguishing the 2015 isolate from the vaccine strain, CL68578. Phylogenetic analysis of HAdV-4 genomic sequences (Figure 3) also showed this isolate to be more closely related to strains RI-67 and CL68578 than to strain NHRC 90339 (GenBank accession no. EF371058), which was isolated at the Coast Guard Recruit Training Center (Cape May, New Jersey, USA) in 2001 and is representative of genome type 4p4, the only p-like variant detected at military training facilities through 2011 (16) (A.E. Kajon, unpub. data). The phylogenetic analysis revealed 2 major clades, recapitulating the original observations and genomic clustering of variants by Li and Wadell (15). The analysis also showed the Smal variant to be closely related to genome type 4a1 and the Smal/XhoI variant to be closely related to genome type 4a2.

### Discussion

Enhanced influenza surveillance by public health laboratories initiated after the emergence of pandemic influenza A(H1N1) in 2009, as well as the wider availability of molecular diagnostic assays for multiple viral pathogens, have resulted in increased diagnostic efforts to determine the etiology of influenza-negative ILI, with consequent increased detection of HAdV-associated ARD. As part of our ongoing collaborative efforts to describe the molecular epidemiology and determine the prevalence of HAdV-associated respiratory disease, we examined HAdV-4 isolates recovered from college students with acute febrile respiratory illness in New York and several adults with severe respiratory disease in other locations in the northeastern United States. Restriction enzyme analysis with enzymes previously used to characterize HAdV strains from military recruits (16) and complete genomic sequencing identified 5 different genomic variants among the characterized clinical
HAdV-4 isolates. Two of these variants, 4a1 and 4a2, had been previously identified in association with outbreaks of febrile respiratory illness in military recruit training facilities in the United States and found to be highly prevalent in the basic training environment nationwide before reinstatement of recruit vaccination protocols in 2011 (16) (A.E. Kajon, unpub. data). The genomic variant 4a1 was isolated from the respiratory specimens of 18 of 36 civilians and 4a2 from the respiratory specimens of 12 of 36 civilians retrospectively examined in this study. Two previously unreported variants closely related to 4a1 (Smal v, n = 4) and 4a2 (Smal/Xho v, n = 1) were identified among the 8 examined cases detected at college 4 in Tompkins County, New York. Surprisingly, a p-like, vaccine-like strain was isolated from a respiratory specimen obtained from an 18-year-old woman (case NY25) at a physician’s office in Ulster County, New York, in February 2015. The genome of this clinical isolate (15–4054; Table 1) had a close similarity to the vaccine strain CL68578, and the NYSDOH epidemiology team confirmed contact between this patient and an active member of the military. However, the mutations distinguishing the genome of this isolate from that of the vaccine strain exclude possible transmission from this source. Evolution of this p-like virus from the vaccine strain is highly likely, considering the ability of HAdV-4 to establish persistent infections in gut lymphoid tissue (36) and that vaccinated persons shed the infectious-nonattenuated vaccine strain in their stool (37).

1997–2011 demonstrated the lack of circulation of vaccine-like strains in the military environment during the 15 years of clear dominance of this re-emerging type as a causative agent of febrile respiratory illness in US recruit training camps (16). Unfortunately, no studies have reported genome typing data for civilian isolates obtained during the same period. A p-like variant designated 4p4 was identified as the only HAdV-4 genomic variant detected among military trainees at the US Coast Guard Training Center in Cape May through 2011 and, albeit with relatively low prevalence, as the only p-like variant circulating at the other 7 military training sites under surveillance (16) (A.E. Kajon, unpub. data).

Conceivably, exposure of the general population to the nonattenuated vaccine strain could have continued through fecal shedding from persons vaccinated during 1971–1997. Another possibility is that the p-like variants could have been circulating among civilian communities at low prevalence since the 1950s, when they were first identified (2). This topic warrants additional research and continued surveillance to further our understanding of the dynamics and routes of transmission of respiratory HAdVs that have the ability to establish persistent infection in the gut lymphoid tissue.

On the basis of the severity of the clinical presentation of some cases in this study, the HAdV-4 vaccine currently licensed for military use should be considered a potentially valuable resource to prevent disease in susceptible populations living in closed communities, such as college settings, summer camps, and long-term care facilities. Our data and reports of cases of severe ARD associated with HAdV-4 infection in Italy and Singapore (38,39) suggest that the role of this HAdV type in the etiology of adult civilian ARD might have been underestimated in the absence of access to molecular (or other) typing resources. Further, in view of the results of this study and previous research documenting the contribution of HAdV infection to influenza-negative ILI (29), the inclusion of HAdV in differential diagnostic test panels would be invaluable to better assess the role of HAdVs as causative agents of severe respiratory illness and to prevent unnecessary treatment of patients with influenza-negative ILI with anti-influenza agents. In addition, while the failure to detect influenza virus does not guarantee the virus was never present, the detection of HAdV in these cases assists in alleviating concerns regarding influenza vaccine failure. Finally, the potential differences in pathogenicity, transmissibility, and fitness between p-like and a-like genomic variants of HAdV-4 that would explain the marked predominance of a-like variants in the examined collections of HAdV-4–positive respiratory specimens representing sampling of ARD in civilian and military populations in the United States over the past 5 decades (16,40) deserve further investigation.

Acknowledgments
We dedicate this work in memoriam to our co-author Marilyn Menegus in celebration of her passion for clinical virology. The authors thank Mathew Shudt for providing technical support with genomic sequencing.

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About the Author
Dr. Kajon is a scientist in the Infectious Disease Program at Lovelace Respiratory Research Institute in Albuquerque, New Mexico, and adjunct faculty at the Department of Molecular Genetics and Microbiology and a member of the Center for Infectious Disease and Immunity at the University of New Mexico. Her primary research interests include molecular epidemiology of adenovirus respiratory infections, natural history of adenovirus infections in transplant recipients, and viral pathogenesis.
SYNOPSIS

References


Address for correspondence: Adriana E. Kajon, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr SE, Albuquerque, NM 87108, USA; email: akajon@lrri.org
During 2004–2014, the Democratic Republic of the Congo (DRC) declared 54% of plague cases worldwide. Using national data, we characterized the epidemiology of human plague in DRC for this period. All 4,630 suspected human plague cases and 349 deaths recorded in DRC came from Orientale Province. Pneumonic plague cases (8.8% of total) occurred during 2 major outbreaks in mining camps in the equatorial forest, and some limited outbreaks occurred in the Ituri highlands. Epidemics originated in 5 health zones clustered in Ituri, where sporadic bubonic cases were recorded throughout every year. Classification and regression tree characterized this cluster by the dominance of ecosystem 40 (mountain tropical climate). In conclusion, a small, stable, endemic focus of plague in the highlands of the Ituri tropical region persisted, acting as a source of outbreaks in DRC.

Plague is a zoonotic disease caused by the gram-negative bacterium *Yersinia pestis* (1). According to World Health Organization (WHO) reports published in 2009 (2) and 2016 (3), >95% of the 15,396 cases reported worldwide during 2004–2014 occurred in Africa, especially in the Democratic Republic of the Congo (DRC, 8,379 [54%] cases); Madagascar (5,583 [36%] cases), Uganda (436 [3%] cases); and Tanzania (191 [1%] cases).

In DRC, plague was first reported in 1928, where J. Winderickx confirmed plague cases in Ituri (Orientale Province), near Lake Albert (4). In 1938, a second focus was discovered near Lake Edward (currently in North Kivu) (5), but no case has been reported there since 1967 (6). Before the 1950s, plague vaccination campaigns and rodent and vector control activities were conducted in both foci (4). Before the 1950s, the total number of notified cases remained low (Figure 1). Control programs then progressively collapsed, and the number of suspected cases notified to WHO dramatically increased in Orientale Province, peaking at 2,000 in 2006 (Figure 1).

Despite the high number of reported cases in DRC since the 1990s, almost no scientific reports have been published about plague foci in DRC. The 2 exceptions are an article describing the plague outbreak in Zobia (Ganga health zone [HZ]) in 2005 (8) and another about the laboratory confirmation of *Y. pestis* during 2 outbreaks (the previous outbreak in Zobia and another in Bole Bole, Wamba HZ, in 2006) (9). The recent events related to the Ebola virus disease outbreak in West Africa demonstrate the importance of remaining vigilant about highly virulent diseases still exhibiting a major epidemic potential (10), such as plague in DRC.

In this study, we aimed to describe the epidemiologic and ecologic characteristics of human plague during 2004–2014 in DRC. We also looked for spatially and temporally grouped cases (i.e., clusters).

**Materials and Methods**

**Study Setting**

We conducted the study in the former Orientale Province, located in northeastern DRC, the only place in DRC where plague was observed during the study period (Figure 2). At that time, the province (500,000 km²; mean population for the period 9.5 million inhabitants) was subdivided into 5 health districts (corresponding to the present eponym 4 provinces): Tshopo, Ituri, Haut-Uele, Bas-Uele, and Kisangani (currently included in Tshopo). The central and western parts of the province were covered by dense and humid tropical forests ranging from 200 m to 500 m in elevation (altitude). The north was covered by savanna vegetation and...
the east by savanna and crops; elevations ranged from 1,000 m in the plateau to >2,500 m in the mountains adjacent to Lake Albert (11). The province generally had an equatorial climate. Precipitation was abundant, ranging from 80 mm per month in Ituri to 200 mm close to the equator. In the eastern highlands, climate was cooler. The north and northeast experienced less rain and had a short dry season (December–February). The heaviest rains usually occurred in October and early November. Fishing, hunting, artisanal mining, and local trade were the main economic activities of the former Orientale Province (12), but in Ituri, residents made a living mainly from farming. During 1997–2003, Ituri was ravaged by armed conflicts that led to thousands of deaths, population impoverishment, and collapse of the healthcare system. The former Orientale Province population comprised 75.5% impoverished persons living in un-sanitary conditions (11).

Data Collection
In DRC, an Integrated Disease Surveillance and Response network surveyed 15 infectious diseases, including plague. Human plague data were collected every epidemiologic week at the HZ level, that is, the fourth health administrative level (the 5 nested health administrative units are State/Province/Health District/HZ/Health Area). The Ministry of Health provided more detailed databases for the initial period of Ganga epidemics in 2005 and Logo epidemics in 2014.

Case Definition
The Ministry of Health provided a database of suspected plague cases; data were consolidated and completed with different patient line-listings and investigation reports performed during outbreaks. Suspected plague cases were defined according to WHO standard protocols (13). Cases were characterized by rapid onset of fever, chills, headache, severe malaise, prostration, with extreme painful swelling of lymph nodes (i.e., buboes) (bubonic form) or cough with blood-stained sputum, chest pain, and difficult breathing (pneumonic form).

All suspected bubonic cases were kept in the database. Because the clinical definition of pneumonic plague is poorly specific, the Ministry of Health provides more accurate definitions during pneumonic outbreaks that account for the specific context (14,15). Pneumonic plague outbreaks have high case-fatality rates (CFRs) in developing countries, reaching almost 100% in the absence of proper patient care (7). Taking into account the observations of Neerinckx et al. (7) regarding CFRs, we applied a restrictive definition of suspected pneumonic plague outbreaks, discarding suspected pneumonic plague cases associated with low CFR (i.e., <5%), and without any biological confirmation. Implausible reductions in CFR, to <2%, were also observed at the end of plague-confirmed outbreaks. In 2004, for Ganga HZ, Bertherat et al. (16) proved that many of these cases resulted from other diseases, such as leptospirosis. We also discarded such cases from the database because, despite the
presence of external medical teams assisting local response, we could not identify any confirmation of any pneumonic plague cases during postoutbreak periods.

An outbreak of pneumonic plague is defined as the presence of a single confirmed case of pneumonic plague. During outbreaks, suspected cases were confirmed by culture or by detection of Y. pestis F1 antigen in bubo aspirates using the rapid diagnostic test (RDT; Institut Pasteur, Antananarivo, Madagascar) or staining sputum smears (Gram and Wayson staining) according to standard protocols (17).

**Population and Environmental Data**

We obtained HZ-level population data. In 2009, the Ministry of Health calculated HZ populations using data from the Expanded Programme on Immunization and from the Leprosy Elimination Program. We adjusted these data for the other years with the annual rate of natural growth (2.5%) (18). We also collected HZ-level environmental co-factors and retained the following co-factors for environmental analyses: elevation, land cover, precipitation levels, and climate type. Elevation data were derived from the Shuttle Radar Topography Mission (http://srtm.usgs.gov/) and monthly precipitation data retrieved from the TRMM (Tropical Rainfall Measure Mission) 3B43 version 7 (http://disc.sci.gsfc.nasa.gov/precipitation/documentation/TRMM_README/TRMM_3B43_readme.shtml). Land cover data were extracted from MODIS Yearly 12Q1 (Moderate Resolution Imaging Spectroradiometer, https://ltdaac.usgs.gov/dataset_discovery/modis/modis_products_table/mcd12q1). We summarized climate
type ecoclimatic zones as defined on the website of the Food and Agriculture Organization of the United Nations (http://www.fao.org/ag/AGAInfo/programmes/documents/livatl2/africaezmaps.htm). These ecosystems are built incorporating repeated measures of NDVI (Normalized Difference Vegetation Index), temperature, rainfall, length of growing period, and elevation. Four ecosystems are present in Orientale Province: ecosystems 32, 33, 38, and 40, presented here as tropical, dry tropical, equatorial, and tropical mountain, respectively (Figure 2). Precise descriptions of these zones are available at http://www.fao.org/ag/AGAInfo/programmes/documents/livatl2/Ecosystems.htm and http://www.fao.org/ag/AGAInfo/programmes/documents/livatl2/afeztables.htm.

We extracted and analyzed geographic data at the HZ level with ARCGIS 9.3 (ESRI, Redlands, CA, USA), using mean value and SD for elevation and precipitation and calculating the HZ proportion covered by each land cover or ecosystem type. We extracted the HZ map from the Health Mapper (19).

**Statistical Methods**

Spatial case clusters were groups of contiguous HZs with significantly more suspected plague cases than the remaining HZs. To investigate clusters, we analyzed the number of cases in each HZ reported during January 2004–December 2014 using spatial scan statistics in SaTScan software (20,21). This approach systematically moves an elliptic scanning window of increasing diameters over the study region. For every diameter, it compares the observed case number inside the window with what would be expected over a random Poisson distribution of cases (22). The maximum allowed cluster size corresponded to 15% of the population of Orientale Province. We computed the Oliveira F coefficient for every HZ located in a likely cluster and obtained the statistical significance for each cluster with 999 Monte Carlo hypothesis testing, with a level of significance at 0.05 (22).

We assessed seasonal characteristics of case time series using seasonal trend decomposition based on local regression (23). We analyzed environmental co-factors using generalized additive models applied to a quasi-Poisson distribution, accounting for overdispersion, with log of population as offset and geographic coordinates as bivariate spline smoothing (24). For multivariate analyses, we assessed environmental patterns using the classification and regression tree method (25). This approach retains only the main co-factors among the collinear factors, thereby generating a tree in which the terminal nodes represent classes of HZs with common characteristics. Incidence ratios between classes issued from classification and regression tree analysis were estimated using the generalized additive model. Statistical analyses were provided by using R software version 3.1.3 (The R Foundation for Statistical Computing, Vienna, Austria).

**Results**

**Global Morbidity and Mortality**

During 2004–2014, a total of 4,630 suspected plague cases and 349 deaths (CFR 7.54%) were recorded in Orientale Province, DRC (Figure 3). These findings differ from
WHO records (8,379 suspected cases and 464 deaths) and from the Ministry of Health database (5,153 suspected cases and 325 deaths) because we used a more restrictive definition of pneumonic plague and completed the database with line listings. Of these 4,630 cases, pneumonic plague accounted for 406 (8.8%) cases and 174 deaths (CFR 42.9%). Five pneumonic plague outbreaks were laboratory confirmed.

**Spatiotemporal Distribution of Human Plague**

Spatial scan analysis showed that almost all suspected plague cases were recorded in 3 clusters: Ganga HZ, Wamba HZ (both located in equatorial forest lowlands), and 5 HZs in the Ituri highlands (Fataki, Linga, Logo, Rethy, and Rimba) (Figure 2). Results for Ganga and Ituri clusters were statistically significant (p<0.05), but the result for Wamba was not (p = 0.053). Wamba and Ganga experienced pneumonic plague epidemics; Ituri highlands recorded almost all bubonic cases.

**Bubonic Plague**

During 2004–2014, among the 4,224 cases and 175 deaths resulting from bubonic plague, 3,369 (79.8%) cases and 127 (72.6%) deaths were reported in the 5 HZs in Ituri highlands (Figure 4). Because of security concerns, bubonic plague was rarely laboratory confirmed. Before 2008, RDTs were distributed (thanks to Institut Pasteur of Madagascar) and used in Rethy, Linga, and Rimba. During 2007–2008, a total of 99 of 201 RDTs were positive. After 2008, the remaining RDTs were saved for outbreak investigations.

In Ituri, bubonic plague cases were regularly notified, with seasonality explaining only 8% of the bubonic case time series variance (Figure 5). In 2007 and 2014, an increase in bubonic cases in Ituri highlands was followed by 2 smaller outbreaks of pneumonic cases. A field assessment in this area in April 2010 by several authors (A.A.A., J.-C.S., S.K.B.S., G.D., B.D., R.P.) highlighted various factors facilitating plague transmission (26,27). In particular, crops replaced cattle farming. Because of frequent thefts, seeds and food were stored in houses instead of within the traditional granaries built outside. The 50 dwellings visited were covered with vegetal roofs; >75% had >1 burrow on the ground; 90% of houses had 1 or 2 rooms; and 23 of 30 live rodents captured were caught inside the houses. Approximately 60% of inhabitants slept on the floor and were subject to flea bites.

**Pneumonic Plague**

We identified 3 main outbreaks of pneumonic plague: 1 in 2005 and 2 in 2006. In 2007 and in 2014, two smaller outbreaks of pneumonic cases were recorded after an increase in bubonic cases in Ituri highlands (Figure 3).

**Ganga HZ, 2005**

Bertherat et al. (8) described the first recorded pneumonic plague outbreak in this region. The first cases were reported in January 2005 in a diamond mining camp near Zobia village in the Ganga HZ; at the same time, Ituri highlands were experiencing an increase in bubonic plague (Figure 3). Among the 89 (62%) patients with known occupations, 55 were miners; the male:female ratio was 4.5:1. The CFR ranged from 40% to 70% during the first weeks and suddenly decreased to <5% after the intervention of Médecins Sans Frontières (from Belgium). With the restrictive case definition, the overall CFR was 50/112 (44.6%). No case of bubonic plague was reported in the pneumonic plague–affected areas. As described by Bertherat et al. (8), 18 of 87 samples were positive by RDT and 32 by Wayson staining; cultures were negative.

**Linga and Rethy HZs, 2006**

A second plague outbreak occurred in May 2006 in Linga and Rethy HZs (Figure 3). These HZs regularly recorded sporadic cases of bubonic plague, in contrast with Ganga HZ. The index case-patient was diagnosed in Buba, Linga HZ. The patient first exhibited buboes, but septic shock developed rapidly, and the patient died within 2 days. His funeral rites enabled the disease to spread quickly. During epidemiologic weeks 20–25, a total of 119 cases of pneumonic plague were recorded; CFR was 36%, with a male:female ratio of 1.1:1. Fifteen of 56 samples were positive by RDT and 9 by Wayson staining; 7 *Y. pestis* isolates were cultured (15).

**Wamba HZ, 2006**

The third plague outbreak started in Wamba HZ in August 2006 (Figure 3) in a gold mining camp. The first identified case was a miner who had recently arrived; pneumonic plague developed on August 14, and he died 3 days later. Once again, the funeral rites enabled plague to quickly spread to the neighboring HZs of Pawa and Boma-Mangbetu. During the first weeks of the outbreak, the male:female ratio was 2.9:1 (28), and CFR was 44% (120 cases, 53 deaths) in these 3 HZs. Of 96 specimens, 23 tested positive by RDT and 2 by Wayson staining; 4 *Y. pestis* isolates were obtained (9).

**Mahagi and Logo HZs, 2007**

An outbreak of both bubonic (163 cases, 14 deaths; CFR 9%) and pneumonic (34 cases, 17 deaths; CFR 50%) plague was recorded in Mahagi and Logo HZs during weeks 15–44, 2007 (Figure 3). Seven samples were positive by RDT.

**Logo HZ, 2014**

The last recorded outbreak of pneumonic plague occurred in Logo HZ (Figure 3) during June 8–July 19, 2014. A total
of 33 cases and 14 deaths were recorded, including 21 pneumatic cases (11 deaths, CFR 52%); 17 cases and 7 deaths were recorded in 3 villages only: Bika, Otha, and Jupathomba (male:female ratio 0.54:1). One patient was tested and confirmed positive by both RDT and culture.

**Environmental Analysis**

We conducted univariate analysis for environmental factors (Tables 1, 2). We retained variables at p<0.02 for multivariate analysis.

Classification and regression tree showed 1 at-risk significant class (class 2, p = 0.014) for bubonic plague, encompassing 7 HZs, characterized by having >72.3% of their territory located in tropical mountain ecosystems (Figure 6). The same 7 HZs formed a non-significant at-risk class (class 2, p = 0.43) for pulmonary cases. The Ituri cluster is included in these 7 HZs. Compared with other ecosystems found in Orientale Province, the tropical mountain ecosystem is characterized by a lower maximum value and a later peak month.
for NDVI, lower average and minimum temperatures, less annual precipitation, shorter growing period, and higher elevation (Figure 2; online Technical Appendix).

**Discussion**

Our study shows that plague remains present in northeastern DRC, with yearlong bubonic cases in the mountains of Ituri and outbreaks of pneumonic cases. These outbreaks, for which the estimated CFRs range from 36% to 52%, occurred in the mountains of Ituri and in the equatorial forests of the province.

Access to health facilities was possible for only 37% of the population in Orientale Province (12), and data completeness often was low except during epidemics. Therefore, plague incidence could be underestimated, especially for sporadic cases. Conversely, overestimation of cases and underestimation of CFR are likely during large epidemics because of the low positive predictive value of the case definition and the lack of systematic biological confirmation of cases (16). To better estimate the reliability of diagnosis in the reported suspected plague cases, Neerinckx et al. (7) suggested comparing the reported CFR with the 12% CFR in Madagascar used as a reference for studies in Africa. Cases were overreported during the 2006 Wamba and Rethy outbreaks (16). We therefore used a

![Figure 5. Time series decomposition using LOESS regression for bubonic plague, Orientale Province, Democratic Republic of the Congo, 2004–2014. Plague did not vary seasonally. The trend was decreasing after 2006. Remainder (residuals) explained 63% of model variance, trend 22% and seasonality 8% only.](image)

### Table 1. Results of univariate analysis of environmental variables of bubonic plague, Orientale Province, Democratic Republic of the Congo, 2004–2014*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>t value</th>
<th>p value</th>
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<td></td>
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<tr>
<td>Tropical</td>
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<td>0.02595</td>
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<tr>
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<td>Tropical mountain</td>
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<td>Elevation, m, mean</td>
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<td>0.001066</td>
<td>4.449</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>MODIS‡</strong></td>
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<td></td>
</tr>
<tr>
<td>Water</td>
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<td>0.309</td>
<td>-5.726</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Evergreen broadleaf forest</td>
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<td>Closed shrublands</td>
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<td>8.429</td>
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<td>0.1004</td>
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<td>&lt;0.001</td>
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<tr>
<td>Urban and built-up</td>
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<td>1.916</td>
<td>-4.159</td>
<td>&lt;0.001</td>
</tr>
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<td><strong>Rain accumulation, mm, TRMM3B43§</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
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<td>0.001643</td>
<td>5.044</td>
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<tr>
<td>SD</td>
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<td>0.268</td>
<td>-4.150</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Environmental variables with p<0.2 were kept for multivariate analysis. Negative estimates represent variables protective against plague; positive estimates represent variables increasing plague risk.
†Ecoclimatic zones defined by the Food and Agriculture Organization of the United Nations (http://www.fao.org/ag/AGAInfo/programmes/documents/livatl2/africaezmaps.htm). These ecosystems are built incorporating repeated measures of Normalized Difference Vegetation Index, temperature, rainfall, length of growing period, and elevation.
‡Moderate Resolution Imaging Spectroradiometer (https://lpdaac.usgs.gov/dataset_discovery/modis/modis_products_table/mod12q1).
Table 2. Results of univariate analysis of environmental variables of pneumonic plague, Orientale Province, Democratic Republic of the Congo, 2004–2014*

<table>
<thead>
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<th>Variable</th>
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</table>

Mean rain accumulation, mm, TRMM3B43§

<p>| | | | | |</p>
<table>
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</table>

*Environmental variables with p<0.2. These variables were kept for multivariate analysis. Negative estimates represent variables protective against plague, positive estimates represent variables increasing plague risk.
†Ecolimatic zones defined by the Food and Agriculture Organization of the United Nations (http://www.fao.org/ag/AGAInfo/programmes/documents/livat2/afirazzmaps.htm). These ecosystems are built incorporating repeated measures of Normalized Difference Vegetation Index, temperature, rainfall, length of growing period, and elevation.
‡Moderate Resolution Imaging Spectroradiometer (https://lpdac.usgs.gov/dataset_discovery/modis/modis_products_table/mcd12q1).

restrictive definition of pulmonary cases according to CFR, discarding cases registered at the end of the epidemic, when CFR dropped dramatically.

In the future, overestimation might be resolved by improving laboratory capacities or using RDT directly in the field. However, such an approach requires a prepositioning of test dipsticks and staff training, supervision, and equipment to appropriately collect and process samples (8). These tasks are difficult to implement in the chaotic context of eastern DRC, especially in gold and diamond mines.

During the study period, cases and deaths were much higher than the 800 cases and 56 deaths recorded in the same area during 1928–1960 (4). The collapse of massive plague control activities might partially explain this difference (29).

Before 1950, the Natal multimammate mouse (Mastomys natalensis) was the more frequent commensal host for plague vectors in this region; identified vectors included Xenopsylla cheopis and X. brasiliensis fleas (29,30). Black rats (Rattus rattus), which live closer to humans, were first observed in the highlands in 1958 (4). The human flea (Pulex irritans), which enables interhuman transmission, was introduced in about the same period and became the most frequent indoor flea in Ituri (29,30). These introductions, associated with armed conflicts in 1997–2003 and other ecologic changes, probably facilitated plague transmission to humans.

The field assessment we performed in April 2010 highlighted at-risk contexts similar to what had already been observed in nearby Uganda, where rodents were abundant in households and residents kept crops inside their huts (31) and had no bedding material (32). In the mining camps in forest areas, explosive outbreaks of pulmonary plague in the absence of preceding sporadic cases might be due to an importation of the disease from an endemic area (33–36). These outbreaks were accelerated by funeral rites, when attendees embraced the corpse. Plague also might have spread because of the precarious life of miner populations settled in overcrowded camps.

Conversely, in the mountains of Ituri, a succession of outbreaks and perennial notification of sporadic bubonic cases since 1928 demonstrated a permanent transmission of the disease. This finding suggests circulation of the plague bacterium in ≥1 zoontic host.

Human plague is an epiphenomenon of zoonotic plague, the incidence of which fluctuates in time and is modulated by numerous factors. In Tanzania, plague in rodents was linked to agricultural practices that lead to increased rodent...
populations but a lack of species diversity (37). In Madagascar, climate differently influenced plague transmission in rats (favored by urban areas, low temperature, and humidity) (38,39) and in wild rodents (favored by semiarid regions and the end of the dry season) (40). In Uganda, fewer fleas on rodents were observed during the dry season (41). Ituri is close to the equator (2°N), which can explain the lack of seasonality in human plague.

Plague in humans has been linked with behaviors and environmental hazards (1,42,43). In DRC, bubonic plague was associated with the tropical mountain ecosystem. This relationship should be interpreted cautiously, however, because all plague-endemic HZs are grouped in a single mountainous area. When a model based on the neighboring Uganda focus was applied, Ituri highlands were considered suitable for plague occurrence (44).

It would be interesting to study the Uganda focus and the adjacent Mahagi district as a single focus. Elevations, dry season temperatures, slopes, and landscapes differ slightly between this Mahagi–Uganda focus, which is located mostly in a dry tropical ecosystem, and the Ituri highland focus, which is even wetter and colder. In Uganda, the risk for plague also increased with wetness (44).

Identifying animal reservoirs or assessing genetic exchanges between \( Y. \) \textit{pestis}\ populations in both foci could be of great interest. The old and possibly extinct
focus near Lake Edward displays the same characteristics as the Ituri highlands. If security improves, it could also be useful to actively search for plague in rodents in this area.

Previous observations showed that the risk for plague tends to increase in highlands covered by savannas or meadows with a relatively dry climate (32,45). In Madagascar, the rural elevated districts act as permanent foci from which less favorable areas develop pulmonary epidemics (40). In DRC, the Ituri cluster, well summarized by a tropical mountain ecosystem, acted as a homeland for the plague. This region combines many of the previously described factors.

Because the Ituri focus is among the most active plague foci in the world and is probably at the origin of outbreaks of pneumonic plague that spread in the forest, extending the epidemiologic and ecologic studies of the plague in this focus is of paramount importance. As emphasized by Stenseth et al., “...plague should be taken much more seriously by the international community than appears to be the case” (46). The present-day priority should include establishment of a local and reactive surveillance system together with the improved rapid biologic confirmation of cases to earlier detect and better contain plague outbreaks (47).

Acknowledgments
We thank S. Moore, L. Osei, B. Faucher, and S. Rebaudet for their diligent editorial revisions.

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About the Author
Dr. Abedi is a medical doctor working at Direction de Lutte Contre la Maladie (Direction for Disease Control), Kinshasa, DRC. His primary research interests include transmissible diseases, particularly cholera, plague, and Ebola virus disease.

References
SYNOPSIS


Address for correspondence: Aaron Aruna Abedi, Direction de Lutte Contre la Maladie, Ministère de la Santé, 39 Av de la Justice, Kinshasa. Democratic Republic of the Congo; email: arunaaaron@yahoo.fr
Liver abscesses containing hypervirulent *Klebsiella pneumoniae* have emerged during the past 2 decades, originally in Southeast Asia and then worldwide. We hypothesized that hypervirulent *K. pneumoniae* might also be emerging in France. In a retrospective, monocentric, cohort study, we analyzed characteristics and outcomes for 199 consecutive patients in Paris, France, with liver abscesses during 2010–2015. We focused on 31 patients with abscesses containing *K. pneumoniae*. DOI: https://doi.org/10.3201/eid2402.170957
This bacterium was present in most (14/27, 52%) cryptogenic liver abscesses. Cryptogenic *K. pneumoniae* abscesses were more frequently community-acquired (p<0.00001) and monomicrobial (p = 0.008), less likely to involve cancer patients (p<0.01), and relapsed less often (p<0.01) than did noncryptogenic *K. pneumoniae* liver abscesses. *K. pneumoniae* isolates from cryptogenic abscesses belonged to either the K1 or K2 serotypes and had more virulence factors than noncryptogenic *K. pneumoniae* isolates. Hypervirulent *K. pneumoniae* are emerging as the main pathogen isolated from cryptogenic liver abscesses in the study area.

Pyogenic liver abscesses are common intra-abdominal infections (1–4), associated with a substantial severity (5). Most (30%–70%) pyogenic liver abscesses result from a biliary origin (6), followed by a portal origin (10%–20%) complicating intra-abdominal diseases, such as appendicitis, diverticulitis, infected gastrointestinal tumors, or chronic inflammatory bowel disease. Less frequently, pyogenic liver abscesses might also occur after abdominal surgery, typically pancreateo-duodenectomy associated with injury of the main hepatic artery or some aberrant hepatic arteries, split liver transplantation, or chemoembolization or ablation of liver tumors, or might result from the superinfection of preexisting hepatic lesions, such as hepatic cysts, tumors (primary or secondary), or hydatid cysts (<2%) (7,8). When none of these mechanisms are found, pyogenic liver abscesses are considered cryptogenic (no obvious cause); such abscesses account for ≈20% of cases in industrialized countries (9–12).

In most instances, pyogenic liver abscesses are polymicrobial, and *Escherichia coli* is typically the most common pathogen involved (13). Since the 1990s, pyogenic liver abscesses caused by specific hypervirulent strains of *Klebsiella pneumoniae* have emerged as a major epidemiologic problem in Southeast Asia and now represent ≥80% of pyogenic liver abscesses in Asia (11,14,15). In the past decade, cases of hypervirulent *K. pneumoniae* pyogenic liver abscesses have been reported worldwide, including in Europe and North America, in patients with no travel history to Asia (16–25).

Typically, hypervirulent *K. pneumoniae* are responsible for severe monomicrobial cryptogenic pyogenic liver abscesses (11) often associated with unusual septic metastatic localizations, such as endophthalmitis or meningitis, in immunocompetent hosts. Colonies of hypervirulent *K. pneumoniae* grown on agar plates have a hypermucoviscous phenotype, as shown by a positive result for a string test (11). These strains express genes encoding for virulence factors, such as the hypermucoviscous phenotype (*prmpA*), iron acquisition systems (*iutA, kfu, ybts*), and the capsular serotypes K1 or K2 (*magA* and *wzi*) (26,27).

After recent observations of hypervirulent *K. pneumoniae* pyogenic liver abscesses at Hôpital Beaujon (Cligny, France) and other medical centers in France (22), we hypothesized that hypervirulent *K. pneumoniae* pyogenic liver abscesses might also represent an emerging disease in France. Thus, we retrospectively analyzed the characteristics of *K. pneumoniae* pyogenic liver abscesses in a monocentric cohort of pyogenic liver abscesses during 2010–2015 and compared the characteristics of cryptogenic and noncryptogenic *K. pneumoniae* abscesses.

Methods

Study Population

We conducted a retrospective, monocentric, cohort study at Hôpital Beaujon, a 500-bed tertiary care university hospital on the outskirts of Paris. This hospital specializes in digestive and liver diseases and is 1 of 5 major regional centers specializing in liver diseases; the hospital serves a population of 12 million persons (~15% of the population of France). We reviewed all records from 2010–2015 in which a primary diagnosis was liver abscess. After records were made anonymous, we reviewed medical charts to confirm a diagnosis of liver abscess, which was defined by the association of typical clinical features (fever, abdominal pain); biologic abnormalities (inflammatory syndrome, increased bilirubin level); and lesions corresponding to an abscess observed by imaging (computed tomography scan, ultrasonography, or magnetic resonance imaging). Only patients with a microbiologically proven pyogenic abscess were included.

All medical records were reviewed by the same group of physicians, including clinicians (B.R., M.L.G., and A.L.) and a radiologist (M.R.), and cases were divided into 4 groups according to the suspected origin of the infection: biliary, portal, postprocedural, or superinfection of underlying liver diseases. In the absence of any of these conditions, pyogenic liver abscesses were considered cryptogenic. We then obtained *K. pneumoniae* pyogenic liver abscesses from each group and analyzed their epidemiologic, clinical, radiologic, and microbiological characteristics and their outcomes.

Identification and Characterization of *K. pneumoniae* Isolates

For all patients included in this study, microbiological identification was available by blood cultures or cultures of abscess drainage. At the time of disease, all strains had been identified by using the API System (bioMérieux, Marcy l’Étoile, France) or matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry (Bruker, Wissembourg, France), and antibiograms had been created. For this study, all available *K. pneumoniae* isolates underwent other phenotypic and molecular tests.
We assessed hypermucoviscosity by using the string test. A positive result for this test was defined as formation of a viscous string >5 mm when bacterial colonies are stretched on an agar plate (28). We determined capsular serotypes K1 or K2 and 8 virulence genes (a plasmid-borne regulator of the extracellular polysaccharide synthesis gene [prmpA], 4 iron-capture systems genes [aerobactin iutA, yersiniabactin ybtS kfu, and entrobackent entD], a capsular fucose production gene [wcaG] [29], an allantoin metabolism gene [allS], and a type-3 fimbrial adhesion gene [mrkD]) by using a multiplex PCR, as described (30). We performed multilocus sequence typing by using the international K. pneumoniae multilocus sequence typing scheme (http://bigdb.web.pasteur.fr/).

Statistical Analysis
We compared characteristics of K. pneumoniae cryptogenic and noncryptogenic pyogenic liver abscesses by using Student t-test or Wilcoxon rank-sum test for continuous variables and χ² or Fisher exact tests for categorical variables, when appropriate. A p value <0.05 was considered significant. We performed statistical analyses by using Stata version 13 (StataCorp LLC, College Station, TX, USA).

Results
A total of 216 patients had pyogenic liver abscesses during the study period. However, after analyzing all medical charts, we found that 199 pyogenic liver abscesses were definitively identified; these abscesses were further studied. Among the 199 abscesses, a microbiological diagnosis was available for 158 abscesses, which constitute the study cohort (Figure 1). Pyogenic liver abscesses were cryptogenic in 27 (17%) of 158 patients. K. pneumoniae was isolated from 31 (20%) of 158 patients and was more frequent in patients with cryptogenic pyogenic liver abscesses (14/27, 52%) than in those with noncryptogenic pyogenic liver abscesses (17/131, 13%; p = 0.00003). K. pneumoniae was the most common causative microorganism in the group with cryptogenic pyogenic liver abscesses (Figure 2).

We analyzed characteristics of patients having K. pneumoniae cryptogenic and noncryptogenic liver abscesses (Table 1). A total of 13/17 patients with K. pneumoniae noncryptogenic pyogenic liver abscesses had a biliary tract origin, and 2/17 showed development of pyogenic liver abscesses after liver transplantation (1 patient after cyst puncture and 1 patient after chemoembolization). Healthcare-related infections (12/17 vs. 0/14; p<0.00001) and cancer (7/17 vs. 0/14; p<0.01) were significantly more frequent for patients with noncryptogenic pyogenic liver abscesses than for patients with cryptogenic pyogenic liver abscesses. We found a tendency toward higher C-reactive protein levels for patients with K. pneumoniae cryptogenic pyogenic liver abscesses. We observed 7 relapses (41%) for the 17 patients with K. pneumoniae noncryptogenic pyogenic liver abscesses, compared with none for the 14 patients with cryptogenic pyogenic liver abscesses (p<0.01) (Table 1).

In the cryptogenic group, 3 patients were of Asian origin, compared with 1 in the noncryptogenic group. Three patients had severe septic metastases. The first of these patients was a 64-year-old woman with diabetes from Sri Lanka who had K1-type K. pneumoniae pyogenic liver abscesses, endophthalmitis, and brain abscesses. She survived after enucleation treatment. The second patient was a 57-year-old man with diabetes from Morocco who had K2-type K. pneumoniae pyomyositis associated with pyogenic liver abscesses. He was cured after 6 weeks of treatment with antimicrobial drugs. The third patient was a 33-year-old man from France who did not have any previous medical condition, but in whom K1-type K. pneumoniae acute tibial periostitis associated with pyogenic liver abscesses developed. He was cured after 6 weeks of treatment with antimicrobial drugs.

Microbiological Analysis
All 14 cryptogenic K. pneumoniae pyogenic liver abscesses but only 6/17 noncryptogenic cases were monomicrobial (p = 0.008). Among the 31 K. pneumoniae pyogenic liver abscesses, data for antimicrobial drug susceptibility were available for 13/14 cryptogenic isolates and 14/17 noncryptogenic isolates. A total of 23 (74%) of 31 isolates had been stored at –80°C and were further characterized: 13 (93%) of 14 from cryptogenic pyogenic liver abscesses and 10 (59%) of 17 from noncryptogenic pyogenic liver abscesses. We determined characteristics of these strains (Table 2).

All 13 K. pneumoniae isolates from cryptogenic pyogenic liver abscesses were confirmed to be hypervirulent K. pneumoniae; 10 had a positive string test result and a K1 (n = 10) or K2 (n = 3) capsular serotype. However, only 3/10 isolates from noncryptogenic pyogenic liver abscesses had a positive string test result (p<0.0005), and only 1 had a K2 serotype (p<0.00005). This K2 isolate had 3 virulence genes (prmpa, iutA, and ybtS); 2 pyogenic liver abscesses in the drainage area of an obstructed bile duct caused by pancreatic cancer developed in the patient infected with this strain.

We found the prmpA gene in all isolates from cryptogenic pyogenic liver abscesses but only in 1 isolate from noncryptogenic pyogenic liver abscesses (p<0.00005). Other virulence genes, such as iutA, ybtS, kfu, wcaG, and allS, were more common in isolates from cryptogenic pyogenic liver abscesses, and entD and mrkD were similarly present in both groups (Table 2). All K1 K. pneumoniae
had sequence type (ST) 23. We further noted new alleles of the gapA and rpoB genes, which resulted in a new ST (ST2395) (Table 2).

*K. pneumoniae* isolates from cryptogenic pyogenic liver abscesses were less likely to be antimicrobial drug resistant than were *K. pneumoniae* isolates from noncryptogenic pyogenic liver abscesses. A total of 54% of the isolates were wild type, and no extended-spectrum β-lactamase (ESBL) was detected in the 13 strains from cryptogenic pyogenic liver abscesses compared with 36% wild-type strains and 21% ESBL-producing strains in the noncryptogenic group.

**Discussion**

We studied 199 patients with liver abscesses managed in a single hospital during 2010–2015 and analyzed in detail 158 patients with microbiologically proven pyogenic liver abscesses. Although our study was retrospective, it had the advantage of particularly homogenous management in a monocentric university hospital specialized in gastroenterology, liver diseases, and abdominal surgery. The main finding was that hypervirulent *K. pneumoniae* are now responsible for most cryptogenic liver abscesses in patients in the Paris region in France.
Pyogenic liver abscesses caused by hypervirulent *K. pneumoniae* represented 14 (8.9%) of 158 liver abscesses, which is more frequent than previously described in Europe (16). Although hypervirulent *K. pneumoniae* have only rarely been described in Europe and North America (18,21,22,28,31), our results indicate that hypervirulent *K. pneumoniae* pyogenic liver abscesses might be considered as an emerging infection in the Paris area and should always be considered in the diagnosis of patients with pyogenic liver abscesses of no obvious origin. This factor is a key point because infections are often severe, metastatic locations might impair prognosis (especially for meningeal or ocular involvement), and immediate treatment with antimicrobial drugs is required.

We found a relatively low proportion (17%) of cryptogenic pyogenic liver abscesses, which might be related to the conditions of our hospital, which is a highly specialized tertiary care hospital. However, similar rates of cryptogenic abscesses were reported in the United Kingdom (7/42, 16.7%) (32). This finding suggests that epidemic diffusion of hypervirulent *K. pneumoniae* in Europe has not yet reached its peak, in contrast to what was observed in other regions, because cryptogenic pyogenic liver abscesses now represent as many as 34% of pyogenic liver abscess cases in Australia (33) and 65% of pyogenic liver abscess cases in Asia (34).

All *K. pneumoniae* isolates from these cryptogenic abscesses were hypermucoviscous (defined as a positive string test result) and belonged to K1 or K2 serotypes, thus having the characteristics of hypervirulent *K. pneumoniae*. Similar to Ye et al. (35), we observed a clonal diffusion of ST23 K1-type hypervirulent *K. pneumoniae*, in contrast to K2-type hypervirulent *K. pneumoniae*, which belong to different sequence types (ST65, ST86, ST380, and ST679) (35).

One patient with noncryptogenic pyogenic liver abscesses was also infected with a strain harboring phenotypic and genotypic characteristics of hypervirulent *K. pneumoniae*. This finding indicated that hypervirulent *K. pneumoniae* might also be involved in noncryptogenic pyogenic liver abscesses.

From a clinical point of view, our description of hypervirulent *K. pneumoniae*–related pyogenic liver abscesses is consistent with previous reports. These abscesses were mostly monomicrobial, rarely harbored organisms that showed antimicrobial drug resistance, occurred in persons with few
concurrent conditions, were not healthcare-related, and were more likely responsible for aggressive inflammatory disease with metastatic infectious locations \((17,36,37)\). Unlike a previous report \((17)\), diabetes did not appear to be a risk factor for hypervirulent \(K.\ pneumoniae\) pyogenic liver abscesses, and Asian patients did not represent the main ethnic group \((21\%\text{ in our study vs. } 50\%\text{ in cohorts in the United States})\). In addition, hypervirulent \(K.\ pneumoniae\)−related pyogenic liver abscesses were usually single and located in the right hepatic lobe \((17)\).

Although hypervirulent strains were involved and metastatic locations were frequent, outcomes were more favorable than for patients with noncryptogenic \(K.\ pneumoniae\) pyogenic liver abscesses, probably because patients with hypervirulent \(K.\ pneumoniae\)−related pyogenic liver abscesses were less likely to be immunosuppressed or have cancer. Thus, host factors seem to outweigh bacterial determinants in the prognosis of pyogenic liver abscesses \((38)\). However, a substantial mortality rate \((4\%–10\%)\) caused by these hypervirulent strains occurred and should not be underestimated \((17,36–38)\). As found in our study, antimicrobial drug resistance was rarely an issue for these hypervirulent strains. Nevertheless, ESBL- and carbapenem-resistant hypervirulent \(K.\ pneumoniae\) have been reported in Taiwan, China, India, and France and might represent an emerging problem in the future \((39–42)\).

The microbiological definition of hypervirulent \(K.\ pneumoniae\) is still ambiguous because none of the phenotypic (string test) or genotypic (capsular serotype and virulence genes) tests alone is specific for hypervirulence \((43–46)\). However, the combination of clinically aggressive monomicrobial cryptogenic pyogenic liver abscesses

### Table 1. Characteristics of 31 patients with cryptogenic or noncryptogenic \(Klebsiella pneumoniae\) liver abscess, Hôpital Beaujon, Clichy, France, 2010–2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>Noncryptogenic liver abscess, n = 17</th>
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</tr>
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<td>63</td>
<td>NS</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>9 (65)</td>
<td>12 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>F</td>
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</tr>
<tr>
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<tr>
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<td>5 (36)</td>
<td>8 (47)</td>
<td>NS</td>
</tr>
<tr>
<td>African</td>
<td>6 (43)</td>
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<tr>
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</tr>
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<tr>
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<tr>
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<tr>
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<td>1 (6)</td>
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<td>Malnutrition</td>
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<td>2 (12)</td>
<td>NS</td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fever</td>
<td>10 (71)</td>
<td>13 (76)</td>
<td>NS</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>6 (43)</td>
<td>8 (47)</td>
<td>NS</td>
</tr>
<tr>
<td>Severe sepsis</td>
<td>4 (29)</td>
<td>4 (24)</td>
<td>NS</td>
</tr>
<tr>
<td>Septic metastasis</td>
<td>3 (21)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>Biological features, median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>229 (246)</td>
<td>96 (64)</td>
<td>NS</td>
</tr>
<tr>
<td>Bilirubin, mg/dL</td>
<td>14 (10)</td>
<td>22 (14)</td>
<td>NS</td>
</tr>
<tr>
<td>Morphologic features</td>
<td></td>
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<tr>
<td>Multiple abscesses</td>
<td>4 (29)</td>
<td>8 (47)</td>
<td>0.3</td>
</tr>
<tr>
<td>Right liver localization</td>
<td>12 (86)</td>
<td>10 (59)</td>
<td>0.1</td>
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<td>Bacteriological features</td>
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<tr>
<td>Polymicrobial</td>
<td>0</td>
<td>11 (65)</td>
<td>0.008</td>
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<tr>
<td>Positive blood culture</td>
<td>8/9</td>
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<td>NS</td>
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<td>Positive pus culture</td>
<td>11/11</td>
<td>11/12</td>
<td>NS</td>
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<tr>
<td>Antimicrobial drug-resistance phenotype</td>
<td></td>
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<tr>
<td>Extended-spectrum β-lactamase</td>
<td>0/13</td>
<td>3/14</td>
<td>0.2</td>
</tr>
<tr>
<td>Drug susceptible</td>
<td>7/13</td>
<td>5/14</td>
<td>NS</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
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<tr>
<td>Death</td>
<td>0</td>
<td>4 (24)</td>
<td>0.08</td>
</tr>
<tr>
<td>Relapse</td>
<td>0</td>
<td>7 (41)</td>
<td>&lt;0.01</td>
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<tr>
<td>Follow-up, d, median (IQR)</td>
<td>177 (445)</td>
<td>391 (1,051)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Values are no. (%) or no. positive/no. tested unless otherwise indicated. NS, not significant \((p>0.05)\). IQR, interquartile range \((IQR \text{ is the difference between the 25th and } 75\text{th percentiles and is shown as a sample value})\).†Some patients had several causes of immunosuppression.
and isolation of positive string test–positive *K. pneumoniae* isolates with K1/K2 capsular serotypes and these genes involved in hypervirulence (e.g., *prmpA* and *iutA*) is highly suggestive of hypervirulent *K. pneumoniae*–related disease. We believe that it is the combination of this typical clinical and microbiological presentation that defines what might be called the hypervirulent *K. pneumoniae* syndrome.

Our study had several limitations. First, this study was monocentric and retrospective, which might limit its epidemiologic scope. Second, information concerning patients’ travel history was missing. Third, because our center is highly specialized in liver diseases, cryptocogenic hypervirulent *K. pneumoniae* pyogenic liver abscesses might be underestimated, and cancer or postsurgical pyogenic liver abscesses are probably overrepresented.

As the main microorganism responsible for cryptocogenic pyogenic liver abscesses, hypervirulent *K. pneumoniae* represents an emerging microorganism in the Paris area. The clinical and molecular differences between *K. pneumoniae* implicated in cryptocogenic and noncryptogenic pyogenic liver abscesses confirm the correlation between clinical and microbiological virulence and define a hypervirulent *K. pneumoniae* syndrome. This finding is a major epidemiologic shift that should be considered in the diagnostic and therapeutic management of patients with pyogenic liver abscesses without underlying or obvious causes.

Given the rapid increase in the prevalence of hypervirulent *K. pneumoniae* pyogenic liver abscesses in other parts of the world, it is possible that France and Europe might experience the same epidemic evolution as occurred in Asia in the early 2000s. Thus, clinicians need to be prepared for this possibility.

### About the Author

Dr. Benjamin Rossi is a senior fellow at Hôpital Beaujon, Clichy, France. His research interests are bacterial drug resistance and virulence.

### References


Address for correspondence: Agnès Lefort, Service de Médecine Interne, Hôpital Beaujon, 100 Blvd du Général Leclerc, 92110, Clichy, France; email: agnes.lefort@aphp.fr

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Alveolar and cystic echinococcosis are emerging and re-emerging in Europe, Africa, and Asia. The expansion of *Echinococcus* spp. tapeworms in wildlife host reservoirs appears to be driving this emergence in some areas. Recent studies suggest a similar phenomenon may be occurring in North America. We describe the context of *Echinococcus* spp. research in North America, with a specific focus on the contiguous United States. Although studies were conducted in the United States throughout the 1900s on various sylvatic and domestic *Echinococcus* spp. tapeworm cycles, data are lacking for the past ≈30 years. We review previous research, provide analysis of more recent focal studies, and suggest that *Echinococcus* spp. tapeworms, in particular *E. canadensis*, may be underrecognized. As a result, we suggest that additional research and surveillance be conducted for these tapeworms in wildlife host reservoirs across the United States.

*Echinococcus* spp. (family Taeniidae, class Cestoda) are zoonotic tapeworms currently infecting 2–3 million persons worldwide and causing US $200–$800 million in annual economic losses related to human infection (1,2). Infection appears to be increasing, reemerging, and geographically expanding in multiple locations across Europe, Asia, Africa, and the Americas (primarily in Latin America), with >200,000 new cases/year (3). *Echinococcus* spp. tapeworms have complex domestic and sylvatic life cycles that affect the health of >40 companion animal, livestock, and wildlife host species (4,5).

The basic *Echinococcus* spp. life cycle involves 2 hosts, where carnivores (wild and domestic) are the definitive hosts and small mammals and ungulates (domestic and wild) are the intermediate hosts (6). From within the small intestine of the definitive host, the mature tapeworm releases immediately infective eggs that are shed with the feces into the environment. Intermediate hosts ingest the eggs as they feed on contaminated vegetation. Once ingested, the oncosphere hatches and penetrates the small intestine to migrate to various organs and tissues, where it develops into one or more hydatid cysts (6). Definitive hosts ingest the cysts when feeding on the viscera of infected intermediate hosts (6). Humans are aberrant dead-end hosts that are infected from accidental ingestion of eggs, typically from interaction with domestic dogs, which act as bridge hosts between wildlife and the human environment. Contamination of the human environment may occur either directly (from feces) or indirectly (eggs carried on paws and fur) (7). Humans may also become infected through foodborne transmission, most often through eating inadequately washed fruits and vegetables (8). Intermediate hosts and humans may develop alveolar, cystic, or polycystic echinococcosis, depending on the parasite species involved (6).

Infection in livestock can cause substantial economic losses, including the condemnation of infected viscera; decreased meat, milk, and wool production; and delayed fecundity, growth, and performance (4). Estimated global economic loss due to infection in production animals is US $1.5–$2 billion annually (2). The global disease burden on wildlife species is unknown, as is the effect that echinococcosis may have on the overall fitness of wildlife animals and populations.

Throughout the early decades of the 1900s, only 1 species of *Echinococcus*, *E. granulosus*, was formally recognized in North America and across the world. Analysis of genetic and phenotypic data, host specificity and preference, and differences in human pathogenicity and tissue tropism has since revealed that *E. granulosus* sensu lato is actually composed of a complex of 10 specific genotypes, G1–10 (9). Subsequently, several of the genotypes or genotype complexes have been elevated to distinct species: *E. granulosus* sensu stricto (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. intermedius* (G6–G7), and *E. canadensis* (G8–G10) (9,10). Genotypes 6–7 are still often grouped with *E. canadensis*; however, distinct host preferences and genetics demonstrate the proper elevation to their own species (10). Additional recognized species of *Echinococcus* are *E. shiquicus*, *E. vogeli*, *E. felidis*, *E. oligarthra*, and *E. multilocularis*. *E. granulosus* sensu stricto and *E. multilocularis* tapeworms cause the 2 most pathogenic forms of disease in humans; *E. multilocularis* infections are the most deadly, given the metastatic nature of the cysts and the related difficulty of treatment.
Although well studied globally, the current presence, prevalence, and transmission dynamics of *Echinococcus* spp. tapeworms in the contiguous United States are currently unknown. Substantial research was conducted between the early 1930s and the 1980s; however, very little research has occurred in the past 3 decades. Given the documented expansion of these tapeworms across many regions of the world, including within Canada, a better understanding of the presence, prevalence, and disease ecology in the United States is needed, particularly because echinococcosis is not a reportable disease. Additional research will provide scientists, veterinary and human medical professionals, and other public health officials with a more complete picture of *Echinococcus* spp. cycles in the United States. Here we set the context for additional research through a synopsis of *Echinococcus* spp. and echinococcosis in humans in North America, with a focused discussion of endemic US *Echinococcus* spp. cycles from the early 1900s to the present. We conclude with recommendations for further research.

**Echinococcus** spp. Tapeworms and *Echinococcosis in Alaska and Canada*

Even before advances in molecular genetics, researchers recognized the form of *E. granulosus* cycling between wild canids and their ungulate prey species in Alaska and Canada as distinct and referred to it as the northern biotype (11). The northern biotype species is now recognized as *E. canadensis*. In the boreal regions of North America, *E. canadensis* are ubiquitous parasites of wild canids and ungulates from the western coast of Alaska through all territories and provinces of Canada, exclusive of its east coast (11–13). Caribou (*Rangifer tarandus*), moose (*Alces alces*), and elk (*Cervus canadensis*) appear to be the most important intermediate hosts, although other ungulate species may become infected. Wolves (*Canis lupus*) and domestic dogs are the most important definitive hosts; however, coyotes (*Canis latrans*) are also competent (11). Rausch’s (11) comprehensive review of *E. canadensis* tapeworms provides extensive information regarding the strongly endemic cycles he and others observed from the 1950s to the late 1990s throughout Alaska, Canada, and other Arctic countries. Likewise, readers are directed to Schurer et al.’s (14,15) reviews of unglulate and wolf infections.

All 3 reviews indicate fairly stable *E. canadensis* cycles and transmission dynamics in the Arctic and sub-Arctic regions of North America. For example, Rausch reported that in a randomly collected sampling of 200 wild canids in the Brooks Range of Alaska, ≈30% were infected with *E. canadensis* tapeworms. This finding is similar to Schurer et al.’s (15) recent report indicating infections in 37% (71/191) of wolves sampled across Canada. Rausch (16) found 24% (24/101) of moose infected in an agricultural region of southern Alberta, and 4% (1/23) of moose infected in the Anchorage area. Schurer et al.’s (14) review of ungulate infections in Canada revealed prevalence of 0%–73% in elk, 1–21% in caribou, 11–38% in moose, and 0.3%–44% in white-tailed deer (*Odocoileus virginianus*), depending on sample location.

*E. multilocularis* tapeworms are similarly endemic in Alaska and Canada, spanning from the northern Arctic regions south into the rural and urban southern provinces of Canada, where urban coyotes and domestic dogs have been confirmed as both definitive and aberrant intermediate (dog) hosts (7,13,17,18). As with *E. canadensis*, Rausch et al. (19) performed important work related to *E. multilocularis* in Alaska, with a particular focus on St. Lawrence Island. Their work revealed heavy infections in Arctic fox (*Vulpes lagopus*) and their small mammal prey species. The mean rate of infection for 1,579 fox examined was 77%; whereas infection in prey animals could range from 10% to 80% (19). On St. Lawrence Island, dog necropsies also revealed a 12% prevalence of infection (20).

Gesy et al.’s (21) recent review of *E. multilocularis* tapeworms across Canada demonstrated coyotes, red fox (*Vulpes vulpes*), wolves, and Arctic fox were all competent hosts, with prevalence differences related to sampling location. For example, their report indicated 37% (10/27) of coyotes and 17% (1/6) of red fox were infected in Quesnel, British Columbia (21). Three dogs in Canada were also recently found to be infected with cysts (7,13,17). The high prevalence in wild hosts along with the new intermediate infections in urban pet dogs has increased concern that the geographic reach of *E. multilocularis* tapeworms is expanding within Canada. As a result, Massolo et al. (22) suggest that much more research is necessary to understand potential public health risks associated with alveolar echinococcosis (AE) in North America.

Both AE and cystic echinococcosis (CE) have been reported in Alaska and Canada, with most infections occurring in Native American populations in Alaska. During 1940–1990, a total of 300 cases of CE were reported in Alaskan Native Americans, with only an additional 3 cases between 1990 and 1999 (23). Pathogenicity of CE in Native American patients appeared to be fairly benign, with smaller, asymptomatic cysts that often resolved without the need for surgery (23). However, Castrodale et al. (23) reported 2 unusually severe cases of CE in Alaska in 1999, in a 51-year-old Caucasian woman and a 17-year-old Native Alaskan woman. The cysts from the latter patient were confirmed as belonging to the species *E. canadensis* (G8) (23). Most AE cases in Alaska also arose in Native American populations, with St. Lawrence Island as an infection hotspot. Jenkins et al. (13) reported 54 human cases during 1947–1986, none during 1986–2010, and potentially 5 during 2010–2014, although the latter infections were more likely to be CE.
Human AE and CE cases in Canada are much less prevalent than those in Alaska. Serosurveillance of indigenous populations in the Saskatchewan, Nunavut, Quebec, and the Northwest Territories of Canada indicated exposure to CE of 0%–48% (20). CE infections in nonindigenous patients are rare and often only incidentally reported. AE cases in Canada are even more rare, with 1 autochthonous case reported before 2013 (20). Subsequent reviews have found 12–16 additional cases; however, Deplazes et al. (20) suggest these numbers are under representative, given the strongly endemic regions present in Canada.

**Echinococcus spp. Tapeworms and Echinococcosis in Mexico**

Far fewer studies have researched the *Echinococcus* spp. tapeworms present in Mexico; however, the primary species appears to be *E. intermedius* (G7), cycling between dogs and pigs (20). The species *E. granulosus* s.s. (G1) and *E. ortleppi* (G5) were also previously reported (20). Prevalence in slaughtered pigs is low, with reports of 0.27%–6.5%. Deplazes et al. (20) provide more extensive information regarding infections in Mexico.

**Echinococcus spp. Tapeworms and CE in the Contiguous United States**

**Domestic Cycles**

Historically, several genotypes of *E. granulosus* s.l. tapeworms were considered endemic and regularly cycling in the Mississippi Valley, the mid-Atlantic states, and multiple western and southwestern states. Franklin and Ward (24) reported that hydatid cysts were found in hogs and cattle in Virginia, Oklahoma, Arkansas, and Louisiana before 1951. They then reported the first discovery in Mississippi, finding that 2 of the 50 dogs they examined in 1951 were infected with *E. granulosus* s.l. tapeworms. Subsequently, Ward (25) reported that 6% of 8,066 slaughtered hogs in Mississippi were infected in 1956 and that 4 of the 9,300 dog intestines he examined over 8 years were infected with 300–700 adult *E. granulosus* cestodes each. At that time, 15 dogs had been confirmed with *E. granulosus* infection, with 5 of them from Mississippi (25). Given our current genetic understanding of *Echinococcus* spp., the species cycling in Mississippi was most likely *E. intermedius* (G7). Infections were also reported in dogs from New York, Kentucky, Georgia, and Tennessee, as well as Washington, DC (25). However, we were unable to find the originally cited papers to report the intermediate hosts involved and thus cannot comment on what species were present in those states.

Throughout the 1900s, an endemic dog/sheep cycle of CE also existed in the Central Valley of California. Through examination of the organs of 22,720 sheep carcasses during December 1967–June 1968, Sawyer et al. (26) found 1,100 infected sheep. Tracebacks conducted where possible demonstrated that infections could range from 5% to 99% of a producer’s entire herd (26). Some of the infected herds originated in Idaho and Utah, while the rest originated from 8 ranches in 4 California counties (26). For the producers with the highest herd prevalence, Sawyer et al. (26) investigated the ranches, took medical histories of the families, tested the onsite dogs for taeniids through arcoleine purging, and tested live sheep serum for evidence of infection. Resident domestic dogs and sheep were infected on 3 of the 4 ranches evaluated, implying local transmission. Local transmission indicates that the dogs were probably allowed to consume viscera of infected sheep, with subsequent egg contamination of the human environment through dog infections (27). Across the same 4 ranches, 3 humans also harbored hydatid cysts; however, only 1 of these cases was confirmed as acquired within California.

In Utah, Loveless et al. (28) investigated dog and sheep infections during 1971–1976, finding an overall prevalence of 11.3% (95/839) in dogs and 9.8% (877/8,994) in sheep. A subsequent study by Anderson et al. (29), with data covering 1971–1982, revealed a prevalence of 9.7% (109/1,120) in dogs and 7.1% (1,116/15,775) in sheep. During 1944–1980, Utah had the highest density of autochthonous human cases in the United States, with 50 persons infected (28). A similar dog/sheep cycle was established in New Mexico and Arizona (30). In those areas, human infections were reported during 1969–1976, revealing 21 confirmed autochthonous cases. Nineteen of the cases occurred in Native Americans of the Navajo, Zuni, and Santo Domingo tribes (30). The source of infection in all of these areas appeared to be contamination of the human environment by infected dogs. Risk factors for human infection included shepherding behavior; companion-animal ownership; home slaughter of sheep; allowing dogs to consume raw viscera; and contact with dogs, sheep, and swine (31,32). Although not confirmed, our current understanding of *Echinococcus* spp. tapeworms suggests that the organism causing the California, Utah, New Mexico, and Arizona cycles was most likely *E. granulosus* s.s.

More recent focal studies have revealed that cycles of *E. granulosus* s.l. continue to occur throughout the United States. For example, in 1994, Hoberg et al. (33) reported an unusual case of 3 cysts in a 14-year-old Thoroughbred horse that lived in Virginia and Maryland. The cysts were not sequenced to determine the genotype; however, those authors assert it was probably related to the species *E. equinus* (G4), as previously found in horses in Europe and Asia.

**Sylvatic Cycles**

Throughout the mid-1900s to late 1900s, a sylvatic coyote/deer cycle occurred concurrently with the domestic sheep/dog cycle in California (34,35). Brunetti and Rosen (34)
analyzed 2,049 deer carcasses collected between 1945–1969 and found an overall infection rate of 1.3%. The infected animals were concentrated in 8 counties in the Central Valley of California. Although unknown, the species involved in the sylvatic cycle may have been *E. canadensis* given the host species involved.

Riley (36) reported a sylvatic cycle between moose and wolves in Minnesota in the 1930s. Ramsey (37) reported that *E. canadensis* infection was found in a moose in northwestern Montana during 1976–1983. In 2009, Foreyt et al. (38) reported the presence of *E. canadensis* infections in wolves, elk, mule deer (*Odocoileus hemionus*), and a mountain goat (*Oreamnos americanus*) in Idaho and Montana; however, the specific genotypes (G8 or G10) were not delineated. In 2012, Lichtenwalner et al. (39) described the finding of an *E. canadensis* (G8)–infected moose in Maine.

The several sylvatic cycles reported in California, Montana, Idaho, and Minnesota indicate that *E. canadensis* tapeworms have been sporadically endemic in wild canid/ungulate populations across the United States since the first discovery in the early 1900s. With no further cases reported, the coyote/deer cycle appears to have ended in California. However, the cestode now appears to be reemerging in a wolf/ungulate cycle in the western United States.

### *E. multilocularis* and AE in the Contiguous United States

*E. multilocularis* tapeworms are also historically endemic in several northern states. During 1965–1969, Leiby et al. (40) examined 7,898 definitive and intermediate host mammals from eastern Montana, North and South Dakota, Minnesota, and Iowa. Among the definitive hosts, the authors found that 8.5% (131/1,540) of red fox and 4.1% (7/171) of coyotes examined were infected. In North Dakota, 55.3% of red fox were found infected in 1965, while only 7.3% were infected in 1968. No domestic cats (n = 35) or dogs (n = 88) were found to be infected (40). However, in a subsequent report, Leiby and Kritsky (41) reported that 2 adult house cats from a homestead in North Dakota were infected with *E. multilocularis* tapeworms in 1971.

Of the numerous potential intermediate hosts examined by Leiby et al. (40), only 3 species, the deer mouse (*Peromyscus maniculatus*), meadow vole (*Microtus pennsylvanicus*), and house mouse (*Mus musculus*), were found to have *E. multilocularis* cysts. Infected deer mice were found in all 5 states, with the highest number, 202/4,209 (4.8%), in North Dakota. Infected meadow voles were found only in Iowa and North Dakota, with 1.9% infected (20/1,033). Only 1/91 (1.1%) house mice examined, also from North Dakota, was infected. The reported infections may be an overly conservative estimate because data were obtained through visual examination of the carcasses for cysts; early or latent cysts may be too small for gross visualization.

In the 1980s, Ballard et al. (42,43) extended the known range of *E. multilocularis* tapeworms into Nebraska, Illinois, and Wisconsin, further indicating the endemic cycle in the north-central United States. Ten (27%) of 36 foxes were infected in Nebraska, 4 (10%) of 40 in Illinois, and 6 (8.3%) of 72 in Wisconsin (42). In the mid-1990s, the known range expanded again when *E. multilocularis* infection was found in Michigan and Ohio (44,45).

In 2000, Hildreth et al. (46) reported the results of a combined study of both wild canids and human trappers in South Dakota. The intestines of foxes trapped between 1987–1991, and of coyotes trapped between 1990–1991, were examined for the presence of *E. multilocularis* tapeworms. Results showed that 74.5% of foxes and 44.4% of coyotes were infected at the time of trapping. Because trappers are a population at extreme risk for AE, given their constant and extended contact with potentially infected wild canids, blood samples were obtained from 115 attendees of the South Dakota trappers meetings in 1990 and 1991. However, despite their high risk, none of the trappers showed evidence of infection.

Only 1 autochthonous case of AE has been confirmed in the contiguous United States, and that occurred in a 56-year-old woman from Minnesota (47). The patient had never lived outside of Minnesota, and her travel history included only California, Hawaii, Florida, and Manitoba, Canada. Gamble et al. (47) asserted that she was most likely to have been infected through interaction with pet dogs allowed to consume rodents on the farm where she grew up.

### Conclusions and Recommendations

In the past 3 decades, there has been little focus on *Echinococcus* spp. tapeworms in the contiguous United States in humans or animals. The apparent lack of concern regarding the potential public health threat posed by echinococcosis is probably due to the rarity of reported human cases. Similarly, since the 1980s, there have only been a handful of reports on the presence and prevalence of *Echinococcus* spp. tapeworms in domestic and wild animals in the lower 48 states. The lack of human cases may reflect low exposure to infected definitive hosts of either domestic or sylvatic origin. Alternatively, it may reflect lack of detection, misdiagnosis, or failure to publish literature regarding new AE and CE cases. Regardless of the reason, however, there is a legitimate concern that the public health risk may increase within the United States in the future, given the continued expansion of human infection in Europe and Asia, as well as an apparent expansion of the range of *E. multilocularis* tapeworms in Canada.

Current surveillance and basic scientific understanding of *Echinococcus* spp. tapeworms and echinococcosis in the United States is particularly lacking. In addition, there are many outstanding questions, including the presence,
prevalence, and geographic distribution of *Echinococcus* spp. tapeworms across the contiguous United States; geospatial, ecologic (both biotic and abiotic), and host population variables influencing sylvatic echinococcosis dynamics; and perhaps most important, what risk these sylvatic and domestic cycles pose to human health. As noted by Massolo et al. (22), Canada appears to be experiencing an increase in the presence and prevalence of *E. multilocularis* tapeworms, with spillover events starting to occur. If this asserted expansion is true, a similar expansion may be occurring in the United States. We support increased surveillance of *Echinococcus* spp. tapeworms in the United States to answer these questions and in particular to focus on the existing and potential public health risks associated with endemic *Echinococcus* spp. tapeworms. To enhance data collection on current and past cases, and further define the human burden of echinococcosis, we support and recommend the use of historically underused data sources, such as the National Inpatient Sample (48). We believe addressing the described data gaps is very important not only because of potential spillover from sylvatic cycles but also because echinococcosis is not reportable in either animals or humans in the United States and the United States does not screen for these types of infections in imported animals. If appropriate surveillance occurs that comprehensively analyzes current endemic cycles, then effective and efficient detection of new or expanding cycles or spillover events would be much more likely to occur, leading to a decrease in potential human cases or other negative public health outcomes.

**About the Author**

Ms. Cerda is an adjunct instructor in the School of Global Environmental Sustainability at Colorado State University. She studies disease ecology and the intersection of human, animal, and ecosystem health.

**References**


Address for correspondence: Jacey R. Cerda, c/o Lora Ballweber, Colorado State University, 200 West Lake St, 1682 Campus Delivery, Fort Collins, CO 80523, USA; email: Jacey.cerda@colostate.edu
We conducted an investigation of *Borrelia miyamotoi* infections in humans and ticks in northeastern China. Of 984 patients reporting recent tick bites, 14 (1.4%) were found to be infected with *B. miyamotoi* by PCR and genomic sequencing. The 14 patients had nonspecific febrile manifestations, including fever, headache, anorexia, asthenia, and arthralgia. Rash, eschar, and regional lymphadenopathy were each observed in 1 patient. Four (28.6%) patients were hospitalized because of severe disease. *B. miyamotoi* was detected in 3.0% (19/627) of *Ixodes persulcatus*, 1 (2.8%) of 36 *Hemaphysalis concinna*, and none of 29 *Dermacentor silvarum* ticks. Phylogenetic analyses based on sequences of a nearly entire 16s rRNA gene, a partial flagellin gene, and the glyc- erophosphodiester phosphodiesterase gene revealed that *B. miyamotoi* identified in patients and ticks were clustered in the group of the Siberian type. These findings indicate that *B. miyamotoi* is endemic in northeastern China and its public health significance deserves further investigation.

*Borrelia miyamotoi* is an emerging pathogen first identified in *Ixodes persulcatus* ticks and rodents from Japan in 1994 (1,2). Since then, this bacterium has been discovered in various other *Ixodes* tick species, including *I. ricinus*, *I. scapularis*, *I. pacificus*, *I. ovariatus*, and *I. pavlovskyi*, all of which are known for biting humans (3). Human infection with *B. miyamotoi* was first reported in Russia in 2011 (4) and subsequently in the United States (5,6), Europe (7,8), and Japan (9). *B. miyamotoi* disease usually manifests as a febrile illness characterized by fatigue, headache, chills, myalgia, arthralgia, and nausea (3,6).

Human-biting ticks are highly prevalent in northeastern China, where various emerging tickborne infections have been found in ticks and humans (10). However, some febrile patients with a recent tick bite could not have infection with the known tickborne pathogen diagnosed. We conducted an investigation of *B. miyamotoi* infections in patients and questing ticks to understand the potential threat of this bacterium to humans in this region.

**Materials and Methods**

**Patients and Data Collection**

Patients reporting a recent tick bite and who saw a doctor at Mudanjiang Forestry Central Hospital during May 2013–June 2015 were enrolled in the study. The hospital is one of the largest in Mudanjiang City, located in Heilongjiang Province of northeastern China (11). We administered a standardized questionnaire to each participant to collect demographic information, medical history, and environmental exposures. We retrieved data on clinical manifestations, underlying conditions, laboratory tests, treatment, and outcomes from medical records. Blood samples were collected on the day when the patient saw a doctor. All participants provided written or oral informed consent.

**Tick Collection**

During the study period, we collected questing ticks in the same region where the infected patients resided by dragging a flannel flag over vegetation. The 2 collection sites are forested highlands with elevations of 410 m and 550 m above sea level and harbor the same types of habitats as those areas where patients presumably were exposed to ticks (Figure 1). Approximately 20–35 ticks were collected per hour per person. All ticks were identified to the species level and developmental stage by an entomologist and stored at −20°C until DNA extraction.

**PCR Detection and Sequencing**

We used 200 μL blood from each patient to extract DNA with the QIAmp DNA Blood Mini Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer’s instructions. We
B. miyamotoi Infections, Northeastern China

ground each tick individually in a 200-μL phosphate-buffered saline buffer and extracted total DNA with the DNeasy Tissue Kit (QIAGEN). We used real-time PCR (rPCR) targeting of the 353-bp partial B. miyamotoi 16S rRNA gene (rrs) for screening blood and tick samples (4). The samples that were positive by rPCR were then subjected to amplification of a nearly full-length rrs, a partial flagellin gene (fla), and a partial glycerophosphodiester phosphodiesterase gene (glpQ) with specific primers (12; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/2/16-0378-Techapp1.pdf). In addition, we tested all samples by PCR for spotted fever group rickettsiae (13), pathogens in the Anaplasmataceae family (14), Borrelia burgdorferi sensu lato (15), and Babesia (11). To avoid risk for contamination, we performed template isolation, PCR setup, and agarose gel electrophoresis in separate rooms, and a negative control (distilled water) was concurrently included in each amplification. All the PCR products were purified with the QIAamp Gel Extraction Kit (QIAGEN) and then directly sequenced on an automated DNA sequencer (3730 DNA Sequencer; Applied Biosystems, Carlsbad, CA, USA). We compared the sequences obtained with previously published sequences deposited in GenBank by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic Analyses

We conducted phylogenetic analyses based on nucleotide sequences of rrs (1,400 bp), fla (506 bp), and glpQ (461 bp) by using the maximum-likelihood method in MEGA software version 6.0 (http://www.megasoftware.net). We applied bootstrap analysis of 1,000 replicates to assess the reliability of the reconstructed phylogenies.

Results

Identification of B. miyamotoi Infection in Patients

During the study period, we screened a total of 984 participants who had a recent tick bite and sought medical care by using a rPCR assay specific for B. miyamotoi. Fourteen patients were positive. Nucleotide sequences of the 353-bp amplicons for all patients were identical to one another and to the corresponding fragment of B. miyamotoi. The nucleotide sequences of nearly the entire rrs were obtained from 5 patients (patients 2, 3, 7, 8, 13) (Table); 4 of these were identical to one another (GenBank accession no. KU749372) and to an FR64b strain from a rat in Japan. The other sequence had 1-bp difference (GenBank accession no. KU749374) (Figure 2). We amplified and sequenced partial fla (GenBank accession no. KU749378) and glpQ (GenBank accession...
The patients with *B. miyamotoi* infection had influenza-like manifestations, such as fever, headache, anorexia, asthenia, and arthralgia (Table). Rash, eschar, and regional lymphadenopathy were each observed in 1 patient. Elevated hepatic aminotransferase levels, increased leukocyte count, and thrombocytopenia occurred occasionally.

Four patients (patients 1–4) (Table) were hospitalized because of severe disease with an irregular fever up to 38.4°C–39.7°C. The median length of hospital stay was 14 days (range 3–20 days). Two hospitalized patients received doxycycline (100 mg 2×/d); the other 2 inpatients and 10 outpatients were not treated with doxycycline because their infections were diagnosed retrospectively. Defervescence occurred 3–5 days after treatment, and all the clinical manifestations disappeared subsequently. No in-hospital deaths or clinically significant sequelae were noted in follow-up observations. Two patients were co-infected with *Candidatus Rickettsia tarasevichiae* (patients 4 and 5) and 1 with *Anaplasma capra* (patient 6). In comparison with the other 11 patients, the 3 patients co-infected with 2 tickborne pathogens had no more complicated symptoms or prolonged course of disease.

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**Table.** Epidemiologic, clinical, and laboratory testing features of 14 patients with *Borrelia miyamotoi* infections, northeastern China, May 2013–June 2015*

<table>
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<tr>
<td>Tick bite location</td>
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<td>Trunk</td>
<td>Neck</td>
<td>Ear</td>
<td>Leg</td>
<td>Trunk</td>
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<td>Scalp</td>
<td>Arm</td>
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<td>5.1</td>
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<td>0.9</td>
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<td>1.7</td>
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<td>1.2</td>
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<td>5.5</td>
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<td>8.3</td>
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<td>173</td>
<td>210</td>
<td>218</td>
<td>NA</td>
<td>79</td>
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<td>NA</td>
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<td>13.9</td>
<td>31</td>
<td>16.7</td>
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<td>Hospital stay (d)</td>
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<td>No</td>
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<td>No</td>
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<tr>
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<td>(3)</td>
<td>(15)</td>
<td>(20)</td>
<td></td>
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</table>

*AC, *Anaplasma capra*; ALT, alanine aminotransferase; AST: aspartate aminotransferase; CRT, *Candidatus Rickettsia tarasevichiae*; NA, not applicable; †, positive; −, negative.

*AC, *Anaplasma capra*; ALT, alanine aminotransferase; AST: aspartate aminotransferase; CRT, *Candidatus Rickettsia tarasevichiae*; NA, not applicable; †, positive; −, negative.

The 14 patients were distributed in 11 counties within the hospital catchment area (Figure 1). Ages ranged from 10 to 65 years (median 44.5 years), and 11 patients were male. All the patients had recent tick bites at various locations: 8 on the trunk, 3 on extremities, and 1 each on the scalp, ear, and neck (Table). Thirteen patients received a tick bite while they collected herbs in the forest, and 1 was bitten at home while taking care of goats that usually roamed in the forest during daytime. The median interval between known tick bite and illness onset ranged from 3 to 40 days (median 8.5 days). All 14 patients were immunocompetent and previously healthy.

no. KU749386) from 7 patients (patients 1, 2, 3, 7, 8, 9, 13) (Table); these sequences were 100% homologous to one another. The fla amplicons from all patients showed 1 bp difference with the corresponding sequence of the FR64b strain from Japan (Figure 3), whereas the glpQ amplicons had an identical sequence to that of FR64b (Figure 4).

**Epidemiologic and Clinical Characteristics of the Patients**

The 14 patients were distributed in 11 counties within the hospital catchment area (Figure 1). Ages ranged from 10 to 65 years (median 44.5 years), and 11 patients were male. All the patients had recent tick bites at various locations: 8 on the trunk, 3 on extremities, and 1 each on the scalp, ear, and neck (Table). Thirteen patients received a tick bite while they collected herbs in the forest, and 1 was bitten at home while taking care of goats that usually roamed in the forest during daytime. The median interval between known tick bite and illness onset ranged from 3 to 40 days (median 8.5 days). All 14 patients were immunocompetent and previously healthy.
Identification of *B. miyamotoi* in Ticks

We captured a total of 692 adult ticks: 627 *I. persulcatus*, 36 *H. concinna*, and 29 *D. silvarum*. We detected *B. miyamotoi* in 19 (3.0%) *I. persulcatus*, 1 (2.8%) *H. concinna*, and no *D. silvarum* ticks (online Technical Appendix Tables 2, 3). We then amplified the nucleotide sequences of nearly the complete *rrs* from 7 *I. persulcatus* ticks. Five of these were identical to one another (GenBank accession no. KU749373) and to those from 4 of 5 human patients tested (GenBank accession no. KU749372); however, the 7 ticks had 1-bp difference with the other 2 *I. persulcatus* ticks (GenBank accession nos. KU749375 and KU749376) and the other patient (GenBank accession no. KU749374) (Figure 2). The *fla* sequences from 6 *I. persulcatus* ticks were 100% homologous (GenBank accession no. KU749379) to those of patients; 1 sequence (GenBank accession no. KU749381) was different by 2 bp (Figure 3). The sequences of *glpQ* from the 8 *I. persulcatus* ticks (GenBank accession no. KU749384) were exactly the same as each other and those from patients (Figure 4).

Genetic Characteristics of *B. miyamotoi*

The phylogenetic trees based on nearly the complete *rrs* (1,400 bp), the partial *fla* (506 bp), and the partial *glpQ* (461 bp) sequences demonstrated that the *B. miyamotoi* identified from patients or ticks in this study were clustered in the same clade as those from Japan and Russia belonging to the Siberian type, which was distinct from both European and American types (Figures 2–4).

Discussion

The *B. miyamotoi* bacterium is a newly described emerging pathogen, which was known to be transmitted to human beings by *Ixodes* ticks in North America and Eurasia (6,16). Since the first identification of human infections in Russia in 2011, patients infected with *B. miyamotoi* have been sequentially diagnosed in the United States, Europe, and Japan (6). In this study, we report *B. miyamotoi* infections in a series of patients and in *I. persulcatus* and *H. concinna* ticks in northeastern China. These findings imply that persons who are exposed to ticks might run a risk of contracting *B. miyamotoi* disease. The dispersed geographic distribution of infected patients (Figure 1) indicates that *B. miyamotoi* might be widely prevalent in this region.

Although no clinically validated test is available for *B. miyamotoi*, the disease can be diagnosed by PCR performed on blood during acute-phase infection through amplifying and sequencing the *rrs*, *fla*, and *glpQ* genes. These PCRs are effective methods because of their high sensitivity and specificity for detecting *B. miyamotoi* DNA in either blood or tick samples, and they provide a valuable tool not only for the diagnosis of human infections but also for identification of *B. miyamotoi* in ticks (3,6). In this study, we used rPCR for detecting the 16S rRNA gene of *B. miyamotoi* in human and ticks. The sensitivity of the assay was reported to be up to 5 × 10^3 copies/mL as determined by using recombinant DNA of the *B. miyamotoi* 16S rRNA gene fragment with a known number of copies (4). We then sequenced all the 353-bp amplicons of rPCR to confirm the specificity of the test. The samples positive for rPCR were subjected to amplification of the nearly complete *rrs* and partial *fla* and *glpQ* genes. The phylogenetic analyses based on the 3 genes revealed that *B. miyamotoi* from patients and ticks were genetically homogeneous to one another and clustered in the same group with those from Japan and Russia, belonging to the Siberian type (3,4,6).

Human cases with *B. miyamotoi* infection have been diagnosed in Russia, the United States, European countries, and Japan (4–9). These reports imply its public health significance, given that human *B. miyamotoi* infection appears
to be comparable in frequency to human granulocytic anaplasmosis and babesiosis in some areas (6,17), and can occasionally lead to severe illness with neuroborreliosis and meningoencephalitis, especially in immunocompromised persons (5,8,18). The most commonly reported clinical manifestations of *B. miyamotoi* infection are fever, fatigue, headache, chills, myalgia, arthralgia, and nausea (6). The 14 patients in our study were immunocompetent and had similar manifestations observed in the previously reported cases in other countries (Table 1). Four patients (28.6%) were hospitalized for severe illness. *B. miyamotoi* infection should be included in the differential diagnosis of patients with a history of tick bite in areas where this pathogen has been identified in ticks or humans.

Up to now, *B. miyamotoi* has been found only in *Ixodes* ticks from wide geographic range, including Japan, Russia, the United States, Canada, and many countries in Europe, including the Czech Republic, Denmark, England, Estonia, France, Germany, the Netherlands, Poland, Sweden, and Switzerland (6,17,19,20). In this study, we detected *B. miyamotoi*...
not only in *I. persulcatus* ticks but also in *H. concinna* ticks captured in the patients’ residences. This finding indicates that both *Ixodes* and *Haemaphysalis* tick species might serve as competent vectors of *B. miyamotoi*. Unfortunately, we did not collect or record the species of the ticks attached to the patients who were seen at the hospital during the study period (May 2013–June 2015). According to our 2016 surveillance data, 93 of the 511 patients in the same sentinel hospital saw a doctor while the tick was still attached. These ticks were 80 (86.0%) *I. persulcatus*, 5 (5.4%) *H. concinna*, and 8 (8.6%) *D. silvarum*, roughly reflecting the abundance of the 3 tick species in the environment.

In conclusion, physicians should be aware of the presence of *B. miyamotoi* in northeastern China and make the differential diagnosis for patients with an exposure to ticks to attain an etiologic testing and effective treatment. The public health significance as well as potentially wider distribution of the emerging disease deserves further investigation.

This study was funded by the National Key R&D Program (2016YFC1201902, 2016YFC1200301, and 2016YFC1201905), the National Natural Science Foundation (81290344 and 81621005), and the State Key Laboratory of Pathogen and Biosecurity (SKLPBS1427) of China. The funders had no role in the study design, data gathering, analysis, interpretation, or writing of the paper. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**About the Author**

Dr. Bao-Gui Jiang is a scientist at the State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology. His research interests are the epidemiology and ecology of tickborne diseases.

**References**


Address for correspondence: Wu-Chun Cao or Wei Liu, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, 20 Dongda St, Fengtai District, 100071, Beijing, P.R. China; email: caowc@bmi.ac.cn or lwbiome@163.com
Plasmid-Encoded Transferable \textit{mecB}-Mediated Methicillin Resistance in \textit{Staphylococcus aureus}

Karsten Becker, Sarah van Alen, Evgeny A. Idelevich, Nina Schleimer, Jochen Seggewiß, Alexander Mellmann, Ursula Kaspar, Georg Peters

During cefoxitin-based nasal screening, phenotypically categorized methicillin-resistant \textit{Staphylococcus aureus} (MRSA) was isolated and tested negative for the presence of the \textit{mecA} and \textit{mecC} genes as well as for the SCC\textit{mec}-\textit{orX} junction region. The isolate was found to carry a \textit{mecB} gene previously described for \textit{Macrococcus caseolyticus} but not for staphylococcal species. The gene is flanked by \(\beta\)-lactam regulatory genes similar to \textit{mecR}, \textit{mecl}, and \textit{blaZ} and is part of an 84.6-kb multidrug-resistance plasmid that harbors genes encoding additional resistances to aminoglycosides (\text{aacA-aphD}, \text{aphA}, and \text{aadK}) as well as macrolides (\text{ermB}) and tetracyclines (\text{tetS}). This further plasmid-borne \(\beta\)-lactam resistance mechanism harbors the putative risk of acceleration or reacceleration of MRSA spread, resulting in broad ineffectiveness of \(\beta\)-lactams as a main therapeutic application against staphylococcal infections.

Staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec})–mediated \(\beta\)-lactam resistance resulting from production of an additional penicillin-binding protein (PBP) 2a drastically limits the treatment options in cases of hospital- and community-related infections by staphylococci, leading to increased illness, death, and socioeconomic costs (1,2). Besides methicillin-resistant coagulase-negative staphylococci, notorious for foreign body–associated infections, methicillin-resistant \textit{Staphylococcus aureus} (MRSA) strains are a global public health priority, despite some countries in Europe reporting stabilizing or decreasing MRSA rates (3–5). Since the initial reports of MRSA in 1961, several epidemic waves have resulted in threats of healthcare-, community-, and livestock-associated MRSA (6–9).

For staphylococci, 2 PBP 2a-encoding genes, \textit{mecA} and \textit{mecC}, including several allotypes, have been described as chromosomally located genetic bases for phenotypic methicillin resistance (10–14). In contrast, \textit{mecB}, originally described as \textit{mecA}\textnormal{\textsubscript{s}}, was reported as part of a probable primordial form of a methicillin resistance gene complex often found in a transposon \textit{mec} complex (Tn6045) in \textit{Macrococcus caseolyticus}, a colonizer of animal skin (15,16). Just recently, a \textit{mecD} gene, most closely related to \textit{mecB}, has been detected in bovine and canine \textit{M. caseolyticus} isolates (17).

The impact of plasmidborne resistance for staphylococci is abundantly demonstrated for \(\beta\)-lactamase–mediated penicillin resistance. Resistance rates are >60% in human \textit{S. aureus} isolates from the general population and >90% from hospital-related cases, regardless of the clinical background (18,19). In contrast to frequent interstrain and interspecies transmission of resistance plasmids by conjugation or transduction, only a relatively low rate of spontaneous horizontal transfer of SCC\textit{mec} elements is assumed, resulting in still-manageable and controllable MRSA rates if prevention measures are adequate (20–24).

However, transferable methicillin resistance might bear the consequence of an almost complete loss of \(\beta\)-lactam drugs as the most efficient class of antibacterial drugs for treatment of staphylococcal infections. Here, we report both a plasmid-encoded, and thereby transferable, methicillin resistance encoded by \textit{mecB} and the occurrence of this gene in an isolate of the genus \textit{Staphylococcus}.

Methods

Strain Detection and Identification

At the University Hospital of Münster, Germany, MRSA is generally cultured, identified, and differentiated by routine microbiological diagnostic methods using dextrose broth enrichment; chromID MRSA selective agar (bioMérieux, Marcy-l’Étoile, France), which contains cefoxitin; VITEK 2 automated system (bioMérieux) applying the antimicrobial susceptibility test card AST-P632; PBP2a detection kit (PBPa Culture Colony Test, Alere, San Diego, CA, USA); \textit{S. aureus}–specific PCR targeting \textit{mecA/ mecC} (GenoType MRSA, Hain-Lifescience, Nehren, Germany); and matrix-assisted laser desorption/ionization
time-of-flight mass spectrometry (Microflex-LT system, MALDI-Biotype 3.0; Bruker Daltonik, Bremen, Germany). In February 2016, an S. aureus isolate (which we numbered UKM4229) was recovered during routine MRSA screening. The isolate displayed a β-lactam–resistant phenotype without carrying the methicillin resistance genes mecA or mecC. For further characterizations, isolate UKM4229 was stored at −80°C and was cultivated on chromID MRSA agar (bioMérieux) at 37°C.

**Genetic Analysis**

We extracted genomic DNA from S. aureus isolate UKM4229 using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. We isolated plasmid DNA with the PrepEase Mini Spin Plasmid Kit (Affymetrix USB, Santa Clara, CA, USA) following the protocol standards. For both plasmid and genomic DNA, we applied lysostaphin (20 µg/mL) (Wakchemie, Steinbach, Germany) for bacterial cell lysis. We performed multilocus sequencing and spa gene typing initially as described elsewhere (25,26) and confirmed our results later by analysis of whole-genome sequencing (WGS) and DNA microarray data (discussed later in this article). We analyzed DNA sequences using RidomStaphType and SeqSphere+ (Ridom GmbH, Münster, Germany). Applying DNA microarray analysis (IdentiBAC Microarray; Alere Technologies GmbH, Jena, Germany), we identified resistance and virulence determinants and checked genotyping results.

**Molecular Confirmation of Methicillin Resistance**

Using PCR, we tested for the presence of methicillin resistance genes mecA and mecC (27,28) as well as mecB. DNA sequences of PCR oligonucleotides are given in Table 1. Oligonucleotides for mecB were made on basis of the plasmid pMCCL2 of M. caseolyticus (GenBank accession no. NC_011996.1). We performed PCR reactions using the following protocol for mecA: 5 min at 95°C; 40 cycles of 0.5 min at 95°C, 0.5 min at 55.5°C, and 0.75 min at 72°C; and final elongation of 7 min at 72°C. The protocol for mecB was 5 min at 95°C; 35 cycles of 0.5 min at 95°C, 0.5 min at 57°C, and 2.5 min at 72°C; and final elongation of 7 min at 72°C. The protocol for mecC: 5 min at 95°C; 40 cycles of 0.5 min at 95°C, 0.5 min at 59.3°C, and 2 min at 72°C; and final elongation of 7 min at 72°C.

**Antibiotic Drug Susceptibility Testing**

We determined the MIC of cefoxitin for S. aureus isolate UKM4229 by the reference broth microdilution method according to the International Organization for Standardization (ISO) 20776-1 guideline (https://www.iso.org/standard/41630.html), as required by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI). Cefoxitin (Sigma Aldrich, Taufkirchen, Germany) was tested in 2-fold concentrations (0.25–128 µg/mL). We subcultured the isolate and incubated it overnight before testing.

We investigated the susceptibility profile of UKM4229 by determining MICs of various β-lactam and non–β-lactam antibiotic drugs (Table 2) using the gradient diffusion method (Etest; bioMérieux) according to the manufacturer’s instructions. As recommended, the inoculated plates were incubated at 35°C for 18 ± 2 hours. In addition, we tested oxacillin using conditions for increased expression of methicillin resistance, as reported for mecA isolates (30): Mueller-Hinton agar supplemented with 2% saline, incubation at 30°C, and prolonged incubation up to 48 h. We investigated the applicability of a commercial automated susceptibility testing device to recognize methicillin resistance due to presence of mecB in S. aureus by using the VITEK 2 system. We evaluated the in vitro activity of the endolysin HY-133 against UKM4229 using the broth microdilution method in accordance with ISO 20776-1 guidance (https://www.iso.org/standard/41630.html), as described elsewhere (31,32). In brief, we tested 2-fold final concentrations of HY-133 ranging from 0.06 µg/mL to 8 µg/mL using 1–5 × 105 CFU/mL suspension of UKM4229 in cation-adjusted Mueller-Hinton broth. The MICs were read after incubation at 35°C for 18 ± 2 h.

We performed all experiments in triplicate on different days and calculated the median MIC values. We used S. aureus ATCC 29213 as a quality control strain on every testing day. For the antibiotic drugs we used, the MICs for the quality control strain were within acceptable limits throughout the testing.

**Whole-Genome Sequencing**

For the PacBio RS II platform (Pacific Biosciences, Menlo Park, CA, USA), we extracted staphylococcal DNA using the Genomic-tip 20/G Kit (QIAGEN) according to the manufacturer’s instructions, except that we

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**Table 1. Oligonucleotides used in study of methicillin resistance genes in Staphylococcus aureus**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Nucleotide sequence, 5' → 3'</th>
<th>Melting temperature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA</td>
<td>mec5</td>
<td>AAAAACTGATGGTAAGGGTTGGC</td>
<td>55.5°C</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td>mec6</td>
<td>AGTTCGCCAGTACCGGATTTCG</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>mecC</td>
<td>mecAL3</td>
<td>TCAAAATGGATTGGTTTCCATTATCA</td>
<td>59.3°C</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>mecAL4</td>
<td>AACTTGGTTATTCAAAAAGTAGGACGA</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>mecB</td>
<td>mecB-for</td>
<td>TTAACATATACACCGGCTTG</td>
<td>57°C</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>mecB-rev</td>
<td>TAAAGTTCTAGGCCACCTCC</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
applied lysostaphin (20 µg/mL) (Wakchemie) for bacterial cell lysis. We sequenced the extracted high-quality, double-stranded DNA (5 µg) using P6-C4 chemistry on

Table 2. Susceptibility to antimicrobial drugs of Staphylococcus aureus isolate UKM4229 from a 67-year-old cardiology inpatient who had no signs of infection, Münster, Germany†

<table>
<thead>
<tr>
<th>Antimicrobial class and agent</th>
<th>Median MIC, µg/mL</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactams</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>1.5</td>
<td>R</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3</td>
<td>R</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>6</td>
<td>S</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>3</td>
<td>S</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>12</td>
<td>R</td>
</tr>
<tr>
<td>Oxacillin†</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>Cephalosporins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>32</td>
<td>R</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>3</td>
<td>S</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>24</td>
<td>S</td>
</tr>
<tr>
<td>Cefepime</td>
<td>6</td>
<td>S</td>
</tr>
<tr>
<td>Ceftobiprole</td>
<td>2</td>
<td>S</td>
</tr>
<tr>
<td>Ceftaroline</td>
<td>0.5</td>
<td>S</td>
</tr>
<tr>
<td>Carbapenems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>Non-β-lactams</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycopeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>Lipoglycopeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telavancin</td>
<td>0.012</td>
<td>S</td>
</tr>
<tr>
<td>Lipopeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td>0.19</td>
<td>S</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.19</td>
<td>S</td>
</tr>
<tr>
<td>Macrolides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;256</td>
<td>R</td>
</tr>
<tr>
<td>Lincosamids</td>
<td>&gt;256</td>
<td>S</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;256</td>
<td>S</td>
</tr>
<tr>
<td>Oxazolidones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>Rifamycins</td>
<td>0.008</td>
<td>S</td>
</tr>
<tr>
<td>Phosphonic acid derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>&lt;0.064</td>
<td>S</td>
</tr>
<tr>
<td>Streptogramins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinupristin/dalfopristin</td>
<td>0.5</td>
<td>S</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12</td>
<td>R</td>
</tr>
<tr>
<td>Glyclycyclines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.125</td>
<td>S</td>
</tr>
<tr>
<td>Folate pathway inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>0.047</td>
<td>S</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>24</td>
<td>R</td>
</tr>
<tr>
<td>Pseudomonic acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mupirocin</td>
<td>0.19</td>
<td>S</td>
</tr>
<tr>
<td>Fusidanes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.094</td>
<td>S</td>
</tr>
<tr>
<td>Bacteriophage endolysins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HY-133</td>
<td>0.25</td>
<td>S</td>
</tr>
</tbody>
</table>

*Tested by using the gradient diffusion method. S, susceptible; R, resistant (according to EUCAST [www.eucast.org] for antibiotic drugs with available breakpoints)

†Conditions: 2% NaCl, 30°C; 18 h/48 h. MIC at regular reading after 18 ± 2 h/MIC after 48 h.

Results

During routine MRSA screening, we recovered an S. aureus isolate UKM4229 from a combined nasal-throat swab of a 67-year-old male cardiology inpatient who had no signs of infection. We isolated colonies with typical appearance for presumptive MRSA from a chromogenic MRSA selective agar and identified them as S. aureus by VITEK 2, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and PCR. Discrepancies between phenotypic detection of methicillin resistance by VITEK 2 and negative results of a PBP2a detection kit, as well as negative mecA and mecC test results, by commercial and in-house PCRs led to the detection of a mecB-encoded methicillin resistance.

S. aureus isolate UKM4229 showed a median MIC of 32 µg/mL for cefoxitin, as determined by broth microdilution and gradient diffusion tests. The MICs of other antibiotics, as well as correspondent interpretative categories, are shown in Table 2; the resistance gene profile is given in online Technical Appendix Table 1 (https://wwwnc.cdc.gov/EID/article/24/2/17-1074-Techapp1.pdf). Optimal oxacillin testing conditions previously reported to increase expression of methicillin resistance in mecC isolates (30) unexpectedly led to lower oxacillin MIC values for UKM4229 (Table 2). A novel anti–S. aureus agent in development, the recombinant phage endolysin HY-133 (Hyglos, Bernried, Germany) (31,32), was also active. VITEK 2 recognized mecB-associated methicillin resistance by oxacillin MIC determination and cefoxitin screening.

Although mecA/mecC PCR did not yield amplicons, mecB-specific PCR applying total and plasmid DNA resulted in a PCR product similar to those of M. caseolyticus isolate AM20CR01 (from a clam; isolate provided by J.E. Rubin, Institute of Veterinary Microbiology, University of Saskatchewan, Saskatchewan, Canada). Comparative analysis of the DNA sequence revealed complete sequence identity (100%) with reported mecB genes located either on plasmid pMCCL2 (M. caseolyticus JCSC5402; GenBank accession no. NC_011996.1) or within the SCCmec-like element of M. caseolyticus JCSC7096 (accession no. AB498756.1) (15,16). Apart
Within the pSAWWU4229_1 plasmid, mecB was flanked by β-lactam regulatory genes similar to mecR, mecI, and blaZ (nucleotide identities: 99.9%, mecRm from M. caseolyticus JCSC7096; 100%, mecIm from M. caseolyticus JCSC7096 and 100%, blaZm from M. caseolyticus JCSC7096). pSAWWU4229_1 contained additional antibiotic drug resistance genes encoding resistance to aminoglycosides (aacA-aphD, aphA, and aadK), as well as macrolides (ermB), tetracyclines (tetS), and streptothricin (sat), all located in the same gene section. This particular region of the plasmid showed similarities with the transposon Tn551 of S. aureus 4578 (GenBank accession no. LC125350.1; blastn 2.7.0+ maximum score 11,064; query coverage 10%; identity 99%) (34). The sequences shared 48.9% nucleotide identity (global alignment using Stretcher [Emboss], Matrix EDNAFULL; gap penalty 16, extend penalty 4). Mating-pore genes or genes responsible for the DNA transfer suggesting self-transmission or mobilization properties of pSAWWU4229_1 were not detected. Genotyping revealed that S. aureus isolate UKM4229 belonged to multilocus sequence typing type ST7 and spa-type t091 (spa-CC 091).

Figure. Circular map of the mecB-carrying plasmid pSAWWU4229_1 from Staphylococcus aureus isolate UKM4229, obtained from a 67-year-old cardiology inpatient who had no signs of infection, Münster, Germany. Arrows indicate annotated genes: the mec-complex is noted in green, antibiotic resistance genes in red, transposase/integrase genes in orange, other genes with known function in violet, and other genes with unknown function in gray.
DNA microarray analysis and WGS revealed the isolate possessed the leucotoxin genes lukF, lukS, lukD, lukE, lukK, and lukY. The isolate belonged to capsule type 8, and the biofilm-associated genes icaA, C, and D were detected. Furthermore, the hlb-converting bacteriophage of immune-evasion cluster type G comprising the enterotoxin encoding genes sep, sak, and scn was present in the genome. Additional information about the virulence profile of this isolate is given in online Technical Appendix Table 2.

Discussion

Recent studies have shown that mobile SCCmec elements have been imported more frequently by different S. aureus clonal lineages than previously assessed (35). Nevertheless, in contrast to the huge diversity of non-MRSA S. aureus clonal lineages (36), comparatively few clonal lineages still dominate the global MRSA population (37). However, an increased transferability of methicillin resistance by a plasmid-encoded course of action would have the capacity to drastically change the MRSA epidemiology. In staphylococci and other members of the phylum Firmicutes, plasmids have contributed enormously to the emergence and spread of antimicrobial resistance, and plasmid-encoded penicillin resistance has reached or exceeded 80% of clinical staphylococcal isolates (38).

In M. caseolyticus, mecB genes have been found within the chromosome as part of an SCCmec element as well as on a plasmid (15,16,39). For S. aureus UKM4229, it was shown that the mecB carrying plasmid pSAWWU4229_1 was distantly related to a macrococal plasmid (pMCLL2 of M. caseolyticus JCSC5402), substantiating a possible gene transfer between the two genera. Because macrococal and staphylococcal species may share the same hosts, mammalian skin and food, an exchange of mobile genetic elements between members of both closely related genera is likely and transmission to mammal-adapted staphylococci is generally to be feared (3). Genotyping of S. aureus UKM4229 revealed spa-type t091, which is relatively common, as 0.92% of the >370,000 submitted spa sequences assigned to ≥17,000 spa types (as of February 2017) of the RIDOM SpaServer database (http://spa.ridom.de/spatypess.html) belong to this spa type.

Routine phenotypic methods for susceptibility testing cannot distinguish between methicillin resistance determinants; thus, mecB-encoded methicillin resistance can remain undiscovered. Moreover, mecB detection is not part of molecular screening approaches. Certain clonal lineages of S. aureus, including MRSA, have emerged as zoonotic pathogens colonizing farm animals and wild animals (40). Tetracycline resistance frequently observed in staphylococci associated with husbandry is another indication for a possible livestock origin of the isolate (41). A putative livestock source of the mecB-encoding plasmid underlines the importance of the One Health concept in combating the spread of antimicrobial drug resistance.

Although the mecB isolate has been tested susceptible toward several agents of non-β-lactam antibiotic drug classes, the generally increased risk, compared to that of a SCCmec transfer, should be taken into consideration in that a mecB-encoding plasmid will be transmitted through horizontal gene transfer to other staphylococcal strains, even to already multidrug-resistant strains. In S. aureus, 2 major means of horizontal gene transfer for plasmids have been described: conjugation and bacteriophore transduction. Here, pSAWWU4229_1 did not harbor the typical genes responsible for conjugation or mobilization, which is, however, a common lack in S. aureus, affecting ≈95% of plasmids (42). In contrast, for most staphylococcal plasmids, a transfer through bacteriophage generalized transduction has been suggested (43,44). Further studies are warranted to underpin this putative threat and to investigate how a plasmidborne methicillin resistance would affect the SCCmec-based methicillin resistance.

For UKM4229, the WGS data revealed that the SCCmec chromosomal attachment site (attB) locus and the neighboring orfX (rlmH) gene were intact, and no integration of an SCCmec element was found.

The mecB isolate was tested to be susceptible to cefotibiprole and ceftaroline. Although cephalosporins with anti-MRSA activity are still active against the majority of MRSA isolates, nonsusceptibility has been already associated with certain MRSA lineages ranging between 3.9% and 33.5% of all MRSA isolates (45–48).

The discovery of plasmid-encoded methicillin resistance in S. aureus of probably macrococal origin in a healthcare setting reveals a novel level of risk of the transfer of broad β-lactam resistance in staphylococci. Further studies are needed to clarify the real prevalence of mecB-mediated methicillin resistance among MRSA and methicillin-resistant coagulase-negative staphylococci in human and animal populations, whether mecA and mecC genes could be found integrated on plasmids, and how the answers to these questions may affect human and animal health.

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supervising the routine diagnostics leading to the isolation and presumptive identification of the isolate, as well as Melanie Bach and Martina Schulte for excellent technical assistance.

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About the Author
Dr. Becker is a professor of medical microbiology at the Institute of Medical Microbiology, University Hospital Münster, Münster, Germany. His research is focused on the epidemiology, pathogenesis, diagnosis, prevention, and therapy of staphylococcal infections. In particular, he has done extensive research on the characterization of MRSA and the staphylococcal small colony-variant phenotype.

References


Address for correspondence: Karsten Becker, University Hospital Münster—Institute of Medical Microbiology, Domagkstr. 10, D-48149 Münster, Germany; email: kbecker@uni-muenster.de
Seoul virus (SEOV) poses a worldwide public health threat. This virus, which is harbored by *Rattus norvegicus* and *R. rattus* rats, is the causative agent of hemorrhagic fever with renal syndrome (HFRS) in humans, which has been reported in Asia, Europe, the Americas, and Africa. Defining SEOV genome sequences plays a critical role in development of preventive and therapeutic strategies against the unique worldwide hantavirus. We applied multiplex PCR–based next-generation sequencing to obtain SEOV genome sequences from clinical and reservoir host specimens. Epidemiologic surveillance of *R. norvegicus* rats in South Korea during 2000–2016 demonstrated that the serologic prevalence of enzootic SEOV infections was not significant on the basis of sex, weight (age), and season. Viral loads of SEOV in rats showed wide dissemination in tissues and dynamic circulation among populations. Phylogenetic analyses showed the global diversity of SEOV and possible genomic configuration of genetic exchanges.

**Hantaviruses** (order *Bunyavirales*, family *Hantaviridae*, genus *Orthohantavirus*) pose a worldwide public health threat and are the causative agents of hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome in the Americas (1). HFRS is caused mainly by Old World hantaviruses, such as Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava–Belgrade virus, and Puumala virus, that are transmitted to humans by inhalation of dust contaminated with rodent excreta (saliva, urine, and feces) or bite by an infected rodent. Annually, 150,000 cases of HFRS are reported (case-fatality rate range <1%–15%) (2). Clinical signs and symptoms include headache, myalgia, abdominal and back pain, nausea, vomiting, diarrhea, proteinuria, thrombocytopenia, hemorrhage, and renal failure (3). The typical disease course consists of 5 phases: febrile, hypotensive, oliguric, diuretic, and convalescent: the phases vary in length from several hours to several days. A difficulty in diagnosis is the extensive incubation period from the time of exposure to the onset of symptoms, which might be as long as 50 days. There are no effective vaccines or antiviral agents against hantavirus infection.

SEOV has a negative-sense, single-stranded, tripartite RNA genome (4). The large (L) segment encodes an RNA-dependent RNA polymerase, the medium (M) segment encodes 2 membrane glycoproteins (Gn and Gc), and the small (S) segment encodes a nucleoprotein. Brown rats (*Rattus norvegicus*) and black rats (*R. rattus*) are the primary reservoir hosts of SEOV and have a worldwide distribution (5,6).

SEOV infections have been reported in Asia, Europe, the Americas, and Africa (7–12). HFRS caused by SEOV is responsible for 25% of clinical cases and is a mild form with a case-fatality rate of <1% in Asia (13). Recently, an outbreak of SEOV-induced HFRS was reported in the United Kingdom among rat owners, breeders, and distributors of the pet animal market (14). In the United States, outbreaks of SEOV infections occurred in 11 states in 2017; there were 17 confirmed SEOV-infected patients (15,16). SEOV was identified in New York, New York, and is considered an urban public health threat (17).

Whole-genome sequencing of SEOV is a prerequisite for tracking SEOV infections and evaluating disease risks for development and implementation of preventive and therapeutic strategies. Acquisition of viral genome sequences plays a critical role in surveillance, identification, and risk mitigation of outbreaks of virus infection (18). Next-generation sequencing (NGS) is a potent tool for defining

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**Multiplex PCR–Based Next-Generation Sequencing and Global Diversity of Seoul Virus in Humans and Rats**

Won-Keun Kim,1 Jin Sun No,1 Seung-Ho Lee, Dong Hyun Song, Daesang Lee, Jeong-Ah Kim, Se Hun Gu, Sunhye Park, Seong Tae Jeong, Heung-Chul Kim, Terry A. Klein, Michael R. Wiley, Gustavo Palacios, Jin-Won Song

Author affiliations: Korea University, Seoul, South Korea (W.-K. Kim, J.S. No, S.-H. Lee, J.-A. Kim, J.-W. Song); Agency for Defense Development, Daejeon, South Korea (D.H. Song, D. Lee, S.H. Gu, S. Park, S.T. Jeong); 65th Medical Brigade/Medical Department Activity–Korea, Seoul (H.-C. Kim, T.A. Klein); US Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, USA (M.R. Wiley, G. Palacios)

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1These authors contributed equally to this article.
virus genome sequences. However, an obstacle for obtaining virus genomic information is ultra-low virus RNA loads in the clinical specimens. To enrich the low amount of viral RNA, we developed a multiplex PCR–based NGS that showed high coverage of HTNV genome sequences from HFRS patients (19).

In this study, we collected 1,269 *R. norvegicus* rats in an urban HFRS-endemic area in South Korea during 2000–2016. We report a robust strategy for whole-genome sequencing of SEOV and provide useful insights into epidemiologic characteristics and phylogeographic diversity of a unique worldwide hantavirus.

**Materials and Methods**

**Ethics**

Human samples were provided after informed consent was obtained. The study was approved and conducted in accordance with ethics guidelines for the Korea University Institutional Animal Care and Use Committee. Live trapping of rats at US military training sites and installations was approved by US Forces Korea in accordance with regulation 40–1 (Prevention, Surveillance, and Treatment of Hemorrhagic Fever with Renal Syndrome). Rats were humanely killed by cardiac puncture, and tissues were collected under isoflurane anesthesia in accordance with procedures approved by Korea University Institutional Animal Care and Use Committee protocol #2010–212.

**Sample Collection**

We tested retrospective HFRS patient serum samples obtained from the Korea Bank for Pathogenic Viruses (Seoul, South Korea). We collected *R. norvegicus* rats during 2000–2016 by using collapsible live-capture traps (Tomahawk Live Trap Co., Hazelhurst, WI, USA, and H.B. Sherman, Tallahassee, FL, USA). Traps were set at intervals of 1–2 m and examined early the next morning over a 1–2-day period at US Army training sites. For the US Army Garrison in Seoul, we used baited live capture traps (Tomahawk Live Trap Co.) or glue boards. Captured rats were submitted to the 5th Medical Detachment/Medical Command Activity–Korea, US Army Garrison (Yongsan, Seoul), and then transported to the College of Medicine, Korea University (Seoul), where they were held in a Biosafety Level 3 laboratory until processing. Live rats were humanely killed by cardiac puncture under isoflurane anesthesia and identified to species by using morphologic criteria and PCR, when required. Serum, lung, spleen, kidney, and liver tissues were collected aseptically and frozen at −70°C until used.

**Indirect Immunofluorescence Antibody Test**

We used an indirect immunofluorescence antibody (IFA) test for serum samples from HFRS patients and live rats. We initially diluted samples 1:32 in phosphate-buffered saline and then tested them for IgG against SEOV. We applied diluted serum samples to slides containing SEOV-infected Vero E6 cells fixed with acetone and incubated wells at 37°C for 30 min. The slides were washed, fluorescein isothiocyanate–conjugated goat antibody to human and rat IgG (ICN Pharmaceuticals, Laval, Quebec, Canada) was added, and slides were incubated at 37°C for 30 min. We then washed the slides again and examined them for virus-specific fluorescence by using a fluorescent microscope (Axio Scope; Zeiss, Berlin, Germany).

**Real-Time Quantitative PCR**

We performed real-time quantitative PCR (qPCR) for total RNA by using the high-capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) in a 10-µL reaction mixture containing 1 µg of total RNA. We used an SYBR Green PCR Master Mix (Applied Biosystems) in a StepOne Real-Time PCR System (Applied Biosystems). We performed reactions at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, and then 1 cycle at 60°C for 1 min. Primer sequences specific for SEOV S segments were SEOV-S719F: 5′-TGCGACTAGCAAAAGACTGG-3′ and SEOV-S814R: 5′-CAGATAAUCTCCAGCAATAGGA-3′.

**Reverse Transcription and Rapid Amplification of cDNA Ends PCR**

We extracted total RNA from serum or lung tissues of seropositive samples by using TRI Reagent Solution (Ambion Inc., Austin, TX, USA). We synthesized cDNA by using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) and random hexamer or OSM55 (5′-TAGTAGTAGTAGAATTC-3′). For initial identification, we used oligonucleotide primers for SEOV L segment as described (20). To obtain the 3′ and 5′ termini genome sequences of SEOV, we performed rapid amplification of cDNA ends (RACE) PCR by using a 3′-Full RACE Core Set and a 5′-Full RACE Core Set (Takara Bio Inc., Shiga, Japan), according to the manufacturer’s specifications. We purified PCR products by using the LaboPass PCR Purification Kit (Cosmo Genetech, Seoul, South Korea). We performed sequencing in both directions of each PCR product by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automated sequencer (Applied Biosystems).

**Multiplex PCR–Based NGS**

We designed multiplex PCR primers for SEOV L, M, and S segments and amplified cDNA by using primers (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/24/2/17-1216-Techapp1.xlsx) and primer mixtures and Solg 2× Uh-Taq PCR Smart Mix (Solgent, Daejeon, South Korea), according to the manufacturer’s instructions.
We performed the first and second enrichments in a 25-µL reaction mixture containing 12.5 µL of 2× Ul reaction pre-mix, 1 µL of cDNA template, 10 µL of primer mixture, and 1.5 µL of distilled water. Initial denaturation was at 95°C for 15 min, followed by 40 cycles or 25 cycles at 95°C for 20 s, 50°C for 40 s, and 72°C for 1 min, and a final elongation at 72°C for 3 min.

We prepared multiplex PCR products by using the TruSeq Nano DNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. We mechanically sheared samples by using an M220 focused ultrasonicator (Covaris, Woburn, MA, USA). The cDNA amplicon was size-selected, A-tailed, ligated with indexes and adaptors, and enriched. We sequenced libraries by using the MiSeq benchtop sequencer (Illumina) with 2 × 150 bp and a MiSeq reagent V2 (Illumina). We imported and analyzed Illumina FASTQ files by using EDGE (21).

**Phylogenetic Analysis**

We aligned and edited virus genome sequences by using the multiple sequence alignment with high accuracy and high throughput algorithm (22). We generated phylogenetic trees by using the maximum-likelihood method in MEGA version 6.0 (23) and models for analysis according to the best fit substitution model (TN93 + gamma + invariant for L segments, general time reversible + gamma + invariant for M segments, and TK2 + gamma for S segments). We assessed support for topologies by bootstrapping for 1,000 iterations. The prototype strain used, SEOV 80-39, was isolated from *R. norvegicus* rats captured in Seoul in 1980.

**Results**

**Retrospective Analysis of HFRS Patient Specimens**

We found that specimens collected in 2002 from 6 HFRS patients were positive for SEOV by ELISA (J.-W. Song and H. Kariwa, unpub. data). We confirmed that the HFRS specimens were serologically positive for SEOV by IFA (Table 1). Titers of SEOV-specific antibody ranged from 1:128 to 1:4,096. Reverse transcription PCR detected the partial sequence of L segment (nt 2946–3335) from 2 HFRS patients (Hu02-180 and Hu02-258).

**Epidemiologic Surveillance of *R. norvegicus* Rats**

We collected 1,269 *R. norvegicus* rats in urban HFRS-endemic areas in South Korea, including the city of Seoul (1,226/1,269) and Gyeonggi (40/1,269), Gangwon (1/1,269), and Jeollanam (2/1,269) Provinces (Table 2). A total of 76 (6.2%) of 1,226 rats collected in Seoul were serologically positive for SEOV. However, we found IgG against SEOV in only 1 (2.3%) of 43 rats collected from the other areas, including Gyeonggi, Gangwon, and Jeollanam Provinces. We detected SEOV RNA in 13 (16.9%) of 77 seropositive *R. norvegicus* rats. Serologic prevalence of SEOV in male rats (7.5%, 43/576) was not significantly different from that in female rats (5.0%, 34/684) (p = 0.0763 by χ² test). Serologic prevalence of SEOV in rats by weight (age) was 6.1% (21/342) in those weighing ≤50 g, 5.9% (22/374) in those weighing 51–100 g, 5.8% (30/521) in those weighing 101–200 g, and 13.8% (4/29) in those weighing 201–300 g. Seasonal prevalence of SEOV infection in rats was 5.8% (11 of 190) in spring (March–May), 4.4% (19/433) in summer (June–August), 6.4% (27/420) in fall (September–November), and 10.0% (19/190) in winter (December–February).

**SEOV RNA Loads in Tissues from Seropositive *R. norvegicus* Rats**

To measure viral load of SEOV RNA in *R. norvegicus* rats, we performed real-time qPCR for seropositive samples from lungs, livers, kidneys, and spleens (Figure 1). Viral load for SEOV showed ranges from tissues of 5 rats (Rn02-15, Rn10-134, Rn10-145, Rn11-44, and Rn11-53) that were positive by serologic and molecular screening (IFA+ PCR+). Rat Rn10-145 showed the highest amount of SEOV RNA in all tissues, followed by rats Rn02-15 and Rn10-134. Rat Rn11-44 showed the highest amount of SEOV RNA in all tissues except liver. Rat Rn11-53 showed the highest amount of SEOV RNA load in lung tissues, but virus RNA was not detectable in liver, kidney, and spleen tissues.

**Multiplex PCR–Based NGS for Retrospective HFRS Patient and *R. norvegicus* Rat Specimens**

We determined viral loads for HFRS patient specimens by using real-time qPCR. Cycle threshold (Ct) values ranged from 27.5 to 36.8 (Table 3). To perform multiplex PCR–based NGS for SEOV, we designed multiplex PCR primers to amplify

---

**Table 1. Laboratory diagnosis of samples from patients with SEOV-induced HFRS, South Korea, 2002**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Onset date</th>
<th>Collection date</th>
<th>IFA titer†</th>
<th>Reverse transcription quantitative PCR result‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEOV Hu02-112</td>
<td>Unknown</td>
<td>Unknown</td>
<td>1:128</td>
<td>–</td>
</tr>
<tr>
<td>SEOV Hu02-180</td>
<td>2002 Feb 9</td>
<td>2002 Feb 15</td>
<td>1:1024</td>
<td>+</td>
</tr>
<tr>
<td>SEOV Hu02-258</td>
<td>2002 Feb 28</td>
<td>2002 Mar 7</td>
<td>1:4096</td>
<td>+</td>
</tr>
<tr>
<td>SEOV Hu02-294</td>
<td>Unknown</td>
<td>Unknown</td>
<td>1:4096</td>
<td>–</td>
</tr>
<tr>
<td>SEOV Hu02-529</td>
<td>2002 May 8</td>
<td>2002 May 13</td>
<td>1:2048</td>
<td>–</td>
</tr>
</tbody>
</table>

†HFRS, hemorrhagic fever with renal syndrome; Hu, human; IFA, indirect immunofluorescence antibody test; SEOV, Seoul virus; +, positive; –, negative.
‡For IgG against SEOV. Antibody titration was tested by using serial 2-fold dilutions starting at a serum dilution of 1:32.
‡For large segment RNA using hantavirus genus–reactive primers.
every 150-bp sequence for the entire SEOV tripartite genome. We recovered genomic sequences of SEOV from 6 SEOV-positive patient samples. We sequenced human sample Hu02-258, which showed the highest viral load (lowest Ct value), for 99.6% of the L segment, 99.7% of the M segment, and 91.6% of the S segment. Recovery rates for SEOV genomic sequences from samples Hu02-180 and Hu02-529 showed a correlation with viral loads. Samples Hu02-112, Hu02-294, and Hu02-668 showed high recovery rates of SEOV S and M segments despite lower viral loads (highest Ct values). However, the L segment showed relatively low coverages (85.0% for Hu02-180, 68.2% for Hu02-294, and 72.7% for Hu02-668).

Using total RNA extracted from rat lung tissues, we determined viral loads by using real-time qPCR. C<sub>t</sub> values ranged from 16.1 to 27.6. We applied multiplex PCR–based NGS for whole-genome sequencing of 4 SEOV strains in the IFA+ PCR+ rats captured in South Korea during 2000–2016. Coverage of genomic sequences of SEOV was 99.1%–99.7% for L segments, 99.2%–99.7% for M segments, and 98.3%–99.4% for S segments. We observed a correlation between C<sub>t</sub> values and multiplex PCR–based NGS coverages (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/2/17-1216-Techapp2.pdf). Whole-genome sequences from Rn10-134, Rn10-145, Rn11-44, and Rn11-53 were obtained with termini sequences of 3′ and 5′ ends. SEOV sequences were deposited in GenBank (accession nos. MF149938–MF149957).

**Figure 1.** Measurement of SEOV RNA loads in different tissues of *Rattus norvegicus* rats, South Korea, 2000–2016. C<sub>t</sub> values were determined for SEOV small segment RNA in lung, liver, kidney, and spleen tissues obtained from 5 rats positive for SEOV IgG and SEOV RNA. Solid horizontal line indicates assay cutoff value. C<sub>t</sub>, cycle threshold; S, small; SEOV, Seoul virus.

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### Table 2. Serologic and molecular prevalence of SEOV in *Rattus norvegicus* rats, by metropolitan area/province, sex, weight, and season, South Korea, 2000–2016*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. rats</th>
<th>Prevalence of IgG against SEOV, no. positive/no. tested (%)</th>
<th>SEOV RNA, no. positive/no. tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region, n = 1,269</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seoul</td>
<td>1,226</td>
<td>76/1,226 (6.2)</td>
<td>13/76 (17.1%)</td>
</tr>
<tr>
<td>Gyeonggi Province</td>
<td>40</td>
<td>1/40 (2.5)</td>
<td>0/1</td>
</tr>
<tr>
<td>Gangwon Province</td>
<td>1</td>
<td>0/1 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Jeollanam Province</td>
<td>2</td>
<td>0/2 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Sex, n = 1,269</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>576</td>
<td>43/576 (7.5)</td>
<td>9/43 (21.0)</td>
</tr>
<tr>
<td>F</td>
<td>684</td>
<td>34/684 (5.0)</td>
<td>4/34 (11.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>0/9 (0)</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Weight, g, n = 1,269</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>342</td>
<td>21/342 (6.1)</td>
<td>2/21 (9.5)</td>
</tr>
<tr>
<td>51–100</td>
<td>374</td>
<td>22/374 (5.9)</td>
<td>3/22 (13.6)</td>
</tr>
<tr>
<td>101–200</td>
<td>521</td>
<td>30/521 (5.8)</td>
<td>7/30 (23.3)</td>
</tr>
<tr>
<td>201–300</td>
<td>29</td>
<td>4/29 (13.8)</td>
<td>1/4 (25.0)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>0/3 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Season, n = 1,269</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring, Mar–May</td>
<td>190</td>
<td>11/190 (5.8)</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Summer, Jun–Aug</td>
<td>433</td>
<td>19/433 (4.4)</td>
<td>2/19 (10.5)</td>
</tr>
<tr>
<td>Fall, Sep–Nov</td>
<td>420</td>
<td>27/420 (6.4)</td>
<td>6/27 (22.2)</td>
</tr>
<tr>
<td>Winter, Dec–Feb</td>
<td>190</td>
<td>19/190 (10.0)</td>
<td>5/19 (26.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>36</td>
<td>1/36 (2.8)</td>
<td>0/1 (0)</td>
</tr>
</tbody>
</table>

*ND, not determined; SEOV, Seoul virus.*
We obtained 9 genome sequences of SEOV S segments from HFRS patients and *R. norvegicus* rats. Phylogenetic analysis of SEOV S segments showed that group A formed a monophyletic lineage with group D (Figure 2). Group C genetically clustered with group E. The phylogeny of group B was distinct from those of groups A, C, D, and E. Group F from mountainous areas in China formed a lineage that was independent from the other groups obtained from rats collected in urban areas.

We obtained 6 genome sequences of SEOV M segments from HFRS patients and *R. norvegicus* rats (Figure 3). Phylogenetic analysis of SEOV M segments showed distinct phylogenetic clusters (groups A–F). These phylogenetic patterns showed that M segments of SEOV had genetic heterogeneity when compared with S segments.

We obtained and phylogenetically analyzed 5 SEOV L segments (Figure 4). The SEOV L segment from an HFRS patient and *R. norvegicus* rats captured in South Korea clustered to form a monophyletic group with SEOV 80-39. SEOV strains from China belonged to a genetic lineage with SEOV DPRK08 from North Korea. SEOV strains from the United Kingdom and Baltimore formed a close phylogenetic group. SEOV IR33 and IR473 obtained from laboratory outbreaks in the United Kingdom were independent from other SEOV strains.

**Discussion**

NGS is a robust tool for obtaining extensive genetic information and completing whole-genome sequences (18). However, molecular enrichment plays a critical role in amplification of pathogen genomic sequences from clinical or animal specimens. Our previous study showed recovery of nearly whole-genome sequences of HTNV from HFRS military patients by using virus-targeted molecular enrichment (19).

In this study, whole-genomic sequencing of SEOV, an etiologic agent of mild HFRS worldwide, was applied to samples from retrospective HFRS patients and seropositive *R. norvegicus* rats by using multiplex PCR–based NGS. Nearly whole-genome sequences of SEOV tripartite RNA, on the basis of SEOV 80-39 (prototype strain), corresponded to viral loads of patient serum samples and rat lung tissues. Phylogenetic analyses of the genome sequence of SEOV tripartite RNA supported worldwide distributions of SEOV and identified 6 genetic lineage groups. Group A contained SEOV strains from northeastern and southeastern China and an SEOV strain from North Korea. Group B contained SEOV strains from Southeast Asia (Singapore and Vietnam) and France. Group C contained SEOV strains from South Korea and Japan and SEOV strain Tchoupitoulas from Louisiana in the United States. Group D contained SEOV strains from Jiangxi and Hubei Provinces in southeastern China. Group E contained strains from the United Kingdom and the United States (New York, NY, and Baltimore, MD). Group F contained SEOV strains from mountainous areas in southeastern China.

We obtained and phylogenetically analyzed 5 SEOV L segments from HFRS patients and *R. norvegicus* rats. The phylogenetic analysis of SEOV L segments demonstrated distinct phylogenetic groups (groups A–F). Group A contained SEOV strains from northeastern and southeastern China and an SEOV strain from North Korea. Group B contained SEOV strains from Southeast Asia (Singapore and Vietnam) and France. Group C contained SEOV strains from South Korea and Japan and SEOV strain Tchoupitoulas from Louisiana in the United States. Group D contained SEOV strains from Jiangxi and Hubei Provinces in southeastern China. Group E contained strains from the United Kingdom and the United States (New York, NY, and Baltimore, MD). Group F contained SEOV strains from mountainous areas in southeastern China.
The close genetic relationship of SEOV in South Korea and Japan was probably caused by geographic distance and historical activities (e.g., commerce and occupation by Japanese forces). The genetic lineage containing strains from Southeast Asia and France might have originated during colonization or on trade routes that extended distribution of SEOV-infected rats. Recently, SEOV outbreaks have been reported in the United Kingdom and United States. Clinical cases showed that SEOV infections were identified among pet owners, breeders, and distributors. The genetic relationship of SEOV between counties probably reflects movement of rats associated with the animal pet market.

The prevalence of hantaviruses (e.g., HTNV and Imjin virus [MJNV]) in natural reservoir hosts has showed sex- and weight (age)-specific differences. However, in our study, the incidence of SEOV in R. norvegicus rats was not dependent on sex and weight (age). Epidemiologic differences in hantavirus infections between A. agrarius and R. norvegicus rats might be, in part, caused by ecologic differences, reservoir host distributions, and behavior (e.g., association with humans). Seasonal circulation of SEOV infection was maintained over 1 year, suggesting...
an enzootic infectious cycle. These observations might suggest that preventive strategies for disease risk mitigation focus on limits of rat populations all year.

Our previous study demonstrated differential amounts of HTNV RNA in lung, kidney, liver, and spleen tissues of rodents collected in areas in which HFRS is prevalent (30). In addition, the genomic RNA load of MJNV, a shrewborne hantavirus, showed various patterns in different tissues in nature (28). IFA+ PCR+ shrews showed high and various loads of MJNV RNA in all tissues. MJNV RNA from IFA–PCR+ shrews was detected in lung but not in kidney, liver, or spleen tissues, indicating an early phase of infection before MJNV-specific IgG was produced (31,32). In our study, rats Rn02-15, Rn10-134, and Rn10-145 showed various amounts of SEOV RNA in all tissues. Rat Rn11-44 had high levels of SEOV RNA in all tissues except the liver. Virus RNA in rat Rn11-53 might reflect the early phase of SEOV infections because of highest viral load in lung tissues but not other tissues. Patterns of SEOV RNA loads might indicate systemic infections in nature and active circulation of virus among rat populations in urban HFRS-endemic areas.

Diversity of virus genomes results from genomic variation or exchanges (33). RNA viruses show high mutation rates caused by deficiencies in proofreading by virus polymerases. Genomic variation also results from a mechanism of host immune evasion (34,35). Genetic exchanges, such as reassortment and recombination, lead to the generation of divergent virus progeny (36). Our previous studies identified reassortment and recombination of hantaviruses, including HTNV and MJNV, in nature (19,28,37). Using nearly
complete sequences of SEOV S, M, and L segments, phylogenetic analyses demonstrated that S segments of group A SEOVs formed a cluster with those of group D SEOVs and that L and M segments of group A SEOVs showed a close phylogenetic relationship with those of group B SEOVs. The S segment of group C SEOVs grouped phylogenetically with group E SEOVs. However, L and M segments of group C SEOVs formed a distant genetic cluster from those of group E SEOVs. Phylogenetic analysis of SEOV S segments showed a differential pattern from that of SEOV M segments, indicating a genome organization compatible with genetic exchanges in nature. To clarify genetic events among SEOV worldwide, whole-genome sequences of the SEOV L segment need to be investigated. Application of multiplex PCR–based NGS will be useful in elucidating phylogenetic patterns of the SEOV L segment.

In conclusion, this epidemiologic survey of R. norvegicus rats in urban HFRS-endemic areas of South Korea identified the prevalence and distribution of SEOV. We applied multiplex PCR–based NGS to whole-genome sequencing of SEOV tripartite RNA from retrospective serum samples from HFRS patients and rat tissues. Phylogenetic analyses demonstrated the global distribution and genetic diversity of SEOV on the basis of nearly complete genome sequences. This study provides useful information for SEOV-based surveillance, disease risk assessment, and mitigation against hantavirus outbreaks.

Acknowledgments
We thank Charles Hong for providing support; Ji Hye Kim for providing experiment support; the Pest Management staff, US Army Garrison, Yongsan, for collecting rats; American soldiers for collecting rodents at US Army training sites/installations; and Ki-Joon Song for providing retrospective HFRS patient serum samples.

This study was supported by the Agency for Defense Development (South Korea) (grant UD160022ID), the National Research Foundation (South Korea) (grant NRF-2017M3A9E4061992) and the Armed Forces Health Surveillance Branch–Global Emerging Infections Surveillance and Response System (Silver Spring, MD, USA).

About the Author
Dr. Won-Keun Kim is a virologist and research instructor at the College of Medicine, Korea University, Seoul, South Korea. His research interests include next-generation sequencing, hantaviruses, and virus–host interactions.

References


Address for correspondence: Jin-Won Song, Department of Microbiology, Korea University School of Medicine, Incheon-ro 73, Seongbuk-gu, Seoul 02841, South Korea; email: jwsong@korea.ac.kr
Staphylococcal toxic shock syndrome (TSS) was originally described in menstruating women and linked to TSS toxin 1 (TSST-1)–producing *Staphylococcus aureus*. Using UK national surveillance data, we ascertained clinical, molecular and superantigenic characteristics of TSS cases. Average annual TSS incidence was 0.07/100,000 population. Patients with nonmenstrual TSS were younger than those with menstrual cases but had the same mortality rate. Children ≤16 years of age accounted for 39% of TSS cases.

**Clinical and Molecular Epidemiology of Staphylococcal Toxic Shock Syndrome in the United Kingdom**

Hema Sharma, Debra Smith, Claire E. Turner, Laurence Game, Bruno Pichon, Russell Hope, Robert Hill, Angela Kearns, Shiranee Sriskandan

In support of improving patient care, this activity has been planned and implemented by Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is jointly accredited by the Accreditation Council for Continuing Medical Education (ACCME), the Accreditation Council for Pharmacy Education (ACPE), and the American Nurses Credentialing Center (ANCC), to provide continuing education for the healthcare team. Medscape, LLC designates this Journal-based CME activity for a maximum of 1.00 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 75% minimum passing score and complete the evaluation at http://www.medscape.org/journal/eid; and (4) view/print certificate. For CME questions, see page 411.

**Learning Objectives**

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

- Assess the clinical features and epidemiology of toxic shock syndrome (TSS) in England, Wales, and Northern Ireland, based on a study using UK national surveillance data
- Identify the molecular epidemiology of TSS in England, Wales, and Northern Ireland
- Discuss superantigen production by dominant TSS strain types and antimicrobial sensitivity of isolates.

**CME Editor**

Dana C. Dolan, BS, Copyeditor, Emerging Infectious Diseases. *Disclosure: Dana C. Dolan, BS, has disclosed no relevant financial relationships.*

**CME Author**

Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. *Disclosure: Laurie Barclay, MD, has disclosed the following relevant financial relationships: owns stock, stock options, or bonds from Alnylam; Biogen; Pfizer.*

**Authors**

*Disclosures: Hema Sharma, PhD, MSc; Debra S. Smith, PhD; Claire E. Turner, PhD; Laurence Game, PhD; Bruno Pichon, PhD; Russell Hope, PhD; Robert Hill, PhD; Angela Kearns, PhD, BSc; and Shiranee Sriskandan, PhD, FRCP, have disclosed no relevant financial relationships.*

**Staphylococcal toxic shock syndrome (TSS)** was originally described in menstruating women and linked to TSS toxin 1 (TSST-1)–producing *Staphylococcus aureus*. Using UK national surveillance data, we ascertained clinical, molecular and superantigenic characteristics of TSS cases. Average annual TSS incidence was 0.07/100,000 population. Patients with nonmenstrual TSS were younger than those with menstrual cases but had the same mortality rate. Children ≤16 years of age accounted for 39% of TSS cases.

**Author affiliations:** Imperial College, London, UK (H. Sharma, D. Smith, C.E. Turner, S. Sriskandan); MRC London Institute of Medical Sciences, London (L. Game); Public Health England, London (B. Pichon, R. Hope, R. Hill, A. Kearns)

DOI: [https://doi.org/10.3201/eid2402.170606](https://doi.org/10.3201/eid2402.170606)

1Current affiliation: University of Sheffield, Sheffield, UK.

2These authors contributed equally to this article.
Staphylococcal Toxic Shock Syndrome in the UK

Most caused by burns and skin and soft tissue infections. Nonmenstrual TSS is now more common than menstrual TSS in the UK, although both types are strongly associated with the tst+ clonal complex (CC) 30 methicillin-sensitive S. aureus lineage, which accounted for 49.4% of all TSS and produced more TSST-1 and superantigen bioactivity than did tst+ CC30 methicillin-resistant S. aureus strains. Better understanding of this MSSA lineage and infections in children could focus interventions to prevent TSS in the future.

Staphylococcal toxic shock syndrome (TSS) is a life-threatening illness characterized by fever, rash, desquamation, organ dysfunction, and shock. In 1980, the use of highly absorbent tampons in the United States triggered an outbreak of menstrual TSS (mTSS) in young women, and TSS incidence peaked at 13.7/100,000 population (1). Changes in tampon manufacture and advice regarding tampon use helped halt the epidemic. TSS is a notifiable illness in the United States; in 2004–2014, average annual incidence varied from 0.03–0.05/100,000 population (2). In the United Kingdom and other countries in Europe, staphylococcal TSS is not a notifiable illness, so the clinical, microbiological, and toxigenic features of TSS remain poorly described.

TSS is attributed to staphylococcal superantigens that cause massive T-cell activation and cytokine release (3). TSS toxin 1 (TSST-1) is associated with 95% of mTSS cases and 50% of TSS cases caused by nonmenstrual infective foci (nmTSS) (4). Although 24 different staphylococcal superantigens have been described, including staphylococcal enterotoxin (SE) and enterotoxin-like superantigens (5), SE types A, B, and C are implicated in remaining nmTSS cases (3,6), despite the lack of data from Europe.

TSST-1 is encoded by the gene tst, which is carried on mobile genetic elements (MGE) named staphylococcal pathogenicity islands (SaPIs) that lie within the S. aureus chromosome. SaPIs are linked to specific S. aureus genetic families, known as lineages (7). Within human S. aureus strains, tst is carried on SaPI1, SaPI2, and SaP68111 (8,9). Known regulators of tst include the S. aureus accessory gene regulator operon (agr) via the effector molecule RNAIII (10), the staphylococcal respiratory response regulator AB (SrrAB) (10), a glucose catabolite repressor CcpA (11), the staphylococcal accessory regulator A, σB (12) and the SaeRS 2-component system (13).

mTSS strains are reported to belong to a single S. aureus lineage (14,15) corresponding to multilocus sequence type–clonal complex (MLST-CC) 30, a lineage prevalent in the United Kingdom (16). Staphylococcal methicillin resistance is mediated by mecA or mecC genes within the mobile genetic element staphylococcal cassette chromosome mec (SCC mec), of which there are 12 types (17,18). Methicillin-sensitive S. aureus (MSSA) and methicillin-resistant S. aureus (MRSA) strains that are members of CC30 carry tst on SaPI2 (19,20).

In this study, we aimed to characterize the clinical and molecular epidemiology of TSS in England, Wales, and Northern Ireland. We further determined superantigen production by dominant S. aureus strain types.

Methods

Case Identification

Public Health England (PHE) requests the referral of all TSS-associated isolates to the national reference laboratory for characterization, including toxin gene profiling. We identified clinician-diagnosed staphylococcal TSS cases from a database of referred S. aureus isolates from England, Wales, and Northern Ireland during January 2008–December 2012 using the search term “toxic shock syndrome.” Clinical and demographic data from the accompanying isolate referral form (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/2/17-0606-Techapp1.pdf) that had been recorded contemporaneously were scrutinized for accuracy by a clinician (H.S.) before inclusion in the study.

We classified TSS cases in patients ≤16 years of age as pediatric. We classified cases in female patients 12–60 years of age as mTSS if the infection was associated with menstruation or positive vaginal culture for S. aureus. We classified the remaining cases as nmTSS. All cases had an associated S. aureus isolate.

The average annual incidence of TSS was calculated as cases per 100,000 population using Office for National Statistics UK population estimates (http://www.ons.gov.uk/ons/datasets-and-tables/index.html) and was based on data from 2009 and later (due to changes in reporting practice from November 2008 prompted by national guidance on toxin-producing S. aureus). We used total population for the United Kingdom excluding Scotland as the denominator for all TSS and nmTSS cases; the total female population 12–60 years of age as mTSS if the infection was associated with menstruation or positive vaginal culture for S. aureus. We classified the remaining cases as nmTSS. All cases had an associated S. aureus isolate.

Molecular Characterization of Isolates

We made MLST-CC assignments on the basis of sequencing the staphylococcal protein A (spa) gene repeat region (21) and referencing spa server (http://spa.ridom.de/mlst.shtml) and MLST (http://saureus.mlst.net) databases. We performed SCCmec detection, typing, and toxin gene profiling (sea-e, seg-j, tst, and pvl only) by multiplex PCR (22,23).

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Antimicrobial Susceptibility Testing
For isolates from 2008–2011 (n = 148; online Technical Appendix Table 1), we determined antimicrobial MICs by agar dilution (24) and interpreted them in accordance with European Committee on Antimicrobial Susceptibility Testing guidelines (http://www.eucast.org). We did not determine antimicrobial susceptibilities for isolates from 2012.

TSST-1 Production
Based on molecular epidemiologic findings, we assessed TSST-1 production in all tst-positive CC30 MSSA isolates from the TSS cohort (n = 81), including TSS isolates associated with bacteremia, skin and soft tissue infections (SSTI), and deep infections. We also assessed TSST-1 production in randomly selected tst-positive CC30 MRSA isolates from non-TSS patients (n = 39, including carriage, bacteremia, and SSTI isolates) that had been submitted to the reference laboratory during the study period (online Technical Appendix Table 1). We quantified TSST-1 in cell-free broth-culture supernatants by Western blot by comparison with purified TSST-1 protein standards (online Technical Appendix).

T-Cell Proliferation
We obtained normal-donor peripheral blood mononuclear cells (PBMC) from an approved subcollection of the Imperial College NHS Trust Tissue Bank (ICHTB reference R12023) from anonymized consenting healthy donors. We incubated PBMC (1 × 10⁶ cells/mL) with cell-free RPMI bacterial supernatants (1:1,000 dilution) prepared from tst-positive CC30 MSSA isolates from the TSS cohort (n = 77; 4 of the isolates did not grow in RPMI) and the randomly selected tst-positive CC30 MRSA isolates (n = 39) that were investigated for TSST-1 production. We cultured the PBMC in RPMI medium (Invitrogen, Hemel Hempstead, UK) supplemented with 10% fetal calf serum at 37°C for 48 h in triplicate (25). We measured proliferation after incorporating 1.0 μCi/well of [3H] thymidine and allowing an additional 6 h incubation.

DNA Sequencing and Analysis
We extracted whole genomic DNA from randomly selected tst-positive CC30 MSSA isolates from the TSS cohort (n = 4) and tst-positive CC30 MRSA isolates (n = 5) (online Technical Appendix Table 1) (26). We prepared libraries using the Nextera-XT DNA Sample Prep Kit (Illumina, Cambridge, UK) and subjected them to MiSeq sequencing (Illumina), generating 150 bp reads. We deposited data in the GenBank short read archive (accession no. SRP082305). We mapped reads to MLST-CC matched reference genomes MRSA252 (GenBank accession no. NC_002952.2 (27) or MN8 (accession no. NZ_CM000952) using SMALT (http://www.sanger.ac.uk/resources/software/smalt/) and determined single-nucleotide polymorphisms (SNPs) by SAMtools and bcftools (28). We performed de novo assemblies using Velvet (https://www.ebi.ac.uk/~zerbino/velvet/) and annotated them using Prokka (http://www.vicbioinformatics.com/software.prokka.shtml). We used Artemis (http://www.sanger.ac.uk/science/tools/artemis) to visualize the mapping of sequence reads to the reference strain and manually confirm all polymorphisms. For targeted ecpA sequencing, we amplified and sequenced DNA using forward primer 1: 5′-CACAGTGTCCGTGGTTGTTA-3′ and reverse primer 1: 5′-TAAGGCGCATCCCTACTGCAC-3′.

Statistical Analysis
We analyzed data with GraphPad Prism 6.0 (GraphPad Software, La Jolla, California, USA). We tested categorical variables using Fisher exact test or χ² test. We summarized nonparametric data by medians and interquartile ranges (IQR) and compared 2 groups by Mann-Whitney U test. We summarized parametric data by means and SDs and analyzed 2 groups by t-test (2-tailed); we considered p<0.05 significant.

Results
Incidence of TSS
During January 2008–December 2012, a total of 195 TSS case isolates were referred to PHE. We excluded 15 cases from the study (duplicate isolates from the same case, 4 cases; isolates submitted for quality control testing, 2 cases; isolates from cases incorrectly recorded as TSS, 9 cases), leaving 180 microbiologically confirmed TSS cases with isolates. Because of missing clinical data, we were unable to classify 3 isolates as mTSS or nmTSS and could not ascertain the sex of 1 patient with nmTSS.

We considered the apparent rise in cases during 2008–2009 an artifact of increased clinical awareness of severe toxigenic S. aureus disease from late 2008, prompted by national guidance on toxin-producing S. aureus (Figure 1). Beginning in 2009, mTSS referrals declined annually, whereas nmTSS cases remained stable. By 2012, cases of nmTSS outnumbered mTSS. Overall, most cases were nonmenstrual (107, 59.4%). Average annual incidence per 100,000 population was 0.07 (95% CI 0.05–0.10) for all cases, 0.09 (95% CI 0.06–0.14) for menstrual cases, and 0.04 (95% CI 0.02–0.06) for nonmenstrual cases.

Clinical Characteristics of TSS Patients
Despite an overall preponderance of female case-patients, we found no gender difference among nmTSS cases (Table 1). The median age of the cohort was 19 years; patients with nmTSS were younger than those with mTSS (median 15.0 vs. 21.5 years; p = 0.01).
Of the TSS cases studied, 39% (71/180) occurred in children ≤16 years of age; one sixth of all TSS case-patients were <1 year of age (Figure 2). The median age of pediatric TSS case-patients was 4 years, with an average annual incidence of 0.14/100,000 children (95% CI 0.08–022). However, among children <1 year of age, the average annual incidence increased to 0.45/100,000 (95% CI 0.26–0.79). Most pediatric nmTSS cases were related to burns (26.8%, 15/56) or SSTIs (25%, 14/56).

Five percent of all patients with TSS had died at the time of referral of the isolate. We found no difference in fatality rate between mTSS and nmTSS cases and no association with age (online Technical Appendix Table 2). The infective focus in nmTSS cases was SSTI (n = 41), primary bacteremia (n = 15), burns (n = 15), deep abscess (n = 13), respiratory tract (n = 10), bone and joint (n = 4), unknown (n = 6), and other sites (n = 3). We found no association between site of infection and S. aureus lineage (online Technical Appendix Figure 1).

Molecular Characteristics of TSS Isolates
Among 180 TSS S. aureus isolates, we identified 88 spa types associated with 15 different MLST-CCs (online Technical Appendix Table 3). The leading cause of both mTSS and nmTSS was CC30 MSSA, accounting for >50% of infections (Figure 3), although we found a stronger association of CC30 with mTSS than with nmTSS (72.9% vs. 36.4%; p<0.0001; online Technical Appendix Table 3). CC30 MSSA was also the leading cause of TSS among pediatric cases (31/71). We identified only 7 MRSA TSS isolates (online Technical Appendix Table 4).

TSS isolates carried 3 superantigen genes on average (online Technical Appendix Table 5). The most common superantigen gene among both mTSS and nmTSS isolates was tst (Table 2; online Technical Appendix Figure 2), with the exception of the other 2 prevalent superantigen genes, seg and sei, that are carried on an enterotoxin gene cluster (egc) along with selm/n/o/u in most S. aureus isolates (5). The tst gene was associated with mTSS (Table 2) and strongly associated with the CC30 lineage of S. aureus (online Technical Appendix Table 6). The superantigen gene sea combined with tst was also linked to mTSS (Table 2), whereas sea alone was associated with CC30 (online Technical Appendix Tables 5, 6).

Antimicrobial Susceptibility of TSS Isolates
Most isolates were MSSA (mecA negative). The rate of resistance to erythromycin was 9.2%; to ciprofloxacin, 8.5%; to tetracycline, 3.5%; and to teicoplanin, 1.4%. For 7 mecA-positive MRSA-TSS isolates, the resistance rate to ciprofloxacin was 57.1%; to erythromycin, 42.6%; and to clindamycin, 14.3%.

As MRSA-related TSS is rarely reported we examined these cases in more detail. All 7 MRSA cases were nonmenstrual, affecting mainly male patients; 3 were associated with SSTIs. The median patient age was 34 (IQR 2.3–64.3) years. Five isolates were identified as CC22-SC, 2 were MRSA-15, and 4 carried sec, corresponding to the healthcare-associated MRSA clade dominant in the UK, EMRSA-15; MRSA-TSS cases showed a clear association with this

![Figure 1. Staphylococcal TSS cases, England, Wales, and Northern Ireland, 2008–2012. The chart depicts the number of cases per year of total, menstrual, and nonmenstrual TSS cases reported to Public Health England. National guidance on toxin-producing Staphylococcus aureus disease affected reporting practice from November 2008. mTSS, menstrual TSS; nmTSS, nonmenstrual TSS; TSS, toxic shock syndrome.](https://example.com/figure1.png)
lineage (online Technical Appendix Table 4). The remaining CC22 isolate carried tst and belonged to a MRSA lineage frequently identified in the Middle East. Only 1 MRSA-TSS isolate was CC30-SCCmecII, corresponding to the UK HA-MRSA clade EMRSA-16. One isolate was CC6-SCCmecII and lacked all superantigen genes tested.

**TSST-1 Production by CC30 S. aureus**

The strong association of CC30 with TSS was unsurprising because of the presence of tst. We measured TSST-1 in broth-culture supernatants from tst-positive CC30 MSSA isolates from the TSS cohort and, for comparison, randomly selected clinical tst-positive MRSA isolates that belonged to the same lineage (CC30) (20,29) (online Technical Appendix Table 1).

Of note, 77/81 tst-positive CC30 MSSA isolates produced detectable TSST-1, compared with 9/39 tst-positive CC30 MRSA isolates. The tst-positive CC30 MSSA isolates produced more TSST-1 than did tst-positive CC30 MRSA isolates, albeit with marked variability (88.5 ± 48.3 vs. 31.4 ± 18.1 ng/mL; p<0.0001; Figure 4, panel A). Furthermore, the superantigenic activity of isolates, measured by T-cell proliferation in response to broth-culture supernatants, of tst-positive CC30 MSSA strains (164,893 ± 36,191 counts/min) was significantly greater than that of tst-positive CC30 MRSA strains (149,653 ± 30,412 counts/min; p = 0.02; Figure 4, panel B).

**tst-positive CC30 MRSA and Mutation in tst Regulator, CcpA**

To ascertain the basis for the observed variability in TSST-1 production among CC30 S. aureus, we subjected 4 tst-positive CC30 MSSA isolates from the TSS cohort and 5 tst-positive CC30 MRSA clinical isolates to whole-genome sequencing. The tst gene, promoter, and regulator sequences, including SarA, SrrAB, agr, and σB, were identical among the 9 sequenced strains and reference isolates (MN8/MRSA252).

We detected mutations in TSST-1 regulator SaeRS in 2/4 tst-positive CC30 MSSA isolates; a synonymous SNP C481T in SaeR in 1 strain and a nonsynonymous SNP in SaeS in another resulted in a change from asparagine to serine at aa residue 218. Because these strains produced abundant TSST-1 (online Technical Appendix Table 1), we did not study these mutations further.
We detected a nonsynonymous SNP in the sequence of regulator *ccpA* in all 5 *tst*-positive CC30 MRSA isolates but not in any *tst*-positive CC30 MSSA isolate. This difference translated into a change from threonine (A_CA) to isoleucine (A_TTA) at aa residue 87/329 (online Technical Appendix Table 1). Including genome-sequenced isolates, 33/39 *tst*-positive CC30 MRSA isolates had *ccpA* (T87I), compared with 0/23 *tst*-positive CC30 MSSA isolates, confirming an association of *ccpA* (T87I) with CC30 MRSA strains. Furthermore, *ccpA* (T87I) was strongly negatively associated with production of TSST-1 in *tst*-positive CC30 *S. aureus*: 26/33 *ccpA* (T87I) isolates did not produce TSST-1, compared with only 1/23 wild-type *ccpA* isolates (*p*<0.0001 by Fisher exact test).

We conducted SCC*mec* typing on a subset of *tst*-positive CC30 MRSA strains (n = 15; online Technical Appendix Table 1). Results demonstrated an association of *ccpA* (T87I) with SCCmecII; 7/11 SCCmecII isolates had *ccpA* (T87I), compared with 0/4 SCCmecIV isolates (*p* = 0.03 by $\chi^2$ test). This finding highlights the possibility that reduced TSST-1 production might be attributable to either SCCmecII or *ccpA* (T87I).

**Discussion**

We provide a substantive national clinical and microbiological overview of staphylococcal TSS cases in the United Kingdom. TSS incidence was 0.07/100,000 population. nmTSS cases now outnumber mTSS cases, and nmTSS affects younger persons. The *tst*-positive CC30 *S. aureus* lineage was linked strongly with TSS and almost all mTSS cases. CC30 MSSA is a prevalent lineage in the United Kingdom (16), so ongoing surveillance and clinical vigilance for TSS are important.

Our findings may underestimate TSS incidence because notification of TSS is voluntary in the United Kingdom and we included only microbiologically confirmed cases. These factors increase diagnostic confidence, but TSS is a syndromic condition not requiring bacteriological confirmation. Overall TSS incidence was low but similar to rates in the United States (2); improvements in care may account for low overall incidence of TSS, because patients may not fulfill all of the criteria required by the case definition of TSS. The overall TSS incidence in children contrasts with findings from a British Pediatric Surveillance Unit study in which a higher incidence of combined streptococcal and staphylococcal TSS cases was reported (30).

The number of cases of mTSS fell from 2009 to 2012, such that nmTSS cases are now more common than mTSS cases, mirroring US trends (31). Patients in our study were younger than in US cohorts (31,32), and nmTSS patients were younger than those with mTSS. Most nmTSS cases occurred in children, with burns and SSTIs as the cause in 51.8% (29/56) of these cases. An association between nmTSS and increased mortality rate has been reported, although a high incidence of bacteremia may have affected the findings of that study (33). It is possible that we did not ascertain all cases of TSS, although we found no difference in reported deaths between mTSS and nmTSS cases or associations with age; the overall death rate was 5%.

The association of TSS, and particularly mTSS, with a single lineage corresponding to CC30 *S. aureus* has been described in diverse geographic localities (14,15). The *tst*-positive CC30 MSSA clone has recently been named epidemic MSSA-ST30 because it is responsible for a substantial...
amount of *S. aureus* disease and is a precursor to the HA-MRSA clone, EMRSA-16, which has been responsible for major national UK MRSA outbreaks (29).

The *tst* gene was the predominant superantigen gene among TSS isolates, excluding *seg* and *sei*, which were also previously implicated in TSS (34). The superantigens *seg* and *sei* are carried on the *egc*, which is widespread in *S. aureus* (5), and are unlikely to have any specific association with TSS. We linked *tst* to mTSS and CC30. Several groups have demonstrated similar associations of staphylococcal superantigen genes with specific lineages (35,36), due to clonal associations, superantigen arrangements, and transmission via mobile genetic elements, although other firm associations linking lineage, superantigen gene carriage, infection type, and disease presentations have not been made. A recent study of atopic dermatitis that examined the relationship of ethnicity and staphylococcal virulence factors found a lack of *tst*-positive *S. aureus* atopic dermatitis in African American persons that was consistent with an absence of *tst*-positive *S. aureus* mTSS among this group, suggesting differences in disease presentation among disparate ethnic groups (37) based on host characteristics. The ethnicity of the patients with TSS referred to PHE in this study was not recorded, and such bacterial genetic associations with disease could not be made but may merit consideration in future studies.

Among MSSA isolates, resistance rates to key antimicrobial drugs were similar to reported UK MSSA bacteremia isolates (38). Notably, teicoplanin resistance was detected, although rarely. This finding circumvents any need to change current recommendations for antimicrobial drugs for TSS that include a bactericidal cell wall inhibitor (e.g., β-lactamase–resistant antistaphylococcal) and protein-synthesis inhibitor (e.g., clindamycin) along with intravenous immunoglobulin for severe cases unresponsive to first-line therapy and source control (39). No vaccines are available to prevent TSS, although a recombinant TSST-1 variant vaccine has shown promise in a recent human clinical trial and was found to be safe and immunogenic (40).

The MRSA-TSS rate in this study was lower than rates in the United States (32), perhaps reflecting the low UK community-associated MRSA prevalence (41). All MRSA cases were nonmenstrual and mostly associated with recognized healthcare-associated MRSA clones, although we did not record the mode of acquisition. Only 1 CC30 MRSA (EMRSA-16) isolate caused TSS, even though CC30 is the main TSS-associated lineage; this finding mirrors the national decline in UK EMRSA-16 over time (42).

Isolates of *tst*-positive CC30 MSSA were more likely to produce TSST-1 in vitro and secreted almost 3 times more TSST-1 than did *tst*-positive CC30 MRSA isolates, which translated into a functional difference in superantigenic activity. We do not know whether such a difference would extend to the in vivo setting. Our study of TSST-1 production was limited by availability of clinical *tst*+ CC30 strains; clinical TSS CC30 MSSA strains were therefore compared with clinical non-TSS CC30 MRSA strains and not to clinical TSS MRSA strains. Thus, more MSSA than MRSA strains were from the genital tract or from burns, potentially confounding phenotypic differences observed. Defining the precise comparator group for TSS CC30 MSSA isolates is challenging because of lack of TSS CC30 MRSA isolates and suitable non-TSS strains referred to PHE.

Bacterial acquisition of antimicrobial drug resistance elements can be associated with a fitness cost. In the United Kingdom, most CC30 HA-MRSA strains carry SCCmecII (EMRSA-16, ST36-SCCmecII) that may reduce cytolytic toxin production and, in association with *fudoh* gene carriage by this element, reduce hemolytic activity and virulence (43,44). Our findings suggest an association between SCCmecII and reduced TSST-1 production that might be linked to a SNP in a regulatory gene, *ccpA*. The resulting mutation in CcpA occurs adjacent to a co-repressor binding site in the transcriptional regulation region (online Technical Appendix Figure 3) that could influence *tst* promoter binding and affect TSST-1 secretion. Such SNPs in virulence regulators may have had a role in shaping the healthcare-associated phenotype of EMRSA-16 (20). New tools that allow manipulation of previously nontransformable lineages such as CC30 will facilitate investigating such genetic mechanisms in *S. aureus* (45).

Our study shows that the ability to produce TSST-1 varies widely within the *tst*-positive CC30 lineage and impaired expression is associated with the presence of SCCmecII and *ccpA* (T87I), underlining the potential for genomic approaches to contribute to greater understanding of patterns of clinical disease. Given the prevalence of *tst*-positive CC30 MSSA causing TSS and its role as a dominant UK lineage of *S. aureus*, active surveillance of this lineage is required. Clarification of the particular modes of transmission, acquisition, and pathogenesis of this lineage may identify susceptible persons, such as younger persons with burns and SSTIs, who might benefit from interventions such as vaccination with recombinant TSST-1 or *S. aureus* screening and decolonization in the future to prevent the occurrence of this life-threatening syndrome.

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Staphylococcal Toxic Shock Syndrome in the UK

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About the Author

Dr. Sharma is a physician in infectious diseases, microbiology, and virology and a clinical research fellow at Imperial College London. Her primary research interests relate to the pathogenesis of staphylococcal disease.

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Address for correspondence: Shiranee Sriskandan, Imperial College London, Department of Medicine, Hammersmith Campus, Du Cane Road, London W12 0NN, UK; email: s.sriskandan@ic.ac.uk
We describe a lethal respiratory outbreak among wild chimpanzees in Uganda in 2013 for which molecular and epidemiologic analyses implicate human rhinovirus C as the cause. Postmortem samples from an infant chimpanzee yielded near-complete genome sequences throughout the respiratory tract; other pathogens were absent. Epidemiologic modeling estimated the basic reproductive number \( R_0 \) for the epidemic as 1.83, consistent with the common cold in humans. Genotyping of 41 chimpanzees and examination of 24 published chimpanzee genomes from subspecies across Africa showed universal homozygosity for the cadherin-related family member 3 \( CDHR3 \)-Y529 allele, which increases risk for rhinovirus C infection and asthma in human children. These results indicate that chimpanzees exhibit a species-wide genetic susceptibility to rhinovirus C and that this virus, heretofore considered a uniquely human pathogen, can cross primate species barriers and threatens wild apes. We advocate engineering interventions and prevention strategies for rhinovirus infections for both humans and wild apes.

Rhinoviruses are antigenically diverse members of the family \textit{Picornaviridae}, genus \textit{Enterovirus}, that cause the common cold (1). Rhinovirus C causes an estimated 50% of all human upper respiratory tract infections (1) and most acute exacerbations of asthma in children (2). More than 160 distinct rhinovirus genotypes have been identified in human populations globally, and extensive antigenic heterogeneity limits cross-protective immunity (3). Accordingly, persons typically acquire several rhinovirus infections annually throughout childhood and continue to acquire new infections throughout adulthood (4).

Although many rhinovirus infections are only mildly symptomatic, rhinovirus C has been associated with influenza-like respiratory symptoms and acute exacerbations of asthma in children (5). The elevated virulence of rhinovirus C derives from its unique reliance on the cadherin-related family member 3 (CDHR3) receptor for host cell binding (6). A single-nucleotide polymorphism in \textit{CDHR3} (rs6967330, C529Y) is associated with a 10-fold increase of RV-C binding and progeny yield and is a major risk factor for rhinovirus C infection and asthma (7). The protective \textit{CDHR3}-C529 nonrisk allele has been detected exclusively in modern humans, whereas archaic Neanderthal and Denisovan humans each expressed the ancestral risk allele (8). The origin of rhinovirus C and the spread of the protective allele among modern human populations is thought to have occurred ≈8,000 years ago (9).

Humans and chimpanzees (\textit{Pan troglodytes}) are closely related species that share many physiologic similarities, including a general predisposition for pathogen exchange, which, in some cases, might lead to human pandemics (10). Anthroponotic (i.e., of human origin) respiratory viruses have caused notable mortality rates in wild chimpanzee populations (11–14), and molecular testing of both postmortem and noninvasive (fecal) samples from wild apes during respiratory disease outbreaks have implicated human parainfluenzoviruses (family \textit{Paramyxoviridae}) as anthroponotic causes (11–14). However, because of the limitations of field-based diagnostics, direct detection of pathogens from lesions of the respiratory tract has proven difficult (12,14). In this study, we were able to identify rhinovirus C, a pathogen not previously known to infect species other than humans, as the causative agent of an epidemic of respiratory disease in chimpanzees by directly sampling the lesions
of a dead animal and by subsequent noninvasive sampling (from feces) and epidemiological modeling.

Methods

Ethics Statement

This study involved only observational, noninvasive research with wild chimpanzees. All animal protocols were approved by the Harvard University Institutional Animal Care and Use Committee (Cambridge, MA, USA; protocol 96-03) and the University of New Mexico Office of Animal Care Compliance (Albuquerque, NM, USA; protocol 11-100726-MCC). These protocols adhered to guidelines and laws set forth by the Weatherall Report on the use of nonhuman primates in research (https://royalsociety.org/policy/publications/2006/weatherall-report/), as well as the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, Office of Animal Welfare, US Department of Agriculture Animal Welfare Act, Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, US Public Health Service, US National Academies of Sciences National Research Council, and US Centers for Disease Control and Prevention.

Sample Collection and Sequence Analysis

Starting approximately in February 2013 and continuing through August of that year, the Kanyawara community of chimpanzees in Kibale National Park, western Uganda, experienced an outbreak of severe respiratory disease. At the onset of the epidemic, the community consisted of 56 chimpanzees <1 week–56.9 years of age. All Kanyawara chimpanzees are individually identifiable and habituated to human observers, such that trained field assistants collected direct data on behavior and respiratory signs daily. We compiled these data into weekly measures of clinical signs (coughing or sneezing, further classified as mild or severe) and deaths. During June–August 2013, we collected fecal samples from 41 chimpanzees. Immediately after observing defecation by a chimpanzee, we placed 5 mL of the fecal sample into an equal volume of RNAlater buffer (Thermo Fisher Scientific, Waltham, MA, USA), homogenized the mixture; and clarified the mixture by centrifugation at 50 µL of fecal suspension to 350 µL of saline; homogenized the mixture, and stored the sample at –20°C for up to 3 months, until export to the United States.

One chimpanzee showing clinical signs (Betty, a 2.2-year-old female) died during the outbreak; her body was recovered immediately after death. An experienced veterinarian (D.H.) performed the postmortem examination and collected samples from her oropharynx, trachea, and lung using swabs (sterile, plastic shaft with Dacron tip) and stored the samples separately in 0.25 mL of RNAlater buffer at –20°C. We homogenized the swab tips, extracted sample RNA, and converted the RNA to double-stranded cDNA in the field. We shipped the cDNA to the United States for unbiased sequencing and pathogen discovery on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using 300-bp paired-end read chemistry as previously described (15).

We analyzed sequence data using CLC Genomics Workbench version 8.5 (CLC bio, Aarhus, Denmark). In brief, we trimmed low-quality bases (phred quality score <30), discarded short reads (<75 bp), and subjected the remaining reads to de novo assembly. We then analyzed raw sequence reads and assembled contiguous sequences (contigs), which we examined for similarity to known viruses in GenBank databases.

From these analyses, we inferred the presence of a rhinovirus, which we subsequently identified as a member of the rhinovirus C species. We performed pairwise sequence comparisons in MEGA7 (16) to assess the genetic similarity of this virus to its relatives, and we evaluated recombination with other rhinovirus C strains by using RDP4 (17). We performed phylogenetic analyses on this virus and all complete rhinovirus polyprotein genes available in the GenBank database. We first aligned genes by codon using the MAFFT algorithm (18) implemented in Translator X (19) and removed poorly aligned regions using the Gblocks algorithm (20). We then used jModelTest (21) to estimate the model of molecular evolution from the data and PhyML (22) for phylogenetic inference.

We tested chimpanzee fecal samples for a suite of respiratory pathogens by multiplex Luminex assay using the NxTAG Respiratory Pathogen Panel (Luminex Corporation, Austin, TX, USA) (23) and confirmed positive results by singleplex PCR. The NxTAG Respiratory Pathogen Panel includes influenza virus A (multiple subtypes), human respiratory syncytial viruses A and B, coronaviruses (multiple subtypes), human metapneumovirus, rhinovirus/enterovirus, adenovirus, parainfluenza viruses 1–4, bocavirus, and the bacterial pathogens Chlamydia pneumoniae and Mycoplasma pneumoniae. In brief, we added up to 50 µL of fecal suspension to 350 µL of saline; homogenized the mixture; and clarified the mixture by centrifugation at 15,000 rpm for 3 minutes, after which we added Luminex internal control MS2 before nucleic acid extraction with the NucliSENS EasyMag kit (bioMérieux, Marcy-l’Étoile, France). We then used 10 µL of eluate in the Luminex assay according to the manufacturer’s specifications. We applied this same assay to swab samples and assessed the viral load of rhinovirus C–positive swab samples using a published real-time quantitative PCR (qPCR) (24).

CDHR3 Genotyping

Given the influence of CDHR3 allelic variants on rhinovirus C pathogenesis in humans, we genotyped the CDHR3 allele of 41 chimpanzees from the Kanyawara community using their fecal specimens. In brief, we extracted DNA from fecal...
samples using the MagMAX Kit (Thermo Fisher Scientific), and we then genotyped the extracted DNA using a qPCR-based allelic discrimination assay (CDHR3 TaqMan SNP Genotyping Assay; Thermo Fisher Scientific). We assayed serial 2-fold dilutions of eluted DNA to identify the dilution yielding the strongest fluorescent signal; then, we tested 2 additional replicates of each sample at that optimal dilution. We also examined the CDHR3 locus of 24 additional chimpanzees from across Africa (including 4 recognized subspecies) using whole genome sequences previously published as part of the Great Ape Genome Project (25).

**Epidemiologic Modeling**

To infer epidemiologic parameters related to transmission, we constructed an SIR (susceptible-infectious-removed) mathematical model. Following Althaus et al. (26), we fit the SIR model to cumulative incidence data and produced maximum-likelihood estimates of epidemiologic parameters (e.g., transmission rate, recovery rate) using the Nelder and Mead optimization algorithm in the optim package in R version 3.3.2 (https://www.r-project.org/). Because the epidemic was triphasic, we fit each of the three 2013 respiratory episodes separately. We assumed a well-mixed population of 50 chimpanzees and did not explore heterogeneity of social interactions to minimize model complexity and provide baseline estimates of epidemiologic parameters (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/2/17-0778-Techapp1.pdf).

**Results**

The epidemic occurred in 3 phases (Figure 1): an early phase (21 days in February–March 2013), during which 24 animals became ill and 1 infant died; a middle phase (22 days in May–June 2013), during which 40 animals became ill and 4 adults died; and a late phase (19 days in August–September 2013), during which 31 animals became ill and none died. Of the ≈56 chimpanzees in the community at the beginning of 2013, five died during the outbreak (1 infant 2.2 years of age and 4 adults 24.0–57.9 years of age), for an overall mortality rate of 8.9%.

A postmortem analysis was conducted on the infant that died in March (peak of the early phase); other animals that died were not recovered in time for such analyses. The postmortem analysis revealed ecchymotic hemorrhages on the lung surfaces, consolidation of the parenchyma of both lungs (approximately two thirds of both lungs affected), mucoid exudate in the bronchi, hepatomegaly, and hepatic congestion. On the basis of these findings, the cause of death was concluded to be severe acute pneumonia. Deep sequencing of swab samples from this chimpanzee’s oropharynx, trachea, and lung yielded 10,722,035 sequence reads, of which 8,918 had high similarity to rhinovirus C. No other pathogens were detected. Subsequent qPCR confirmed infection with RV-C, with viral loads of $7.41 \times 10^6$ copies/swab in the oropharynx, $1.05 \times 10^7$ copies/swab in the trachea, and $1.74 \times 10^6$ copies/swab in the lung; these values are comparable to the average viral load ($7.76 \times 10^6$ copies/mL) found in rhinovirus C–infected children with acute wheezing illness treated in an emergency department (27). Further sequencing yielded a near-complete rhinovirus C genome consisting of a complete polyprotein gene of 6,450 bases, a complete 3′ untranslated region (UTR) of 35 bases excluding the poly(A) tail, and a partial 5′-UTR of 480 bases. The viral polyprotein sequence was 94.59%
similar at the nucleic acid level and 99.99% similar at the amino acid level to its closest known relative, a rhinovirus C45 sequence (GenBank accession no. JN837686) from a 2-month-old male patient who had a history of mild nasal congestion and discharge in the United States in 2000. RDP4 analyses showed the chimpanzee-derived sequence to be a rhinovirus C45-C11 recombinant, with a breakpoint in the 5′-UTR (Figure 2). In phylogenetic analyses, the virus clustered within known rhinovirus C genotypes, appearing as a sister taxon to the reference rhinovirus C45 isolate (Figure 3). The chimpanzee-derived strain was designated RV-C45-cpz1-2013 to indicate its presumptive serotype and discovery in a chimpanzee in 2013 (GenBank accession no. KY624849).

We confirmed that all swab samples from the deceased infant chimpanzee were positive for rhinovirus C by Luminex assay. Two of the 41 fecal samples collected during the epidemic were positive for enteroviruses, but subsequent molecular typing using published methods (28) demonstrated these to be non-rhinovirus enteroviruses, which occur in wild apes without known clinical significance (14,29,30). Fifteen samples were also positive for adenoviruses by Luminex assay. Subsequent typing of these viruses by PCR and direct sequencing of a portion of the hexon gene according to published methods (31) was successful for 11 samples. The resulting sequences (GenBank accession nos. KY624838–KY624848) were identical or closely related to adenoviruses found in fecal samples from apparently healthy wild chimpanzees and other nonhuman primates (32–35) (online Technical Appendix Figure 2).

Genotyping of chimpanzee DNA from fecal samples showed all 41 animals to be homozygous for the CDHR3-Y529 allele, which is associated with increased susceptibility to rhinovirus C and wheezing illness in humans (7). Similarly, all 24 chimpanzee genomes from the Great Ape Genome Project (25) were homozygous for the CDHR3-Y529 allele.

Epidemiologic modeling of the 2013 chimpanzee respiratory epidemic yielded daily transmission rate estimates of 0.85, 0.44, and 0.62 (average 0.68) and duration of infection estimates of 1.6 days, 5.8 days, and 2.1 days (average 3.2 days) for the 3 phases of the epidemic. Together, these parameters equate to basic reproductive numbers (R0 values) of 1.38, 2.56, and 1.56 for the 3 phases of the epidemic, with an overall R0 estimate of 1.83 (online Technical Appendix Figure 1).

Discussion

Rhinovirus C is unusual among rhinoviruses because of its clinical severity, unique biochemistry of receptor attachment, and role in modern human evolution (9). The analyses we present show that rhinovirus C is also distinguished by its ability to cross primate species barriers and cause severe disease. Although experiments conducted in the 1960s showed rhinoviruses A and B to be capable of infecting chimpanzees in captivity, infections were mild and self-limiting (36,37). Among other enteroviruses, only poliovirus has been implicated as a cause of death in wild chimpanzees (38). By showing that rhinovirus C is highly pathogenic in wild chimpanzees, our findings expand the known host range and clinical consequences of one of the most common causes of human respiratory disease.

Figure 2. Recombination between viral genotypes rhinovirus C45 and C11 leading to RV-C45-cpz1-2013, the strain identified in the Kanyawara chimpanzee community, Uganda, 2013. Analyses were performed in RDP4 (17) on aligned rhinovirus C genome sequences of 36 known genotypes. Each alignment entry encoded the full or nearly full polyprotein gene sequence, but some sequences were missing fragments (<400 bp) of their respective 5′-UTRs (a seq, yellow box at left). The 3′ poly(A) tail was not included. A recombination event between the 2 viruses shown (GenBank nos. JN837686 and EU840952) is the most likely event among all full alignment comparisons (window size 20 bps) according to 6 of the 9 RDP4 algorithms. The average p values were RDP 2.8 × 10−81, GENECONV 3.0 × 10−79, MaxChi 1.4 × 10−18, Chimaera 2.1 × 10−21, SiScan 3.6 × 10−24, and 3Seq 1.5 × 10−27. BootScan, PhyloPro, and LARD made no call for these particular parents. Purple box in the 5′-UTR denotes the 99% breakpoint confidence level (combined). Dashed box indicates the position of the most likely swapped fragment. The (Monte Carlo corrected) probability for this event is 2.8 × 10−81. The virus map is scaled to the alignment. Conf, confidence level; RV-C, rhinovirus C; Δ seq, missing sequence; UTR, untranslated region.
Variant RV-C45-cpz1-2013 occupies an unremarkable phylogenetic position among rhinovirus C genomic variants from humans around the world (Figure 3). Although this virus is a recombinant between 2 genotypes, C11 and C45, it is unlikely that this property is related to its origin in a chimpanzee host. Indeed, rhinoviruses often recombine; the parent strains of RV-C45-cpz1-2013 and the genomic location of the recombination event are similar to those described previously (3). RV-C45-cpz1-2013 also shares with other rhinovirus C variants the capacity to infect the lower respiratory tract and cause severe disease. Viral load values from this animal’s oropharynx (7.41 x 10^6 copies/swab), trachea (1.05 x 10^7 copies/swab), and lung (1.74 x 10^6 copies/swab) are comparable to the average viral load (7.76 x 10^6 copies/mL) found in rhinovirus C–infected children with acute wheezing illness treated in an emergency department (27). Although published estimates of rhinovirus epidemiologic transmission parameters are sparse, the values estimated for the 2013 chimpanzee outbreak (transmission rate 0.68/d; duration of infection 3.2 days) are close to those of common cold viruses in humans (transmission rate 0.74/d; duration of infection 3.0 days) (39). Despite this similarity, these values are likely similar for many acute respiratory viruses.

Epidemics of respiratory disease have been recorded in ape populations across Africa, but the causes have often remained undiagnosed. For example, during 47 years of wild chimpanzee observation in Gombe, Tanzania, respiratory disease accounted for 48% of illness-related deaths and was the predominant cause of lethal illness in this population (40). When noninvasive diagnoses were attempted in other populations, human paramyxoviruses were sometimes implicated (11–14). However, rhinovirus C could have been missed in outbreaks for which the cause was never identified. Standard molecular diagnostics for rhinoviruses might have failed to detect rhinovirus C, which is genetically divergent from rhinoviruses A and B (28). Furthermore, rhinoviruses are adapted to the respiratory tract and are acid sensitive, such that virions dissociate below pH 6.0 (41). Because of this property, rhinoviruses are unlikely to survive gastrointestinal transit, and their nucleic acids are unlikely to be found in feces, except during acute and fulminant infections (42). Noninvasive diagnostics of ape respiratory disease outbreaks might, therefore, be inherently limited in detecting rhinoviruses, which is unfortunate given that the remote locations in which these endangered apes live often preclude invasive diagnostics (12).

In humans, the CDHR3-Y_529 allele is associated with increased susceptibility to rhinovirus C infection (7). This elevated risk results from increased cell surface expression of CDHR3, enhanced viral binding to the cellular receptor, and increased progeny yields (6, 7). GenBank searches of all published nonhuman primate genome sequences suggest that modern humans are the only primate species in which the CDHR3-C_529 nonrisk allele occurs (A.C. Palmenberg, unpub. data). In agreement with this finding, all chimpanzees genotyped from the Kanyawara community and across Africa were homozygous for the CDHR3-Y_529 risk allele. The fact that only modern humans (and not Neanderthals or Denisovans) possess the nonrisk allele implies that resistance to rhinovirus C infection was selected for only in modern humans (9). These results highlight a species-wide susceptibility to rhinovirus C infection among chimpanzees.

Our inference about rhinovirus C as the likely cause of the epidemic is based on clinical samples from a single animal early in the outbreak. We consider it unlikely, however, that rhinovirus C was an incidental finding. The virus was found in this animal’s lung, which was a site of severe pathology, at clinically relevant titers, and paramyxoviruses and other respiratory pathogens were not identified in these

Figure 3. Phylogenetic tree of rhinovirus C variants. The tree was constructed from a codon-based alignment (6,234 positions) of the new chimpanzee-derived sequence identified in the Kanyawara chimpanzee community, Uganda, 2013 (indicated by the asterisk and chimpanzee silhouette), and all human-derived RV-C complete polyprotein gene sequences available in GenBank as of December 18, 2016, with rhinoviruses A and B from the RefSeq database included as outgroups. We created alignments using the MAFFT algorithm (18) implemented in the computer program Translator X (19), with the Gblocks algorithm (20) applied to remove poorly aligned regions. We constructed trees using the maximum-likelihood method implemented in PhyML (22), with best-fit models of molecular evolution estimated from the data by using jModelTest (21). Circles on nodes indicate statistical confidence on the basis of 1,000 bootstrap replicates of the data (closed circles 100%; open circles >75%). Scale bar indicates nucleotide substitutions per site. GenBank accession numbers and other details of the RV-C sequences included in the analysis are in online Technical Appendix Table (https://wwwnc.cdc.gov/EID/article/24/2/17-0778-Techapp1.pdf).
samples or in fecal samples from the 41 other animals. All adenoviruses sequenced from fecal samples were similar or identical to adenoviruses that have been documented in apparently healthy wild chimpanzees and other primates (32,34,35,43). Finally, clinical signs during the chimpanzee outbreak bore a striking resemblance to those caused by rhinovirus C in susceptible humans, and epidemiologic transmission parameter estimates were consistent with the common cold in human populations (39). Nevertheless, we cannot rule out adenoviruses or other undetected co-infecting agents as contributing factors because such infections can enhance the clinical severity of rhinovirus C (1). In this regard, we note that simian immunodeficiency virus, which can cause immunodeficiency in wild chimpanzees (44), was not detected and is thought to be absent from this chimpanzee population (45).

Rhinovirus C is genetically diverse and common among the human populations of sub-Saharan Africa (9). Kibale National Park, wherein the home range of the Kanyawara community lies, is frequented by researchers, tourists, and persons living at the periphery of the park, and chimpanzees sometimes leave the park to raid crops in local villages. Accordingly, myriad pathways exist by which the Kanyawara chimpanzees could have been exposed to rhinovirus C from humans. Current guidelines for visiting wild apes in Uganda and other countries are based in part on generalized risks for respiratory disease transmission from humans (e.g., quarantine periods for arriving travelers are mandated at Kanyawara) (46). We suggest that specific consideration of rhinovirus C and its particular biologic attributes might improve such guidelines. For example, rhinovirus virions are nonenveloped and might therefore persist in the environment for extended periods of time, even maintaining infectivity after desiccation (47).

Long-term records from Kanyawara indicate that respiratory disease outbreaks of varying severity have occurred approximately 2 times per year for at least the past decade, with fatalities occurring every ≈2 years, typically among young animals <5 years of age and adults >35 years of age (R.W. Wrangham, unpub. data). Observations also suggest that respiratory disease outbreaks have occurred in other chimpanzee communities in Kibale at the same times as outbreaks in the Kanyawara community. Kibale contains ≈1,500 chimpanzees in ≈10–20 interconnected communities; that such a population could sustain rhinovirus C or other similar infectious agents in cycles of within-group and between-group transmission is possible, at least for limited time periods (39). If respiratory viruses of human origin can circulate independently in wild chimpanzee populations of sufficient size, this fact would be troublesome not only for chimpanzee conservation but also for human health, in that chimpanzees could also serve as a reservoir for human infections.

Although the mandate that researchers and tourists visiting wild apes wear facemasks is controversial, this practice has successfully reduced the transmission of rhinoviruses and other co-infecting agents that exacerbate rhinovirus clinical severity in hospital settings (48). Similarly, proper hygiene and the use of hand sanitizer effectively reduce the amounts of rhinovirus on human fingers (49). Decades ago, captive chimpanzees were used as study subjects in biomedical research intended to evaluate putative rhinovirus prevention and treatment options (36,37,50). Our results suggest that these investigations could ultimately benefit the wild relatives of the chimpanzees that were the original subjects of these laboratory experiments. We advocate building upon existing data to engineer novel interventions and prevention strategies for rhinovirus infections in both humans and wild apes.

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About the Author
Mr. Scully is a doctoral student at Harvard University in Cambridge, Massachusetts. In his research, he seeks to elucidate the ecologic, epidemiologic, and molecular barriers to cross-species pathogen transmission among human and nonhuman primates in systems ranging from Plasmodium to viruses.

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Address for correspondence: Tony L. Goldberg, University of Wisconsin–Madison, Department of Pathobiological Sciences, 1656 Linden Dr, Madison, WI 53706, USA; email: tony.goldberg@wisc.edu

RESEARCH

Spread of Meropenem-Resistant
*Streptococcus pneumoniae*

Satoshi Nakano, Takao Fujisawa, Yutaka Ito, Bin Chang, Yasufumi Matsumura, Masaki Yamamoto, Miki Nagao, Shigeru Suga, Makoto Ohnishi, Satoshi Ichiyama

After the introduction of pneumococcal conjugate vaccines, the incidence of pneumococcal infections due to meropenem-resistant serotype 15A-ST63 strains increased in Japan. By using whole-genome sequencing and comparing sequences with those of clones from the United Kingdom, the United States, and Canada, we clarified the traits of the serotype 15A-ST63 clone. Our analysis revealed that the meropenem-resistant serotype 15A-ST63 strains from Japan originated from meropenem-susceptible strains from Japan. Recombination site prediction analysis showed that the meropenem-resistant strain-specific recombination regions included the *pbp1a* and *pbp2b* regions. A detailed analysis of the composition of these genes indicated that resistance seems to be caused by *pbp1a* recombination. The *pbp1a* gene in meropenem-resistant isolates was identical to that in multidrug (including meropenem)–resistant serotype 19A-ST320 pneumococci, which have spread in the United States. The global spread of pneumococci of this lineage is noteworthy because serotype 15A is not included in the currently used 13-valent pneumococcal conjugate vaccine.

*Streptococcus pneumoniae* is a common pathogen that causes various types of bacterial infections, such as pneumonia, otitis media, occult bacteremia, and meningitis (1). *S. pneumoniae* is enclosed in a complex polysaccharide capsule that can be used to classify strains into serotypes. So far, at least 92 structurally and serologically distinct serotypes have been recognized (2). To prevent invasive pneumococcal diseases, vaccines currently in use in various regions of the world are 7-, 10-, and 13-valent pneumococcal conjugate vaccines (PCVs) that target a subset of the serotypes. Although these vaccines have decreased the total number of cases of invasive pneumococcal disease, they have also caused a shift in serotype (i.e., increased rate of identification of non-PCV serotype pneumococci) in areas where the vaccines have been introduced (3–6). Public health officials are concerned about the increased incidence of non-PCV serotype pneumococcal infections and the spread of resistant strains that are not covered by the currently used PCVs.

In Japan when PCV13 was in use (2012–2014), we conducted a nationwide pneumococcal infection surveillance study among children (7). We observed an increase in multidrug (penicillin, macrolide, and meropenem)–resistant pneumococcal isolates of serotype 15A and sequence type (ST) 63 (serotype 15A-ST63), which is not covered by PCV13. Similarly, several surveillance studies in other countries also reported an increase in serotype 15A-ST63 pneumococcal infections, including infections caused by penicillin-resistant strains (8–16) but not meropenem-resistant serotype 15A pneumococcal strains. A pneumococcal strain of serotype 15A-ST63 from Sweden was submitted as a resistant clone to the Pneumococcal Molecular Epidemiology Network (PMEN) collection (Sweden15A-25, ATCC BAA-661) (17); however, the penicillin MIC for Sweden15A-25 differs from that for serotype 15A-ST63 from Japan. Although the submitted PMEN strain is slightly resistant to penicillin (MIC 0.064), most serotype 15A-ST63 isolates from Japan are more resistant to penicillin. Although the introduction of PCVs is thought to have driven the increase in non-PCV pneumococcal infections, the mechanism by which the strains have become resistant remains unclear.

In general, pneumococcal resistance to penicillin and cephalosporins (including carbapenems) is caused by mutations in penicillin binding proteins (PBP), especially PBPs 1a, 2b, and 2x (18). Although PBP genes of sensitive pneumococci are well conserved, PBPs of resistant...
isolates are encoded by highly variable genes containing sequence blocks that are generally referred to as mosaic genes; these genes are generated by recombination events (19–21). Therefore, tracking the sequences of PBP genes is useful for predicting resistance to antimicrobial drugs and following pneumococcal epidemiologic trends. In various countries, meropenem is widely used to treat severe infectious diseases; however, infectious diseases caused by pathogens resistant to meropenem (carbapenems) have been increasing (22). Although carbapenem resistance has been noted mainly in Enterobacteriaceae, resistance in other pathogens, including S. pneumoniae, should be noted because such broad-spectrum antimicrobial drugs are often used empirically.

Our aim with this study was to clarify the genetic characteristics of meropenem-resistant serotype 15A-ST63 strains isolated in Japan. In addition, using whole-genome sequencing data, we mapped the evolution of the clone by revealing the genetic associations among drug-resistant serotype 15A-ST63 pneumococcus isolates from different areas of the world (Japan, United Kingdom, United States, and Canada).

Materials and Methods

Bacterial Isolates

From January 2012 through December 2014, we conducted a nationwide, prospective surveillance study among children in Japan with and without invasive pneumococcal disease (7). From 154 medical institutions, we collected isolates from patients with (343 isolates) and without (286 isolates) invasive pneumococcal disease. Among these 629 isolates, we obtained 52 serotype 15A-ST63 isolates, including 35 meropenem-nonsusceptible (MEM-NS) isolates and 17 meropenem-susceptible (MEM-S) isolates. With regard to MEM-NS non–serotype 15A isolates, we obtained 66 isolates comprising 8 serotypes (6A, 6B, 6D, 15B/C, 19A, 19F, 23F, and 35B) and nontypeable serotypes. Following the 2008 Clinical and Laboratory Standards Institute guidelines (23), we performed susceptibility testing for meropenem by using the broth microdilution method and defined the MEM-NS MIC as ≥0.5 mg/L and the MEM-S MIC as <0.25 mg/L.

Whole-Genome Sequencing

Of the isolates described above, we obtained read data for 24 MEM-NS serotype 15A-ST63 isolates, 10 MEM-S serotype 15A-ST63 isolates, and 32 MEM-NS non–serotype 15A (6A, 6B, 6D, 15B/C, 19A, 19F, 23F, 35B, and untypeable) isolates (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/2/17-1268-Techapp1.pdf). To achieve a uniform distribution of regions and times where and when the isolates were identified, we randomly selected 24 MEM-NS serotype 15A-ST63, 10 MEM-S serotype 15A-ST63, and 32 MEM-NS non–serotype 15A isolates from the isolate repository (Table 1). In addition, we obtained read data for the PMEN 15A-25 strain (Sweden15A-25, ATCC BAA-662). We used a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) to extract total genomic DNA from

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<tr>
<th>Serotype and sequence type</th>
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<td>23</td>
<td>43</td>
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</table>
To compare the sequences of the transpeptidase regions of and Pilus Detection PBP Profiles, Antimicrobial Resistance Genes, phylogenic tree based on the putative point mutations out-

To create a phylogenic tree, we used Genealogies Unbiased Phylogenomic Analyses ST63 isolates from Japan with those of isolates from the United Kingdom, the United States, and Canada, we downloaded whole-genome read data from the Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra/) (online Technical Appendix Table 1) (5,8,24,25). To confirm that the data were from serotype 15A-ST63 isolates, we conducted a de novo assembly by using SPAdes (26). The contigs were analyzed by using BLAST+ (27) to verify the presence of the pneumococcal serotype 15A–specific region (online Technical Appendix Table 5) (5), followed by multilocus sequence typing (MLST) (http://pubmlst.org/spneumoniae/), which used the extracted subsequences of each allele from the contigs. We used the draft genome data that contained serotype 15A–specific genes from ST63 isolates for the subsequent analysis.

Phylogenic Analyses To create a phylogenetic tree, we used Genealogies Unbiased By recomBinations In Nucleotide Sequences (Gubbins) (28), which identifies recombination sites and constructs a phylogenetic tree based on the putative point mutations outside of the regions. First, we created 2 phylogenic trees. Although there were no ST63 isolates or isolates that were closely related to ST63 with non–serotype 15A, to prove that there were no serotype switch events from non–serotype 15A to serotype 15A, we created the first tree with isolates from Japan only. To reveal the genetic associations among serotype 15A-ST63 isolates from Japan and elsewhere around the world that are increasing in incidence, we created the second tree with only serotype 15A-ST63 isolates from Japan and other regions in the world. After obtaining the second phylogenetic tree, to predict the recombina-

Whole-Genome Sequencing Statistics The sequencing statistics are shown in online Technical Appendix Table 2. With use of the 2,078,953-bp S. pneumoniae G54 chromosome (reference sequence GenBank accession no. NC_011072.1), serotype 15A-ST63 and PMEN15A-25 isolate genomes were sequenced at an average depth (± SD) of 49.33 (± 9.65) and an average coverage of 95.60% (± 3.75%). The average numbers of contigs and \( N_{\text{avg}} \) (bp) of isolates from Japan sequenced in this study were 109.2 (SD ± 42.2) and 70,819 (SD ± 10,701), respectively.

Phylogenomics The phylogenetic tree created by using all Japan and global isolates classified these isolates into 11 clusters (online Technical Appendix Figure 1). All of the Japan serotype 15A isolates were included in the same cluster, and none of the non–serotype 15A isolates were included in this cluster. This fact indicated that none of the non–serotype 15A iso-

PBP Profiles, Antimicrobial Resistance Genes, and Pilus Detection To compare the sequences of the transpeptidase regions of pbp1a, 2b, and 2x of all isolates, we extracted each PBP transpeptidase region from all obtained contigs by using BLAST+. We allocated PBP transpeptidase type numbers to these contigs by using previous PBP sequence data from the United States (5,29–31). To predict a causal PBP transpeptidase type for meropenem resistance in serotype 15A-ST63 isolates from Japan, we identified recombination sites within these serotype 15A isolates by using Gubbins. In addition, we identified the presence of the \( \text{ermB} \), \( \text{ermTR} \), \( \text{mefA} \), \( \text{mefE} \), \( \text{tetM} \), \( \text{tetO} \), \( \text{rrgA-1} \) (pili1), and \( \text{pilB-1} \) (pili2) genes and searched for mutations within the \( \text{folA} \) and \( \text{folP} \) genes by using the assembled contigs (5). Details of the genomic analysis process are described in the online Technical Appendix.

PBP Recombination Sites that Could Cause Meropenem Resistance Using all of the clade I isolates, we predicted the recombination sites that caused meropenem resistance in the
MEM-NS serotype 15A-ST63 isolates from Japan. This analysis revealed 19 recombination sites that were specific to all MEM-NS isolates (Figure 2; online Technical Appendix Figure 2). One of these recombination sites included the whole \textit{pbp1a} gene, and another overlapped with a part of the nucleotide sequence of \textit{pbp2b}. The recombination site covering \textit{pbp1a} included 8,384 bp (positions 326417–334800 of the reference strain \textit{S. pneumoniae} G54) (online Technical Appendix Figure 3). All MEM-NS serotype 15A-ST63 isolates had the same nucleotide sequence of \textit{pbp1a} as those of MEM-NS serotype 19A, 19F, 23F, 6A, 6B, and nontypeable isolates from Japan. The recombination site that overlapped with the \textit{pbp2b} gene was a 1,970-bp region (positions 1523469–1525438 of the reference strain \textit{S. pneumoniae} G54) (online Technical Appendix Figure 3). Because of this recombination, a portion of the nucleotide sequence of \textit{pbp2b} was replaced with the current sequence, resulting in development of a novel MEM-NS serotype 15A-ST63 \textit{pbp2b} gene that was not found in other isolates in this study or in the public database.

**Comparison of the PBP Profiles and 3 Conserved Amino Acid Motifs**

\textit{pbp1a}

All Japan MEM-NS serotype 15A-ST63 isolates had type 13 \textit{pbp1a}, which has been identified mainly in multidrug-resistant serotype 19A and 19F isolates from the United States (Table 2; online Technical Appendix Tables 1, 6) (5). Of 32 Japan MEM-NS non-serotype 15A isolates, 18 also had type 13 \textit{pbp1a}. These 18 isolates included 10 serotype 19A isolates, 3 serotype 19F isolates, and isolates of 5 other serotypes. All Japan MEM-S serotype 15A-ST63 isolates had type 24 \textit{pbp1a}, which was identified in the PME-N15A-25 isolate and in penicillin-intermediate-resistant and MEM-S serotype 15A isolates in the United States. Of
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86 global isolates, 74 also had type 24 \textit{pbp1a} and the other 12 had novel \textit{pbp1a} genes. All 24 Japan MEM-NS serotype 15A-ST63 isolates had the same SSMK motif (Table 3). All 10 Japan MEM-S serotype 15A-ST63 isolates had the STMK motif.

\textbf{pbp2b}

The Japan MEM-NS serotype 15A-ST63 isolates had a specific novel \textit{pbp2b} type that was not found in the US \textit{pbp2b} profile list (Table 2; online Technical Appendix Tables 1, 7) (5). In the reference \textit{pbp2b} type database, type 74 \textit{pbp2b} was the closest to the novel type from Japan, which possessed 5 aa sequence mutations. Japan MEM-NS non–serotype 15A isolates showed 8 \textit{pbp2b} types, and the types differed from each other mainly by serotype. All Japan MEM-S serotype 15A-ST63 isolates showed type 27 \textit{pbp2b}, which was identified in penicillin-intermediate-resistant and MEM-S serotype 15A isolates in the United States. Of 86 global isolates, 83 also had type 27 \textit{pbp2b}. All sequenced isolates from Japan, including MEM-NS and MEM-S serotype 15A-ST63 isolates and MEM-NS non–serotype 15A isolates, had the same SVVK, SSN, and KTG motifs (Table 3).

\textbf{pbp2x}

In contrast to the \textit{pbp1a} and \textit{pbp2b} type profiles, the \textit{pbp2x} type profile showed a more complicated distribution. The most prevalent \textit{pbp2x} type in the Japan MEM-NS serotype 15A-ST63 isolates was type 43 (22/24 isolates), and 5 of 10 Japan MEM-S serotype 15A-ST63 isolates also had the type 43 \textit{pbp2x} gene (Table 2; online Technical Appendix Tables 1, 8). This type was identified in a serotype 19F-CC177 isolate from the United States that was resistant to penicillin and susceptible to meropenem (5). The other 2 MEM-NS serotype 15A-ST63 isolates each had a novel \textit{pbp2x} type. Among the global isolates, the most prevalent...
was identified in 12 of the isolates from the United Kingdom, and 35 (12/86) and type 179 (12/86). Similar to the tetM type 15A-ST63 isolates had a deletion of 2 nt at codon 339 in tetM, generating a premature stop codon; resistance gene substitution). All 16 of these isolates also had a mefE. However, 16 isolates (5 from the United States and 1 from Canada) had only the folP insertion.

**Discussion**

After the introduction of PCVs, serotype 15A pneumococcal infections and colonization increased in many countries (7–16). According to previous molecular studies, most serotype 15A isolates belonged to ST63, which has been named Sweden15A-25 (PMEN15A-25) in the PMEN and shows strong macrolide resistance. The PMEN database and previous studies regarding serotype 15A-ST63 strains indicate that most isolates that were closely related to PME-N15A-25 were susceptible (MIC ≤0.06 mg/L) or showed intermediate resistance (MIC 0.12–1.0 mg/L) to penicillin (5,9,10,32). Although data for meropenem susceptibility of these isolates are limited (5,7), no studies have demonstrated the meropenem resistance of this strain. Thus, the spread of this strain so far seems to be limited to Japan. However, this finding is of concern for several reasons. One reason is that the fact that serotype 15A is not included in the currently used PCV13; therefore, the increased incidence would continue under the current vaccine pressure. In addition, penicillin, meropenem, and macrolide resistance may cause the strain to spread rapidly. In fact, after the introduction of PCV7, multdrug-resistant serotype 19A-CC320/271 spread rapidly and widely in the United States (33).

Several previous studies revealed the emergence of serotype-switched new strains that showed resistance to several antimicrobials (33–35). Most of the mechanisms underlying the emergence of new resistant strains are

### Table 2. Penicillin binding protein profile of *Streptococcus pneumoniae* serotype 15A-ST63 isolates from Japan, 2012–2014*

<table>
<thead>
<tr>
<th>Clone (no.)</th>
<th>pbp1a (no.)</th>
<th>pbp2b (no.)</th>
<th>pbp2x (no.)</th>
<th>pbp1a:pbp2b:pbp2x (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-NS-19A-ST320 (4)</td>
<td>13 (4)</td>
<td>11 (4)</td>
<td>16 (4)</td>
<td>13:11:16 (4)</td>
</tr>
</tbody>
</table>

*MEM-NS, meropenem-nonsusceptible; MEM-S, meropenem-susceptible; ST, sequence type.

### Table 3. PBPs 1a, 2b, and 2x transpeptidase conserved amino acid motif profile of *Streptococcus pneumoniae* isolates from Japan, 2012–2014*

<table>
<thead>
<tr>
<th>Clone (no.)</th>
<th>pbp1a (no.)</th>
<th>pbp2b (no.)</th>
<th>pbp2x (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-S-15A-ST63 (10)</td>
<td>STMK (10)</td>
<td>SRN (10)</td>
<td>KTG (10)</td>
</tr>
<tr>
<td>MEM-NS-15A-ST63 (24)</td>
<td>SSMK (24)</td>
<td>SRN (24)</td>
<td>KTG (24)</td>
</tr>
<tr>
<td>MEM-NS-non15A (32)</td>
<td>SSMK (32)</td>
<td>SRN (32)</td>
<td>KTG (32)</td>
</tr>
<tr>
<td>PMEN15A-25 (1)</td>
<td>STMK (1)</td>
<td>SRN (1)</td>
<td>KTG (1)</td>
</tr>
</tbody>
</table>

*MEM-NS, meropenem-nonsusceptible; MEM-S, meropenem-susceptible; PBP, penicillin binding protein; PMEN, Pneumococcal Molecular Epidemiology Network; ST, sequence type.*
associated with the recombination of the cps region flanking \( pbp1a \) and \( pbp2x \); resistant strains switched their serotypes via recombination of the cps region, or susceptible strains gained \( pbp1a \) and/or \( pbp2x \) resistance genes with the cps region. In the MEM-NS serotype 15A-ST63 isolates investigated in this study, the recombination sites that caused meropenem resistance included the \( pbp1a \) and \( pbp2b \) regions but did not include the cps region; thus, the serotype switch did not occur. The nucleotide sequence of \( pbp1a \), including the transpeptidase region found in MEM-NS serotype 15A-ST63 isolates, was 100% identical to that of the meropenem-resistant serotype 19A-ST320 strain that is prevalent in the United States (type 13 \( pbp1a \)) (5). This serotype 19A-ST320 strain was also recovered in our previous surveillance study in Japan (7), and the transpeptidase region of the isolates was the same as that of MEM-NS serotype 15A-ST63 isolates. This finding suggests that the MEM-S serotype 15A-ST63 strain gained the meropenem resistance–related \( pbp1a \) gene to become the MEM-NS serotype 15A-ST63 strain. Of note, this \( pbp1a \) type was identified in several Japan MEM-NS serotype isolates, such as 19F, 23F, 6A, 6B, and nontypeable isolates. According to previous PBP profile data from the United States (5), type 13 \( pbp1a \) was found in serotype 19A-ST320 only, and there were no widely spread PBP types across many resistant lineages.

In Japan, broad-spectrum oral cephalosporin, fluoroquinolones, and macrolides have been frequently prescribed. The inappropriate use of antimicrobial drugs may provide selective pressure and cause the spread of meropenem-resistant strains by the transfer of the meropenem resistance–related \( pbp1a \) gene.

The Japan MEM-NS serotype 15A-ST63 isolates had a novel, specific \( pbp2b \) type. The contribution of this \( pbp2b \) type to meropenem resistance is not clear. One of the MEM-NS serotype 15A-ST63–specific recombination sites overlapped the \( pbp2b \) region; therefore, this recombination may have caused meropenem resistance. However, all of the MEM-NS and MEM-S serotype 15A-63 isolates had the same sequences in each of 3 conserved amino acid motifs of \( pbp2b \). We believe that this result reduced the likelihood that \( pbp2b \) recombination is associated with meropenem resistance. In addition, we identified 17 other MEM-NS serotype 15A-ST63–specific recombination sites that occurred outside of \( pbp1a \) and \( pbp2b \). It is possible that these recombination events could result in meropenem resistance by non-PBP mutations.

We note the usefulness of PBP typing for predicting drug resistance and tracing the geographic genetic trends in pneumococci. MLST has been widely used in epidemiologic studies of pneumococci to trace genetic trends and to predict serotype switch events that occasionally lead to the development of antimicrobial drug–resistant clones. However, as in our studies, MLST is unable to predict recombination events that occur without a serotype switch, even if the recombination leads to the development of resistance. These facts highlight the value of PBP typing, and these types of data would support future studies of pneumococci.

All analyzed isolates were positive for \( ermB \) and \( tetM \); however, several isolates had a 2-nt deletion in \( tetM \), which generated a premature stop codon. This deletion was mainly identified in the UK isolates, and only 1 Japan isolate showed this deletion. This deletion was also identified in PMEN15A-25, which was recovered in Portugal in 1998. Considering that \( tetM \) generally exists on a transposable element, these results may imply that the origins of \( tetM \) differ from those of isolates from Japan, the United States, and Europe; \( tetM \) may exist on different transposable elements in each region, which may have been imported from different sources. In addition, the complete conservation of \( ermB \) and \( tetM \) may indicate that these genes contribute to its global spread.

This study had limitations. First, we examined fewer Japan MEM-S than MEM-NS serotype 15A-ST63 isolates, which may have reduced the accuracy of the recombination site prediction. However, the 2 obtained phylogenetic trees, one that was constructed by using all isolates of serotype 15A-ST63 and another that was constructed by using only Japan serotype 15A-ST63 isolates, resulted in similar clade Is. Therefore, we believe that the effect of the small number on the result was low. Second, we analyzed serotype 15A-ST63 isolates recovered from only 4 countries with a reference isolate. Future studies that include many isolates from other countries will provide additional insights into the spread and evolution of the serotype 15A-ST63 strains.

In conclusion, MEM-NS serotype 15A-ST63 pneumococci have spread in Japan after the introduction of PCV7 and PCV13. This strain originated from the MEM-S serotype 15A-ST63 strain that was prevalent in Japan; MEM-S serotype 15A-ST63 became MEM-NS serotype 15A-ST63 because of the recombination of the \( pbp1a \) region. The causative \( pbp1a \) fragment seemed to have been transferred from the MEM-NS serotype 19A-ST320 strain, and the fragment was identified in many meropenem-resistant serotype isolates in Japan. The Japan and North America serotype 15A-ST63 strains seemed to lack the original \( tetM \) gene that had a premature stop codon. The global spread of this lineage is noteworthy because serotype 15A is not included in the currently used PCV13.

Acknowledgments
We are grateful to Toshiaki Ihara for his substantial contribution to the Pneumocatch surveillance study and to the other members of the Pneumocatch surveillance study group.
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About the Author
Dr. Nakano is an assistant professor at the Department of Infection Control and Prevention, Kyoto University Hospital, and the Department of Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine. His research interests focus on molecular microbiology and epidemiology.

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Address for correspondence: Satoshi Nakano, Kyoto Daigaku Igakubu Fuzoku Byoin, 54 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto, Kyoto 606-8507, Japan; email: snakano@kuhp.kyoto-u.ac.jp
Role of Environmental Factors in Shaping Spatial Distribution of Salmonella enterica Serovar Typhi, Fiji

Ruklanthi de Alwis, Conall Watson, Birgit Nikolay, John H. Lowry, Nga Tran Vu Thieu, Tan Trinh Van, Dung Tran Thi Ngoc, Kitione Rawalai, Mere Taufa, Jerimaia Coriakula, Colleen L. Lau, Eric J. Nilles, W. John Edmunds, Mike Kama, Stephen Baker, Jorge Cano

Fiji recently experienced a sharp increase in reported typhoid fever cases. To investigate geographic distribution and environmental risk factors associated with Salmonella enterica serovar Typhi infection, we conducted a cross-sectional cluster survey with associated serologic testing for Vi capsular antigen–specific antibodies (a marker for exposure to Salmonella Typhi in Fiji in 2013. Hotspots with high seroprevalence of Vi-specific antibodies were identified in north-eastern mainland Fiji. Risk for Vi seropositivity increased with increased annual rainfall (odds ratio [OR] 1.26/quintile increase, 95% CI 1.12–1.42), and decreased with increased distance from major rivers and creeks (OR 0.89/km increase, 95% CI 0.80–0.99) and distance to modeled flood-risk areas (OR 0.80/quintile increase, 95% CI 0.69–0.92) after being adjusted for age, typhoid fever vaccination, and home toilet type. Risk for exposure to Salmonella Typhi and its spatial distribution in Fiji are driven by environmental factors. Our findings can directly affect typhoid fever control efforts in Fiji.

With an estimated disease burden of 20.6 million cases in low- and middle-income countries in 2010, typhoid fever remains an enteric disease of public health concern (1,2). Typhoid fever cases largely arise in low- and middle-income countries because marked improvements in water, sanitation, and sewage removal have helped reduce typhoid fever incidence in most industrialized countries (3–6). Salmonella enterica serovar Typhi, the causative agent of typhoid fever, is specific to humans and is typically transmitted by the fecal–oral route between humans, that is, through the ingestion of contaminated food and water (3,7). Typhoid fever infections are usually acute, although for ≈3%–5% of cases, Salmonella Typhi causes an asymptomatic and persistent (chronic) infection. These infected persons are commonly referred to as typhoid fever carriers and are capable of shedding bacteria and sustaining transmission within the community (3,8).

Pathogenicity of Salmonella Typhi is conferred by virulence factors, such as Vi polysaccharide. The Vi polysaccharide is an outer capsular antigen that enables greater human infectivity than those Salmonella Typhi strains not expressing the antigen (9). Because of the highly antigenic nature of Vi, infection with Vi-positive Salmonella Typhi strains elicits Vi-specific antibodies in humans (10). Therefore, detection of Vi-specific IgG can be used to measure Salmonella Typhi exposure, either past or chronic infection(s) (11). Furthermore, current human-approved typhoid fever vaccines are primarily Vi antigen based (e.g., Vi polysaccharide and Vi conjugate vaccines) (12). Despite antigenicity of the Vi polysaccharide, antibodies and immunity conferred by the Vi vaccine are short lived (13).

Fiji is an archipelago of >300 islands in the Pacific Ocean; most of its population is on the 2 islands of Viti Levu and Vanua Levu. During 1991–2000, <5 typhoid cases/100,000 persons were reported per year, mostly in Vanua Levu (14,15). However, since 2005, the number of typhoid fever cases has been increasing (16), reaching a peak of >50 cases/100,000 persons/year after widespread destruction and flooding caused by Cyclone Tomas in 2010. As a result, the Fiji Ministry of Health increased surveillance and implemented additional prevention strategies, such as vaccination against typhoid fever in the worst affected regions (17,18).
The risk factors for transmission of *Salmonella Typhi* in Fiji are only partially understood. Inadequate handwashing practices, poor sanitation, lack of access to safe water, and dumping of untreated waste/sewage are believed to contribute to this transmission (17,19). In addition, every year during November–April, Fiji experiences powerful cyclones, which have led to destruction of homes and contamination of water sources by extensive rainfall and flooding, followed by an increase in diarrheal diseases (20,21). Although flooding has been shown to lead to outbreaks of other foodborne and waterborne diseases (22–24), a direct link between flooding and increased typhoid fever incidence has not been confirmed in Fiji.

Public health efforts to control typhoid fever have been hampered by the lack of information regarding the epidemiology, spatial distribution, and risk factors for typhoid fever exposure in Fiji. Therefore, we used the presence of Vi-specific antibodies as a biomarker for typhoid fever exposure and used geospatial and statistical approaches to identify environment-associated risk factors in the general population of Fiji. Because of the yearly occurrence of cyclones in Fiji, we gave special attention to the potential contribution of flooding (and flood-promoting factors) to seropositivity of *Salmonella Typhi* Vi antigen.

### Methods

#### Study Design

This study was a cross-sectional cluster survey with an associated serologic analysis, which was conducted across 3 divisions in Fiji: Northern, Central, and Western. We excluded administrative areas where the 2010 typhoid vaccination campaign (18) had been implemented. We divided the country into healthcare coverage areas (nursing zones) and selected them by using probability proportional-to-size random sampling based on census data. We then selected cluster sites (communities) within nursing zones by using random list sampling, followed by random sampling of households within community cluster sites by using community health worker censuses or a modified Expanded Program on Immunization sampling of the World Health Organization (25) and then random sampling of a person per household. We excluded children <1 year of age. Community visits and data collection took place during September–December 2013, and entailed questionnaire administration, blood sample collection, and geolocation of surveyed households. Geographic coordinates were collected by using handheld geographic positioning system (GPS) devices at the house of a participant or the nearest community center. We calculated sample size at α = 0.05 by using expected seroprevalence informed from a previous study (26). Further details on study design and sampling have been reported by Watson et al. (26).

Informed consent was obtained in writing or by thumb print from all adult participants and parents or guardians of participating children. Written consent was obtained from children ≥12 years of age. This study was approved by the Fiji National Research Ethics Review Committee (#201303) and the London School of Hygiene and Tropical Medicine Ethics Committees (#6344 and #9187).

#### Survey Data

Information for 44 variables was collected during the cross-sectional survey as described (26). We then selected 13 survey variables for this typhoid fever risk factor analysis on the basis of potential environmental risk factors of interest and potential confounding covariates (26). These variables included age, education, self-reported typhoid fever vaccination status, type of toilet at home, type of sewage system, work location, urbanization, and several flooding-related variables (Figure 1; online Technical Appendix Table 2, https://wwwnc.cdc.gov/EID/article/24/2/17-0704-Techapp1.pdf).

#### Vi-Specific Serologic Analysis

We determined Vi-specific antibody levels by using an ELISA adapted from Rondini et al. (27). In brief, we coated ELISA plates with Vi polysaccharide antigen, blocked with nonfat milk buffer, incubated with alkaline phosphatase–conjugated anti-human IgG, and incubated with participant serum samples (dilution 1:200). We detected antibody binding by using p-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Louis, MO, USA), and measured absorbance at 405 nm. As reported by Watson et al. (26), we used a cutoff of ≥64 ELISA units for a Vi-seropositive result.

#### Geospatial Mapping and Clustering

We estimated the geographic centroid of each community by averaging latitude and longitude coordinates of households sampled within each community and computed typhoid fever seroprevalence for each georeferenced community by using the Vi-seroimmune status of participating persons who resided in each community. We obtained confirmed typhoid fever case incidence data from the Fiji Ministry of Health and mapped per subdivision. All geographic coordinates of communities were displayed in the local projected coordinate system (Fiji Map Grid 1986).

We used Global and Anselin local Moran I tests to identify statistically significant spatial clusters and conducted by using GeoDa version 1.6.7 (online Technical Appendix) (28,29). Vi seroprevalence was log-transformed, separate row-standardized spatial weight matrices were calculated on the basis of an inverse-distance relationship, and global and local spatial associations were analyzed within each division.
Environmental Variables

We downloaded administrative boundaries from the Fiji Global Administrative Divisions Map (30). The largest administrative boundaries are known as divisions (i.e., Central, Western, Northern, and Eastern), and the island of Viti Levu is composed of Central and Western Divisions, and the island of Vanua Levu is the Northern Division. Smaller island groups comprise the administrative Eastern Division (in which samples were not collected for this study). Divisions are divided further into 14 subdivisions.

We obtained geospatial environmental data (topography data [elevation and slope], climate data [annual rainfall, rainfall during the wettest month, total rainfall for the cyclone season], hydrology data [rivers and creeks], and soil data [soil type according to composition and drainage quality]) (31,32) from the University of South Pacific (Suva, Fiji). Euclidean distance maps of straight-line distance to major rivers and creeks were generated from hydrology maps and for poorly drained soils from soil maps. We also provide additional details of spatial data used in the study (online Technical Appendix Table 3).

We generated a deterministic flood-risk model based on the principle that depressions and poorly drained soils are more likely to collect rainwater and be flooded (33). We also provide additional details on development of this flood-risk map (online Technical Appendix Figure 1).

Except for rainfall variables, which we extracted at the community level, we extracted remaining environmental data at the individual geospatially coded household level by using bilinear interpolation. We performed all geospatial processing and mapping by using ArcGIS version 10.2 (Esri, Redlands CA, USA).

Multilevel Mixed-Effect Logistic Regression

We identified risk factors for Vi antigen seropositive status by using multilevel mixed-effects logistic regression (also known as a generalized linear mixed-effect model) by including environmental and individual-related covariates as fixed-effect and a random intercept. First, we ran a null multilevel mixed-effects logistic model with typhoid fever seroimmune status (binary variable) as the dependent variable. We generated the variance partition coefficient and a
caterpillar plot (online Technical Appendix Figure 2) by using community residuals.

We tested 16 environmental covariates (Table 1) in the univariable analysis. Regarding continuous independent variables, if analysis showed at least moderate evidence of an association with seropositivity (p<0.05), we then used the variable in multivariable analysis as a continuous variable. However, if analysis showed weak or no evidence of an association with typhoid seropositivity (p>0.05), we then divided the continuous variable into quintiles (online Technical Appendix Table 3) that were retested in the univariable model separately as categorical or ordered-categorical variables. We tested all continuous variables associated with Vi antigen seropositivity with p<0.10 for categorical variables. We tested all continuous variables univariable model separately as categorical or ordered-categorical variables included the variable in multivariable analysis as a continuous variable. If analysis showed weak or no evidence of an association with typhoid seropositivity (p>0.05), we then used the variable in multivariable analysis as a continuous variable. However, if analysis showed strong evidence of an association with seropositivity (p<0.05), we then used the variable in multivariable analysis as a continuous variable. If analysis showed weak or no evidence of an association with seropositivity (p>0.05), we then used the variable in multivariable analysis as a continuous variable.

In addition to 5 environmental variables (Table 1), we confirmed several nonenvironmental risk factors (i.e., age, education, self-reported typhoid fever vaccine status, type of home toilet, type of sewage system, and knowing persons who have had typhoid fever) for Salmonella Typhi Vi antigen seropositivity as significant risk factors by univariable analysis (online Technical Appendix Table 2). We included these factors in the multivariable analysis. We developed parsimonious regression models by using a backward stepwise variable selection approach, eliminating 1 variable at a time on the basis of the highest p value in a likelihood ratio test and retaining only variables with p<0.05. We validated the final fitted multivariable statistical model by using the Hosmer-Lemeshow test and by generating predicted typhoid seroprevalence values for sampled communities (online Technical Appendix Figure 2). We analyzed data by using Stata version 14 (StataCorp LLC, College Station, TX, USA).

**Boosted Regression Trees Modeling**

We developed a base model by using the location of communities (latitude and longitude) and those variables that

| Table 1. Association between environmental factors and typhoid fever seropositivity by univariable multilevel mixed-effects logistic analysis, Fij* |
| ---------------------------------|-----|----------------|-----------------|------------------ |
| Survey data                      |     |                | Odds ratio (95% CI) | p value |
| Is there a stream nearby?        | 1,508 | Binary | 1.09 (0.82–1.46) | 0.528 |
| No (0)                           | 616   |       |                  |       |
| Yes (1)                          | 892   |       |                  |       |
| No. times house has flooded in past 3 y |       | Categorical | 1.00 (referent) | NA |
| 0                               | 1,380 |       |                  |       |
| 1–2                             | 97    |       | 0.87 (0.52–1.47) | 0.604 |
| 3–5                             | 6     |       | 0.89 (0.15–5.13) | 0.897 |
| No. times land has flooded in past 3 y |       | Categorical | 1.00 (referent) | NA |
| 0                               | 1,264 |       |                  |       |
| 1–2                             | 174   |       | 1.13 (0.77–1.66) | 0.534 |
| 3–5                             | 58    |       | 1.21 (0.66–2.22) | 0.542 |
| Work location†                  | 1,359 | Categorical |                  |       |
| Indoors                         | 636   |       |                  |       |
| Outdoors                        | 267   |       |                  |       |
| Both                            | 456   |       |                  |       |
| Urban                           | 500   |       |                  |       |
| Periurban                       | 247   |       |                  |       |
| Rural                           | 763   |       |                  |       |
| Geospatial data                 |       |       |                  |       |
| Elevation, by quintiles         | 1,462 | Ordered, categorical | 1.02 (0.90–1.15) | 0.793 |
| Slope, by quintiles             | 1,462 | Ordered, categorical | 1.04 (0.93–1.15) | 0.519 |
| Temperature, by quintiles       | 1,462 | Ordered, categorical | 0.95 (0.84–1.07) | 0.398 |
| Annual rainfall, by quintiles†  | 1,462 | Ordered, categorical | 1.13 (1.01–1.28) | 0.039† |
| Rainfall in wettest month, by quintiles | 1,462 | Ordered, categorical | 1.15 (1.02–1.30) | 0.020‡ |
| Rainfall during cyclone season, by quintiles | 1,462 | Ordered, categorical | 1.14 (1.01–1.29) | 0.029‡ |
| Distance to major rivers, by quintiles | 1,462 | Ordered, categorical | 1.07 (0.95–1.20) | 0.255 |
| Distance to major rivers and major creeks, km† | 1,462 | Continuous | 0.99 (0.99–1.00) | 0.081 |
| Distance to major rivers and major and minor creeks, by quintiles | 1,462 | Ordered, categorical | 0.96 (0.86–1.07) | 0.439 |
| Distance to poorly drained soils (major and secondary flood plains), by quintiles | 1,462 | Ordered, categorical | 0.92 (0.80–1.06) | 0.275 |
| Distance to poorly drained soils (major flood plains only), by quintiles | 1,462 | Ordered, categorical | 1.00 (0.87–1.17) | 0.949 |
| Distance from modeled flood-risk area, by quintiles† | 1,462 | Ordered, categorical | 0.90 (0.78–1.03) | 0.134 |

*NA, not applicable.
†Variables included in the multivariable multilevel analysis.
‡Variables strongly associated with typhoid fever seroimmune status by univariable analysis (p<0.05).
were found to be associated with Vi antigen seropositivity by univariable analysis. We conducted a simplification of the base model by removing redundant or noninformative variables and used an ensemble of 50 boosted regression trees (BRT) models with 11 of the most influential predictors and random sampling of 1,305 samples (a minimum of 750 sampled at 1 time) to estimate relative contributions and marginal effect plots of the most influential variables (additional details on the BRT model in the online Technical Appendix). We conducted BRT modeling in R version 3.2.2 (http://www.R-project.org) by using the gbm package (34).

Results

Detection of Typhoid Fever Hotspot Communities

Approximately one third of serum samples (485/1,516) were seropositive for Vi-specific antibodies (online Technical Appendix Table 1). Vi antigen seroprevalence for sampled communities in Fiji ranged from 8% to 65%; estimates were 35% for the Central Division and 24% for the Western Divisions (Figure 1). Furthermore, although the Northern Division (Vanua Levu) has a smaller population, it had a Vi antigen seroprevalence of ≈40%.

Global Moran I analysis showed strong evidence of geographic clustering of Vi antigen seroprevalence for sampled communities in the Western Division (I = 0.49, p = 0.002) and weak evidence for the Central (I = 0.08, p = 0.08) and Northern (I = –0.42, p = 0.10) Divisions. The Anselin Local Moran I test showed that, although Vanua Levu had high typhoid fever seroprevalence, there was no apparent typhoid fever hotspot clustering for communities on this island (Figure 2, panel A). However, 4 high-high (hotspot) seroprevalence cluster communities were detected in the northern and northeast regions of the Western and Central Divisions (Figure 2, panel B, C), whereas coldspots were detected primarily in the Western Division (Figure 2, panel B).

Multilevel Univariable and Multivariable Analyses

Univariable analysis identified 4 environmental variables (work location, annual rainfall, rainfall during the wettest month, and rainfall during the cyclone season) and 4 nonenvironmental variables (age, education, sewage disposal, typhoid fever vaccination status) as having a significant association with Vi antigen seropositivity (p<0.05) (Table 1; online Technical Appendix Table 1). Furthermore, we found suggestive evidence of an association with Vi antigen seropositivity (0.1>p>0.05) for several other environmental and nonenvironmental variables (urbanization, distance to major rivers and major creeks, toilet type, knowing persons who have had typhoid fever) (Table 1; online Technical Appendix Table 2).

We included 1 rainfall variable and all other environmental and nonenvironmental factors with at least a suggestive association (p<0.01) in the multivariable multilevel logistic regression analysis (Table 1; Technical Appendix Table 2). We also included proximity to modeled flood-risk areas as a fixed-term in the final fitted multivariate model regardless of its evidence of association on the univariable analysis because other environmental factors (such as rainfall and proximity to rivers) might have confounded the univariable analysis. The final multivariable statistical model contained 6 variables that explained the variation in Vi antigen seropositivity for sampled persons and communities.

After we adjusted for potential confounders (age, typhoid fever vaccination, and flush toilets), we found that annual rainfall showed a positive association (odds ratio [OR] 1.26/quintile increase; p<0.001). We also found that distance to major rivers and major creeks (OR 0.89/km increase; p = 0.031) and to modeled flood-risk areas (OR 0.80/quintile increase; p = 0.006) showed negative associations with Vi antigen seropositivity (Table 2).

The fitted model not only explained fixed-effect variation across persons, but also some of the variation across sampled communities. Comparison of the null and final models showed a reduction in the variance partition coefficient from 7.6% (p<0.0001) to 2.1% (p<0.0001), which indicated that the final statistical model explained 72% of the variation in seropositivity between communities. We validated the final multivariable model fitted by using the Hosmer-Lemeshow test and found that predicted proportions computed for the individual level were not significantly different from those for the observed proportions (p = 0.558) (online Technical Appendix Figure 2).

### Table 2. Association between social and environmental factors and typhoid fever seroimmune status in multivariable multilevel model, Fiji*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annual rainfall, by quintiles</td>
<td>1.26 (1.12–1.42)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distance to major rivers and major creeks, km</td>
<td>0.89 (0.80–0.99)</td>
<td>0.031</td>
</tr>
<tr>
<td>Distance to modeled flood-risk areas, by quintiles</td>
<td>0.80 (0.69–0.92)</td>
<td>0.002</td>
</tr>
<tr>
<td>Age of participant, y</td>
<td>1.03 (1.02–1.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vaccination status</td>
<td>1.62 (1.02–2.57)</td>
<td>0.041</td>
</tr>
<tr>
<td>Type of toilet at home</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flush</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Water seal/pour flush</td>
<td>1.0 (referent)</td>
<td>NA</td>
</tr>
<tr>
<td>Pit (with or without slab) and bucket</td>
<td>1.66 (1.16–2.38)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>1.51 (0.91–2.52)</td>
<td>0.110</td>
</tr>
</tbody>
</table>

*Multivariable model was run by using 1,338 observations in 61 communities. NA, not applicable.
**Boosted Regression Tree Modeling**

We estimated that age, GPS location, and the 3 environmental factors (distance to major rivers and creeks, distance to flood-risk areas, and annual rainfall) were the major predictors of Vi antigen seropositivity in Fiji (Table 3). These 6 covariates accounted for ≈90% of the estimated relative contribution to Vi antigen seropositivity.

The marginal effect plot for age showed that most exposure to *Salmonella* Typhi occurred in persons <40 years of age and rates plateaued in persons >60 years of age (Figure 3, panel A). Distances <1,300 m to major rivers and major creeks were predicted to increase Vi antigen seropositivity, with distances <200 m showing the greatest effect (Figure 3, panel B). Annual rainfall had a minimal effect on Vi antigen seropositivity until ≈1,700 mm, above which the risk increased dramatically (Figure 3, panel C). Furthermore, shorter distances to modeled flood-risk areas showed some contribution to typhoid fever seropositivity (Figure 3, panel D).

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**Figure 2.** Local clustering of seroprevalence of typhoid fever in divisions in Fiji. Local Anselin Moran I analysis conducted for each division separately by using an inverse-distance weighting for the communities within 3 divisions. A) Northern, B) Western, and C) Central. High-high clusters (hotspots) are communities with high seroprevalence of antibodies against *Salmonella enterica* serovar Typhi Vi capsular antigen that are near other communities with high seroprevalence. Low-low clusters (coldspots) are communities with low seroprevalence of antibodies against *Salmonella Typhi* Vi antigen that are near other communities with low seroprevalence. Red ovals indicate locations of the typhoid outbreak in 2016 after Cyclone Winston and hotspots detected by local clustering.
In the past 2 decades, Fiji has observed a steady increase in confirmed typhoid fever cases \( (16–18) \). However, little is known about the geospatial distribution and underlying risk factors of typhoid fever in Fiji. Our study demonstrated a spatially heterogeneous exposure to typhoid fever across Fiji, with Vanua Levu island showing the highest seroprevalence. High-seroprevalence communities (hotspots) were detected only in Viti Levu, whereas typhoid fever appeared to be more homogeneously distributed in Vanua Levu, suggesting a different transmission pattern on the 2 islands. Annual rainfall and proximity to major rivers, creeks, and potentially floodable areas were major environmental risk factors for serologic evidence of exposure to *Salmonella Typhi* in Fiji.

### Table 3. Relative contributions of predictor variables from an ensemble of 50 boosted regression tree models for typhoid fever seropositivity developed with cross-validation on data from 1,305 samples and 11 variables, Fiji

<table>
<thead>
<tr>
<th>Variable</th>
<th>Data type</th>
<th>Relative contribution, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Continuous</td>
<td>33.0 (31.1–34.8)</td>
</tr>
<tr>
<td>Longitude, °E</td>
<td>Continuous</td>
<td>15.5 (14.7–16.0)</td>
</tr>
<tr>
<td>Distance from major rivers and creeks, m</td>
<td>Continuous</td>
<td>14.5 (13.6–15.3)</td>
</tr>
<tr>
<td>Annual rainfall, mm</td>
<td>Continuous</td>
<td>9.3 (8.5–10.0)</td>
</tr>
<tr>
<td>Distance from flood-risk areas, m</td>
<td>Continuous</td>
<td>7.7 (6.8–8.4)</td>
</tr>
<tr>
<td>Latitude, °S</td>
<td>Continuous</td>
<td>6.9 (5.6–7.9)</td>
</tr>
<tr>
<td>Education</td>
<td>Categorical</td>
<td>4.2 (3.8–4.6)</td>
</tr>
<tr>
<td>Urbanization</td>
<td>Categorical</td>
<td>3.3 (2.9–3.8)</td>
</tr>
<tr>
<td>Typhoid fever vaccination</td>
<td>Binary</td>
<td>2.3 (2.1–2.5)</td>
</tr>
<tr>
<td>Sewage disposal</td>
<td>Categorical</td>
<td>1.8 (1.5–2.2)</td>
</tr>
<tr>
<td>Toilet type at home</td>
<td>Categorical</td>
<td>0.8 (0.6–1.2)</td>
</tr>
</tbody>
</table>

### Discussion

In the past 2 decades, Fiji has observed a steady increase in confirmed typhoid fever cases \( (16–18) \). However, little is known about the geospatial distribution and underlying risk factors of typhoid fever in Fiji. Our study demonstrated a spatially heterogeneous exposure to typhoid fever across Fiji, with Vanua Levu island showing the highest seroprevalence. High-seroprevalence communities (hotspots) were detected only in Viti Levu, whereas typhoid fever appeared to be more homogeneously distributed in Vanua Levu, suggesting a different transmission pattern on the 2 islands. Annual rainfall and proximity to major rivers, creeks, and potentially floodable areas were major environmental risk factors for serologic evidence of exposure to *Salmonella Typhi* in Fiji.

The Vi antigen seroprevalence distribution closely resembled the typhoid fever case incidence pattern reported by the Fijian Ministry of Health during 2008–2013 (online Technical Appendix Figure 3). Vanua Levu and northeastern Viti Levu had the highest typhoid fever burden. In April 2016, after Cyclone Winston hit Fiji, there was a sudden outbreak of typhoid fever in the villages of Qelekuro and Nabulini \( (35) \), which are located in northeastern Viti Levu. This latest typhoid fever outbreak in Fiji supports our findings of high-risk areas for *Salmonella Typhi* exposure, particularly in northeastern Viti Levu (Figure 2, panel A), and reinforces the hypothesis of increased exposure to typhoid fever caused by environmental anomalies in the aftermath of a cyclone.
Similar to our findings, other studies have found positive associations between diseases transmitted by the fecal–oral route (such as cholera and typhoid) and waterborne diseases (such as leptospirosis) with heavy rainfall and proximity to major rivers (36–40). Heavy rains in Fiji, particularly during the cyclone season (November–April) (27), might lead to overflowing of septic tanks and contamination of the local environment and drinking water sources. Furthermore, our study indicated proximity to major rivers and creeks as a risk factor for acquiring Salmonella Typhi, probably because major rivers and creeks are used in Fiji (similar to many other middle-income countries) for washing clothes, taking baths, and swimming (41). In addition, streams near populated areas can become contaminated by cyclones or heavy rains that cause overflowing of sewage and waste systems. Therefore, future studies investigating environmental risk factors should sample surrounding water sources for water quality assessment.

Incidences of many foodborne and waterborne diseases have been shown to increase soon after heavy flooding (22–24,42). Fiji had outbreaks of typhoid fever and leptospirosis after devastation and flooding caused by cyclones (16,18,40,43). Our multivariate model demonstrated an increased risk for Salmonella Typhi infection for persons living closer to the modeled flooding areas. Annual cyclone season and heavy rainfall, combined with most of the population in Fiji living in low-lying coastal areas, make exposure to flooding a common phenomenon and a potential conduit of Salmonella Typhi transmission.

A major strength of this study was the unbiased, individual-level assessment of environmental factors specific to each participant on the basis of their residential GPS coordinates. Furthermore, the large sample number analyzed enabled inclusion of a large number of independent variables (major nonenvironmental risk factors and environmental variables) in the statistical modeling.

However, despite many strengths, the study also had several limitations. Although Vi antigen–specific antibodies were measured as a proxy for Salmonella Typhi infection, the exact role and dynamics of Vi antigen–specific antibodies after Salmonella Typhi infection remain unclear. For example, antibodies against Vi antigen have been found to be short-lived, more often associated with chronic carriage (11,13), and produced as a result of typhoid fever vaccination. Furthermore, geospatial cluster analysis was partially hampered by an uneven distribution of surveyed communities. To mitigate this potential spatial bias, we conducted spatial clustering analysis separately for each division.

Our study was an in-depth investigation of the spatial epidemiology of typhoid fever in Fiji and flooding as a risk factor for transmission of Salmonella Typhi. Findings of this study can be used to improve future typhoid fever control programs. Recent outbreak detection in high seropositivity areas (35) suggests that serosurveillance for IgG against Vi antigen offers potential for identification of areas and communities at higher risk for typhoid fever. This spatial epidemiology analysis suggests flood-prone areas and other communities near major rivers and creeks or in high-rainfall areas could be prioritized for stricter flood control and typhoid fever preventive measures, such as improved sanitation, provision of secure water sources, and typhoid fever vaccination campaigns.

Acknowledgments
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About the Author
Dr. de Alwis is an immunoen epidemiologist at the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam. Her primary research interests involve deciphering mechanisms of protective humoral responses to tropical pathogens and population-level investigations of human serologic responses to natural infections or vaccinations.

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Distribution of *Salmonella Typhi*, Fiji


Address for correspondence: Ruklanthi de Alwis, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam; email: rdealwis@oucru.org

etymologia

**Parvovirus [pahr’ vo-vi”res]**
Eduardo Kaiser Ururahy Nunes Fonseca

Viruses of the family *Parvoviridae* (Latin *parvum* [meaning small or tiny]) are among the smallest viruses described, 18–28 nm in diameter. There are 2 subfamilies of the family *Parvoviridae*: *Parvovirinae* and *Densovirina* (Latin *denso* [thick or compact]). *Parvovirinae* may infect humans, but *Densovirina* infect only arthropods. Structurally, these viruses are non-enveloped, icosahedral viruses that contain a single-stranded linear DNA genome.

The small size of these viruses might account for their late discovery. In 1974, the first pathogenic human parvovirus was discovered and named B19 from the coding of a serum sample, number 19 in panel B, that gave anomalous results during testing for hepatitis B. Although human B19 infections are more often asymptomatic or lead to mild rash illnesses and arthralgias, they can also cause severe anemia in fetuses and in persons with underlying hemoglobinopathies.

Sources


Address for correspondence: Eduardo Kaiser Ururahy Nunes Fonseca, Escola Paulista de Medicina, Rua Dr. Diogo de Faria, 1201 Apto 178, São Paulo 04037-004, Brazil; email: edukaiser_unf@hotmail.com

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Yersinia pestis Survival and Replication in Potential Ameba Reservoir

David W. Markman, Michael F. Antolin, Richard A. Bowen, William H. Wheat, Michael Woods, Mercedes Gonzalez-Juarrero, Mary Jackson

Plague ecology is characterized by sporadic epizootics, then periods of dormancy. Building evidence suggests environmentally ubiquitous amebae act as feral macrophages and hosts to many intracellular pathogens. We conducted environmental genetic surveys and laboratory co-culture infection experiments to assess whether plague bacteria were resistant to digestion by 5 environmental ameba species. First, we demonstrated that Yersinia pestis is resistant or transiently resistant to various ameba species. Second, we showed that Y. pestis survives and replicates intracellularly within Dicyostelium discoideum amebae for ≥48 hours postinfection, whereas control bacteria were destroyed in <1 hour. Finally, we found that Y. pestis resides within ameba structures synonymous with those found in infected human macrophages, for which Y. pestis is a competent pathogen. Evidence supporting amebae as potential plague reservoirs stresses the importance of recognizing pathogen-harboring amebae as threats to public health, agriculture, conservation, and biodefense.

The etiologic agent of plague, Yersinia pestis, is a gram-negative cocccobacillus and a facultative intracellular pathogen. Y. pestis exhibited the highest overall mortality rate of any infectious disease from its earliest recorded emergence through 1941 (1). During 2010–2015, a mean of 650 cases were reported globally each year, with a case fatality rate of 23%–41% (depending on manifestation, treatment efficacy, and medical care), rising to 66%–100% when adequate medical care was not promptly received (2). Y. pestis primarily infects small ground-dwelling mammals, specifically of the taxonomic order Rodentia, but maintains high spillover potential to other vertebrates, including humans, caused by its high virulence and fleaborne transmission. Epizootic plague is typically vectored by multiple flea species and is transmitted within and between meta-populations of hosts by flea bites (Figure 1). Plague ecology is characterized by sporadic epizootics, followed by 2–5-year cryptic dormancy periods (3–9). Despite much information on epizootic transmission mechanisms, little is known about the origin of re-emergent plague cases in wild animal populations (Figure 1). Plague among wild animals commonly re-emerges in plague foci after multiple years of inactivity, despite ongoing biosurveillance and attempts at detection during interpandemic periods. The existence of environmental plague reservoirs has been theorized for >80 years (3–13). Various avenues of recent research suggest that soil-dwelling amebae may be competent environmental reservoirs of Y. pestis. Amebae are a taxonomically diverse group of phagocytic organisms residing in every major lineage of eukaryotes. Amebae are pervasive in soil and water environments and are recognized for their ability to harbor pathogens that drastically affect ecological communities (14–19). Free-living ameba cycle between 2 distinct life-states: trophozoites, an active, mobile, feeding state; and cysts or spores, a robust dormant state induced in part by adverse environmental conditions.

Ameba reservoir potential for Y. pestis is indicated by 4 major factors: the ability of related Y. enterocolitica and Y. pseudotuberculosis bacteria to persist in protozoan amebae (20–22); correlative data indicating plague epizootics temporally follow periods of increased precipitation known to reanimate ameba cysts (5,23,24); the demonstrated ability of Y. pestis to express various proteins enabling escape of the phagolysosome in a diverse array of phagocytic cells including human macrophages (25–27); and prior associations between Y. pestis and soil amebae, Vermamoeba (formerly Hartmanella) rhysodes and Acanthamoeba castellanii, that demonstrate intracellular persistence up to 5 days (13,28,29). Amebae display a high degree of functional homology with mammalian macrophages, leading to the description of amebae as feral macrophages. The ameba reservoir hypothesis is compelling for many pathogens with unexplained sporadic occurrence and cryptic dormancy periods as supported by a growing catalog (>225) of intracellular pathogens capable of surviving and/or replicating within amebae under diverse conditions (14,17,18,30).

Author affiliations: Colorado State University, Fort Collins, Colorado, USA (D.W. Markman, M.F. Antolin, R.A. Bowen, W.H. Wheat, M. Gonzalez-Juarrero, M. Jackson); Burrell College of Osteopathic Medicine, Las Cruces, New Mexico, USA (M. Woods)

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Yersinia pestis in Potential Ameba Reservoir

We tested the hypothesis that 5 species of environmentally ubiquitous amebae demonstrate reservoir potential for Y. pestis. We implemented field and laboratory investigations to assess environmental co-occurrence of study ameba species with plague epizootics; experimental infection prevalence in amebae; experimental infection intensity; intra-ameba bacterial location; bacterial viability postphagocytosis; and bacterial replication inside trophozoite amebae. We discuss the potential for D. discoideum ameba to act as interepizootic reservoirs, the functional homology between phagocytic amebae and mammalian macrophages, and the ability of ameba to exert selective pressure on the evolutionary trajectory of pathogen virulence and transmission mode. Further, we stress the importance of recognizing pathogen-harboring amebae as potential threats to global health, agriculture, conservation, and biodefense.

Materials and Methods
By using field experiments, we molecularly assessed the co-occurrence of amebae and Y. pestis in prairie dog burrows in the Pawnee National Grassland of northeastern Colorado, USA. This grassland is an established plague focus that has exhibited recurrent plague epizootics since ≈1940 (31). We used molecular analyses of soil and ameba cultured from the soil to identify candidate ameba species that may act as reservoirs for plague persistence.

Plague-Endemic Soil Isolates
We selected 24 prairie dog burrows from 8 prairie dog colonies, which can contain hundreds of animals, on the basis of suspected plague presence indicated by sustained decreases in population size during a 3-week observation period in August 2016 (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/24/2/17-1065-Techapp1.pdf). We selected individual burrows within the colony boundaries on the basis of apparent prairie dog activity (feces, freshly excavated soil, and noncollapsed burrow structure) and along a gradient from the center of the colony to the periphery. We collected soil by attaching 50-mL conical tubes to a 6-m flexible metal probe, maneuvering the probe into the prairie dog burrow to maximum achievable depth, and using the probe to scrape soil into the tubes. We sealed viable soil samples (>20 mL from >3 m deep) and stored them at 22°C until processing within 12 hours.

Cultivation of Amebae from Soil
We isolated amebae from soil in plague-affected prairie dog burrows by using modified culture methods (32) (online Technical Appendix Figure 2), incubated culture plates at 28°C, and observed for changes daily. We supplemented liquid medium with gentamicin (200 μg/mL) after 72 hours or at earliest detection of any bacterial growth. We aseptically transferred ameba cultures without bacterial contamination to 25-cm² tissue culture flasks in ameba-specific media containing penicillin/streptomycin. We identified ameba by using multiplex and endpoint PCR after extracting DNA by using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) (33,34) (online Technical Appendix Figure 3).

Bacterial Strains and Culture Conditions
We cultured Y. pestis strains from frozen stocks in lysogeny broth (LB) medium. We used 2 strains of Y. pestis throughout the study: a nontransformed prototypical strain of Y. pestis CO92 and a recombinant gfp-expressing strain, Y. pestis CO92 pgm+, pCD1, pGFPuv, amp+, from the Centers for Disease Control and Prevention (Fort Collins, CO, USA). We cultured the transformed strain by using 100 μg/mL carbenicillin to maintain selective pressure for retention of gfp plasmids. Culture conditions

Figure 1. Infection pathways for plague. During plague epizootics, transmission occurs through flea vectors within meta-populations of ground-dwelling rodents. It is unknown by what route or mechanism Yersinia pestis is maintained during interepizootic periods of plague quiescence. Previous research on fleas has not strongly supported their reservoir potential across interepizootic periods (3). The experiment and analysis of this study test the hypothesis that ameboid species demonstrate reservoir potential for Y. pestis. If Y. pestis is maintained within ameba reservoirs, we suspect that epizootic recrudescence may occur when infected soilborne amebae enter the bloodstream of naive rodent hosts (by entering wounds from antagonistic host-to-host interactions or burrowing activities). Amebae typically lyse when incubated at 37°C and simultaneously release their intracellular cargo, potentially initiating an infection.

We tested the hypothesis that 5 species of environmentally ubiquitous amebae demonstrate reservoir potential for the maintenance of Y. pestis. We implemented field and laboratory investigations to assess environmental co-occurrence of study ameba species with plague epizootics; experimental infection prevalence in amebae; experimental infection intensity; intra-ameba bacterial location; bacterial viability postphagocytosis; and bacterial replication inside trophozoite amebae. We discuss the potential for D. discoideum ameba to act as interepizootic reservoirs, the functional homology between phagocytic amebae and mammalian macrophages, and the ability of ameba to exert selective pressure on the evolutionary trajectory of pathogen virulence and transmission mode. Further, we stress the importance of recognizing pathogen-harboring amebae as potential threats to global health, agriculture, conservation, and biodefense.

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simulated a mammalian host environment (37°C for 24 h to stationary phase) and then an extra-host environment (28°C for 24 h) to activate phenotypically plastic expression profiles. We monitored bacterial growth spectrophotometrically at OD600.

**Ameba Strains and Culture Conditions**

We obtained stocks of *A. lenticulata* (ATCC 30841), *A. castellanii* (ATCC 30234), *A. polyphaga* Linc-Ap1 (CCAP 1501/18), and *V. vermiformis* (ATCC 50237) from the American Type Culture Collection (Manassas, VA, USA) and the Culture Collection of Algae and Protozoa (https://www.ccap.ac.uk/) and DictyBase (http://dictybase.org/). We axenically cultivated ameba stocks with genera-specific media in T25 tissue culture flasks at 28°C and verified them to be axenic by using standardized methods (19,35–37).

**Co-culture Experiments**

**Intraameba Infection Prevalence and Intensity Assays**

We individually co-cultured laboratory ameba species with *Y. pestis* by using established methods (21). We adjusted viable ameba trophozoite densities to 5 × 10⁴ trophozoites/mL in triplicate 25-cm² tissue culture flasks and combined *Y. pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) cultures with ameba flasks (excluding ameba controls), resulting in 5 × 10⁴ viable *Y. pestis* cells/mL and a multiplicity of infection (MOI) of 100 on the basis of OD 600 calculations. We incubated co-cultures at 28°C for 4 h before removing infected amebae, ameba controls, and bacteria controls from the surface of the flasks and washing them 3 times with Page amoeba saline (PAS) at 100 × g for 5 min (36). We then exposed amebae to gentamicin (100 μg/mL) for either 1 or 4 h to eliminate residual extracellular bacteria, then washed them 3 more times to remove antibiotic drug residue. Finally, we removed the supernatant from the last wash, concentrated it via centrifugation (4,400 × g for 10 min), and plated it on LB agar to ensure no extracellular bacteria persisted.

We fixed aliquots of each infected ameba treatment in 4% paraformaldehyde for 15 min before washing (4,400 × g, 5 min) and resuspending in 1× PAS for microscopic analysis. We used a fluorescent confocal microscope (Zeiss LSM 510 with ZEN 2009 SP2 software [Carl Zeiss GmbH, Thornwood, NY, USA]) to determine mean infection prevalence (the percentage of amebae containing ≥1 intracellular *Y. pestis* bacterium across 16 fields of view per treatment replicate). We determined mean infection intensity and its distribution by quantifying the number of intracellular bacteria per individual infected ameba, verified by z-stack confocal microscopy across 16 fields of view per treatment replicate. We used 1-way measured analysis of variance (ANOVA) on prevalence and intensity means across all 5 amebae species. We log-transformed data as necessary to meet model assumption and used least-squared mean analyses with Tukey’s adjustments for pairwise comparisons.

**Ultrastructural Description of Intraameba Bacterial Location**

We used *Y. pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) in similar co-culture infection assays with *A. castellanii* (MOI 100 in PAS at 28°C). We co-cultured bacteria for durations of 10 min, 30 min, and 24 h to assess proximal and delayed effects of phagocytosis on bacterial cell viability and intraameba bacterial location. After co-culture, mixtures were fixed in standard electron microscopy fixative for 2 h, then washed 2 times in 0.1 M cacodylate buffer. We then shipped fixed samples in 0.1 M cacodylate buffer to the Cryo-electron Microscopy Laboratory at the University of Texas Medical Branch (Galveston, TX, USA) for transmission electron microscopy (TEM) according to standardized procedures. We determined bacterial location within amebae by ultrastructural analysis of transmission electron micrographs and studied intracellular bacterial morphology to assess ameba-mediated bacterial lysis as measured by cell size, shape, and apparent membrane integrity.

**Intraameba Bacterial Survival and Quantification of Intraameba Bacterial Replication**

We performed intraameba bacterial survival and replication assays in triplicate across 5 ameba species (*A. castellanii*, *A. lenticulata*, *A. polyphaga*, *D. discoideum*, and *V. vermiformis*); 2 bacteria species (*Y. pestis* CO92 and *Escherichia coli*); 5 postinfection time points (0, 1, 4, 24, and 48 h); and 3 antibiotic drug exposure periods (0, 1, and 4 h) for removing extracellular bacteria postinfection. We used *E. coli* as an ameba-susceptible control bacterium. We performed co-cultures in 200-μL volumes within 96-well plates at a MOI of 100 in 1/2× dilute ameba growth medium at 28°C for 1 h and used control ameba and bacteria as monocultures. After initial infection, we removed the supernatant of each well, washed wells 3 times with PAS, exposed them to gentamicin (100 μg/mL), washed 3 times more, and incubated them in PAS. PAS was verified to be bacteriostatic to *Y. pestis*, thereby precluding extracellular bacterial replication and continuous ingestion by amebae. We lysed infected ameba trophozoites selectively with 100 μL 0.5% sodium deoxycholate for 5 min before serially diluting and plating on LB agar. We incubated plates at 28°C for 48 h before counting CFUs to determine bacterial survival and replication after phagocytosis by amebae. The 0.5% sodium deoxycholate lysing detergent had no effect on CFU counts in bacterial monoculture controls (data not shown).
Results

*Y. pestis* and 5 species of amebae co-occur in soils of prairie dog burrows undergoing plague epizootics. We cultured a wide diversity of amebae from soil within plague-positive prairie dog burrows in northeastern Colorado and identified live amebae of each study species (online Technical Appendix Figure 3). Among 8 prairie dog colonies, 24 burrows sampled yielded 15 heterogeneous ameba cultures free of bacteria or fungi. We identified ≥1 *Acanthamoeba* spp. from 86.6% of cultures (n = 13), *D. discoideum* from 53.3% of cultures (n = 8), and *V. vermiformis* from 6.6% of cultures (n = 1).

*Y. pestis* is phagocytosed by amebae with heterogeneous prevalence and intensity. Representative fluorescent confocal micrographs of *A. castellanii* and *D. discoideum* illustrate differences in infection intensity and prevalence (Figure 2). ANOVA F-test results indicate significant differences in infection prevalence (or phagocytic efficiency) among ameba species (p = 0.0231) (Table). Repeat experiments maintained relative ranking of mean infection intensity and infection prevalence across ameba species (A. castellanii, n = 1,441; A. lenticulata, n = 1,156; A. polyphaga, n = 737; D. discoideum, n = 624; and V. vermiformis, n = 528). Pairwise comparisons indicate *V. vermiformis* has significantly lower infection prevalence than *A. lenticulata* (p = 0.0344). Infection prevalence ranged from 24.07% in 1 replicate of *V. vermiformis* to 54.83% in 1 replicate of *A. lenticulata*.

Infection intensity was also significantly different among ameba species (p = 0.0014) (Table). Pairwise comparisons showed *V. vermiformis* has a significantly lower infection intensity than both *A. lenticulata* (p = 0.0014) and *A. polyphaga* (p = 0.0082) and that *D. discoideum* has a significantly lower infection intensity than *A. lenticulata* (p = 0.0455). These findings demonstrate genus-level differences in infection intensity. Infection intensity frequencies followed a strong negative binomial distribution (Figure 3). Each ameba species had several high-intensity outliers ranging up to a maximum of 84 intracellular bacteria observed in 1 *A. lenticulata* ameba (Figure 3).

*Y. pestis* resides in digestive and central vacuoles of both *D. discoideum* and *A. castellanii* amebae. Green fluorescent protein expressed by intracellular *Y. pestis* co-localizes with ameba vacuoles (Figure 2). TEM micrographs depict intracellular *Y. pestis* maintaining cellular shape and apparent membrane integrity inside *A. castellanii* ameba for ≥24 h postinfection (Figure 4). Ultrastructural analysis of TEM images reveals *Y. pestis* persistence within the niche of a tight-fitting vacuolar membrane visually similar to *Yersinia*-containing vacuoles (YCVs) observed in infected macrophages (27).

*Y. pestis* can survive inside *D. discoideum* amebae for ≥48 hours, but we found genus-level differences in intramaeba survival of *Y. pestis* (Table). The bacterium did not survive beyond 24 h postinfection in *A. castellanii*, *A. lenticulata*, *A. polyphaga*, or *V. vermiformis*. However, *Y. pestis* co-cultured with *D. discoideum* exhibited consistent intracellular survival for ≥48 h postinfection under variable treatment conditions (Table; Figure 5). *Y. pestis* exhibited significantly higher survival/recoverability when co-cultured with amebae as compared to *Y. pestis* monoculture controls (p<0.001). *Y. pestis* monoculture controls yielded...
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a mean of 17 CFUs after 1 h of gentamicin exposure with no recoverable control bacteria across all other treatments. Conversely, E. coli bacteria did not significantly persist under any treatment conditions when co-cultured with ameba (p<0.001). Uninfected ameba control lysates consistently yielded zero bacteria across all ameba species and treatments (data not shown). All Y. pestis co-cultures exposed to antibiotics had supernatants free of extracellular bacteria. Y. pestis had no cytopathic effect on any of the tested ameba species as verified by comparing ameba abundance between co-culture treatments and ameba monoculture controls (data not shown).

Y. pestis replicates intracellularly in D. discoideum amebae for ≥48 hours postinfection (Table; Figure 5). In D. discoideum, the abundance of viable intracellular Y. pestis was significantly greater at each successive time point (24 and 48 h postinfection) after 1 h of antibiotic drug exposure (p = 0.01 and p = 0.002, respectively). Additionally, the abundances of viable Y. pestis in D. discoideum at 24 and 48 h postinfection were significantly greater than immediately after the 4-h antibiotic treatment (p = 0.008 and p = 0.001, respectively). After 48 h postinfection, viable intracellular Y. pestis was only recovered from D. discoideum treatments. Because the data did not meet standard ANOVA assumptions of normality despite transformation attempts, we used a nonparametric Kruskal-Wallis rank-sum test to compare treatment means by species. Results indicated that the increased abundance of Y. pestis in D. discoideum was significant compared with all other species at 48 h postinfection (p<0.001).

Discussion

We demonstrate that Y. pestis (CO-92) can survive and replicate intracellularly within the social, heterogamous ameba

<table>
<thead>
<tr>
<th>Species</th>
<th>Dormant state</th>
<th>Infection prevalence*</th>
<th>Infection intensity†</th>
<th>Intracellular survival‡</th>
<th>Intracellular replication</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean, % SEM</td>
<td>Mean, % SEM</td>
<td>24 h Mean, % SEM</td>
<td>48 h Mean, % SEM</td>
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<td>0 0</td>
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<td>10 11.55</td>
<td>0 0</td>
</tr>
<tr>
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<td>5.36 0.37</td>
<td>31.66 22.04</td>
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</tr>
<tr>
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<td>39.24 3.13</td>
<td>3.57 0.97</td>
<td>270 19.92</td>
<td>226.67 22.71</td>
</tr>
<tr>
<td>Vermamoeba vermiformis</td>
<td>Cyst</td>
<td>29.61 3.4</td>
<td>1.84 0.13</td>
<td>10 9.66</td>
<td>0 0</td>
</tr>
</tbody>
</table>

*Mean percentage of amebae containing >1 intracellular bacterium.
†Mean no. of intracellular bacteria per individual infected ameba.
‡Mean no. of surviving intracellular bacteria (relative to control) in experiments with 1 and 4 h of antibiotic drug exposure.
§We observed no replication in the intraameba replication assay, which we used to count intraameba bacterial colony-forming units before and after co-culture. However, we observed probable but nondefinitive mitotic bacterial replication in the TEM micrographs (Figure 4, panel A).

Figure 3. Boxplots of infection intensity across ameba species after experimental infection with Yersinia pestis. Infection intensity frequencies followed a strong negative binomial distribution. Median infection intensities (horizontal lines inside boxes): AC = 3, AL = 4, AP = 3, DD = 2, VV = 1. Red diamonds denote mean infection intensity (Table). Each ameba species had several high-intensity outliers ranging up to a maximum of 84 intracellular bacteria observed in 1 A. lenticulata ameba (note broken y-axis). AC, Acanthamoeba castellanii (n = 1,441); AL, A. lenticulata (n = 1,156); AP, A. polyphaga (n = 737); DD, Dictyostelium discoideum (n = 624); VV, Vermamoeba vermiformis (n = 528).
Yersinia pestis in Potential Ameba Reservoir

D. discoideum, whereas Y. pestis is only transiently resistant to 4 species of free-living and cyst-forming amebae (A. castellanii, A. lenticulata, A. polyphaga, and V. vermiciformis). Relative to E. coli controls, Y. pestis demonstrated significantly increased survival and replication within amebae despite the 4 cyst-forming amebae successfully killing the bacteria by using unidentified mechanisms.

Amebae cultured from soil in prairie dog colonies with active plague epizootics confirm that ameba species used in our experiments co-occur spatially and temporally with Y. pestis under natural conditions. Interactions between amebae and Y. pestis could select for increasingly ameba-resistant phenotypes, considering the transient resistance already observed in 4 cyst-forming ameba species. Other research has demonstrated the potential for amebae to affect pathogen transmission mode, alter virulence, and act as training grounds for intracellular pathogens by selecting for traits enabling macrophage invasion or avoidance (17,38).

Genus-level differences in ameba infection intensity and infection prevalence confirm that various ameba species have greater reservoir potential than others. In accordance with super-spreader theories, a minority of individual ameba harboring atypically high numbers of intracellular bacteria may be disproportionately causative for pathogen maintenance and re-emergence.

We observed a shorter duration of survival for Y. pestis in A. castellanii compared with prior experiments (24 h vs. 5 d in 13), likely from differing co-culture conditions and ameba strains. Incubation temperatures differed between this and prior experiments (28°C vs. 4°C and 25°C in 13). Many Y. pestis virulence factors are temperature regulated and may differentially facilitate cellular invasion, inhibition of the phagosomal pathway, and intracellular persistence (1,17,38). Additionally, A. castellanii (ATCC 30234)
used in this study was originally derived from yeast cultures in London in 1930, whereas *A. castellanii* (ATCC 300010), used by Benavides-Montaño et al. (13), was originally isolated from California soil in 1957 and enabled longer intracellular survival of *Y. pestis*. Intracellular survival may be affected by traits acquired by co-evolution between ameba- and resistant bacteria in soil environments (17).

In macrophages, *Y. pestis* recruits host Rab1b protein to the phagosome, resulting in inhibition of phagosome acidification and disruption of the remaining phagolysosomal metabolic pathway (26,27,39–41). *Y. pestis* then establishes a replicative niche within the YCV, characterized by a tight-fitting vacuole that expands commensurately with bacterial replication (27). Examination of TEM micrographs shows that intracellular bacteria are localized within form-fitting vacuolar membranes, similar to the YCVs found in macrophages (Figure 4).

The successful intracellular survival of *Y. pestis* in *D. discoideum* for \(\geq 48\) h demonstrates that *Y. pestis* is an ameba-resistant bacterium. This classification supports the potential for *D. discoideum* or related ameba species to be environmental reservoirs of plague. Intracellular survival of the observed duration is consequential given that typical interactions between bacteria and phagocytic cells result in bacterial death in <40 min (27). Most phagocytosed bacteria cannot survive digestive processes characteristic of phagocytic cells including phagolysosome fusion and acidification, or the subsequent recruitment of endosomal lytic factors (26,27,41). Ongoing research assesses the maintenance of viable *Y. pestis* through the entire *D. discoideum* life cycle, including transmissible dormant spores.

Ameba-resistant pathogens often replicate in vacuoles before escaping into the cytosol or outside of the phagocytic cell entirely. In addition to viable intracellular persistence, we observed active intracellular replication of *Y. pestis* (CO-92) in *D. discoideum* (Figure 5) and possible, but unconfirmed, replication of *Y. pestis* (CO92 pgm+, pCDD1, pGFPUv, amp+) in *A. castellanii* (Figure 4A). Analysis of TEM micrographs proved inconclusive for identifying the bacterial division septum; thus, only *D. discoideum* has conclusively demonstrated intracellular replication of *Y. pestis*. *Y. pestis* CFUs recovered from within *D. discoideum* increased significantly \((p = 0.001–0.01; \text{Figure 5})\) in successive postinfection time points across both antibiotic treatment conditions (except in 1 instance where *Y. pestis* increased nonsignificantly \([p = 0.1624; \text{Figure 5})\]). The consistent absence of extracellular bacteria in all *D. discoideum* co-cultures indicates resistance to digestion and the exploitation of an intrameba replicative niche.

Intracellular replication of *Y. pestis* in macrophages occurs within YCVs, and the formation of YCVs requires metabolic pathway inhibition by recruitment of Rab1b GTPases. Orthologous mechanisms are likely the cause for observed *Y. pestis* replication and survival within amebae. We searched for macrophage Rab1b by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against full genome sequences of each study ameba species and located homologous genetic sequences (99.8% similarity) within *A. castellanii* and *D. discoideum* (GenBank accession nos. XM_004347056.1 and XM_637217.1, respectively [42,43]). Future research should attempt to establish whether these ameba sequences are functionally orthologous to those identified in macrophages and whether the presence of particular host GTPases is diagnostic of ameba permissiveness to intracellular bacteria.

Results of this study support the reservoir potential of environmental ameba but do not definitively prove that this mechanism occurs in situ. Further research is necessary to determine if the maximum duration of intraameba *Y. pestis* survival corresponds with the durations of cryptic zoonotic persistence that are characteristic of plague dynamics. Increasing evidence for dormant or viable but nonculturable forms of *Y. pestis* may provide explanations underlying hypothesized multyear survival in ameba spores or cysts (12,25,44–46). Outcomes of this research prompt questions regarding evolutionary selection imposed by ameba on environmental pathogens and applications of the ameba reservoir model for other cryptic environmental pathogens. Further research into ameba-mediated pathogenesis and persistence will offer practical insights for public health, conservation, agricultural management, and biodefense.

In conclusion, the mechanisms underlying plague re-emergence following dormancy have eluded researchers for centuries (1,11). Plague persistence within soilborne microorganisms has been hypothesized as an elusive maintenance mechanism (6,11,12,25). We demonstrated spatiotemporal co-occurrence of plague bacterium and various ameba species during an active plague epizootic. Further, we observed the persistence of viable and replicative *Y. pestis* in *D. discoideum* amebae for \(\geq 48\) hours postinfection and persistence of *Y. pestis* in 4 cyst-forming ameba species for \(\leq 24\) hours postinfection, whereas ameba-susceptible control bacteria were eliminated by amebae in \(<1\) hour. Thus, *Y. pestis* are respectively ameba-resistant and transiently ameba-resistant under the tested infection conditions. *Y. pestis* bacteria resided within ameba structures that were visually analogous to YCVs observed in infected macrophages. These results encourage research into the eco-evolutionary interactions between pathogenic bacteria, amebae, and host immune factors. The reservoir potential of amebae and their shared infection-permissiveness with phagocytic macrophages show promise in explaining the cryptic properties underlying interepizootic plague transmission and persistence.
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About the Author
Mr. Markman is a PhD candidate at Colorado State University in the Department of Biology and is a concurrent research fellow for the National Defense, Science, and Engineering Graduate program of the US Department of Defense and the Vice President for Research program of Colorado State University. He collaborates with the CDC in Fort Collins, CO while researching cryptic bacterial persistence mechanisms that facilitate epidemics.

References


Address for correspondence: David W. Markman, Colorado State University, Department of Biology, 1878 Campus Delivery, Fort Collins, CO 80523, USA; email: david.markman@colostate.edu
New Parvovirus Associated with Serum Hepatitis in Horses after Inoculation of Common Biological Product


Equine serum hepatitis (i.e., Theiler’s disease) is a serious and often life-threatening disease of unknown etiology that affects horses. A horse in Nebraska, USA, with serum hepatitis died 65 days after treatment with equine-origin tetanus antitoxin. We identified an unknown parvovirus in serum and liver of the dead horse and in the administered antitoxin. The equine parvovirus-hepatitis (EqPV-H) shares <50% protein identity with its phylogenetic relatives of the genus Copiparvovirus. Next, we experimentally infected 2 horses using a tetanus antitoxin contaminated with EqPV-H. Viremia developed, the horses seroconverted, and acute hepatitis developed that was confirmed by clinical, biochemical, and histopathologic testing. We also determined that EqPV-H is an endemic infection because, in a cohort of 100 clinically normal adult horses, 13 were viremic and 15 were seropositive. We identified a new virus associated with equine serum hepatitis and confirmed its pathogenicity and transmissibility through contaminated biological products.

Equine serum hepatitis (i.e., Theiler’s disease or idiopathic acute hepatitis) is a serious and often life-threatening disease of horses that was first described in 1919 in South Africa by Sir Arnold Theiler. Theiler observed hundreds of cases of a highly fatal form of hepatitis after experimental vaccination studies to prevent African horse sickness during which infectious virus was administered simultaneously with convalescent equine antiserum (1). The incidence of fulminant hepatitis among horses receiving antiserum in outbreaks of Theiler’s disease has been reported to be 1.4%–2.2% (1,2). Theiler’s disease has been described in horses in many areas of the world after treatment with a variety of equine serum products, including tetanus antitoxin (3–8), botulinum antitoxin (9), antiserum against Streptococcus equi (4,10), pregnant mare’s serum (4), and equine plasma (1,2,5,11). The clinical disease has a high rate of death, but some horses survive, and survivors have not been reported to have evidence of persistent liver disease (5,8).

Recently, 3 new flaviviruses were identified in horses (9,12,13). The first was the nonprimate hepacivirus (NPHV), later called equine hepacivirus (2,12), which is most closely related to hepatitis C virus (14). Natural NPHV infection in horses is reported to cause temporary elevation in liver enzymes, and negative-strand viral RNA was detected within hepatocytes (15–17). The 2 other flaviviruses of horses are Theiler’s disease–associated virus (TDAV) and equine pegivirus (EPgV), both members of the Pegivirus genus (14). TDAV was identified during an outbreak of acute clinical hepatitis in horses, 6 weeks after prophylactic administration of botulinum antitoxin of equine origin (9). EPgV is reported to be a common infection of horse populations of the United States and Western Europe, is not hepatotropic, and has not been associated with hepatic disease (13,16,18).

We identified a new parvovirus in the serum and liver of a horse that died in Nebraska, USA; the virus was also present in the tetanus antitoxin administered to the horse 65 days before disease onset. We acquired the complete viral genome from that horse and, considering its phylogenetic
analysis, tentatively named the virus equine parvovirus hepatitis (EqPV-H). We describe the discovery of EqPV-H, its complete genome, infection prevalence, and virus transmission by inoculation of a commercial equine serum product resulting in hepatitis, thereby confirming EqPV-H association with equine hepatitis.

Materials and Methods

Sample Collection from the Index Horse

On November 6, 2013, a horse living in Nebraska, USA, was treated prophylactically with tetanus antitoxin (manufactured in Colorado, USA) 65 days before the onset of clinical signs of liver failure. After the horse died of Theiler’s disease in January 2014, serum and liver samples were collected and frozen or kept on ice before being shipped from the clinic of origin to Cornell University (Ithaca, NY, USA) for viral diagnostic testing. The remaining antitoxin in the vial that had been administered to the horse was shipped on ice for virologic testing. Aliquots of lots of commercial tetanus antitoxin to be used in the experimental inoculation were tested by PCR for EqPV-H, TDAV, NPHV, and EPgV as described previously (9,12,13).

Unbiased Amplification and High-Throughput Sequencing

We prepared liver suspension using ≥100 mg of liver tissue in 1 mL of phosphate-buffered saline and 3-mm steel beads using tissue lyser (QIAGEN, Hilden, Germany). We centrifuged liver suspension and the antitoxin at 5,000 rpm for 10 min to remove the cell debris and filtered clarified supernatant through a 0.45-µ filter (Millipore, Burlington, MA, USA) and treated with nucleases to digest free nucleic acids for enrichment of viral nucleic acid. We performed sequencing library preparation, sequencing, and bioinformatics as described previously (19).

Complete Genome Sequencing and Genetic Analysis of EqPV-H

We acquired the complete genome of EqPV-H using a primer walking approach as previously described by us for several new animal parvoviruses, including bocaviruses (20–22). The genome of EqPV-H episome is available in GenBank (accession no. MG136722). To determine the sequence relationship between EqPV-H and other known parvovirus species, we used ≥1 representative virus member, including the reference genome from each species and its translated protein sequences, to generate sequence alignments. We generated a phylogenetic tree showing sequences used for the comparison and their GenBank accession numbers (Figure 1, panel A). The evolutionary history was inferred by using the maximum-likelihood method based on the Le_Gascuel_2008 model (23). The tree with the highest log likelihood is shown. We conducted evolutionary analyses in MEGA (24). DNA secondary structures of genomic termini were predicted by Mfold (25).

PCR and Serologic Assays for EqPV-H

We aligned the nonstructural (NS) protein and virion protein (VP) of EqPV-H to all known parvovirus proteins. We used nucleotide and amino acid motifs showing relative conservation among different virus lineages to make primers for PCR amplification. We performed screening tests for EqPV-H and related variants. All PCR mixtures were amplified using MasterMix Gold 360 master mix (catalog no. A3898881; Applied Biosystems, Foster City, CA, USA) and 2 µL of extracted nucleic acids. The EqPV-H NS gene PCR used primer pair EqPV ak1 (5′-GGAGAAGACGGCAACAAATGCA-3′) and EqPV ak2 (5′-AGACATTTTCGGCGGTGAC-3′) in the first round of PCR and the pair EqPV ak3 (5′-GGCGAACAATGCGAGGCTTCGAC-3′) and EqPV ak4 (5′-GGCGGTACGACGCTGTATAC-3′) in the second round of PCR. The EqPV-H VP gene PCR used primer pair EqPV ak5 (5′-GGCCTCTCATTCTGATCC-3′) and EqPV ak6 (5′-GGGATTATCTGTCATTTGTTGGGAC-3′) in the first round of PCR and the pair EqPV ak7 (5′-GGCCTCTCATTCTGAGTTGCTGGCC-3′) and EqPV ak8 (5′-GGCCTCTCATTCTGAGTTGCTGGCC-3′) in the second round of PCR.

Luciferase Immunoprecipitation System Assay for EqPV Serology

We cloned the C terminus of the EqPV-H capsid protein into pREN2 plasmid for making Renilla luciferase fused antigen for a Luciferase Immunoprecipitation System (LIPS) assay. In brief, we amplified the VP1 gene of EqPV-H using primers EqPV LIPSF1 (5′-AGTAAAGTCAATGACCAACCA-3′) and EqPV LIPS1 (5′-GGATCTGAGTTGAGAACAACCA-3′) and EqPV LIPS1 (5′-GGATCTGAGTTGAGAACAACCA-3′) and EqPV-LIPS XhoI (5′-GAGCTCGAGTCAGAACTGACGAACAAATGCA-3′) and EqPV LIPSR1 (5′-GGATCTGAGTTGAGAACAACCA-3′). We sequenced PCR product and then used it as a template to make inserts for LIPS assay using primers with flanking restriction sites, EqPV-LIPS BamHI (5′-GGGATCCCATGCTTTAACCCTATGAC-3′) and EqPV-H LIPS XhoI (5′-GAGCTCGAGTCAGAAACTGACGAACAAATGCA-3′). Inserts were subsequently sequenced, digested, and ligated into pREN-2 expression vector. Details of LIPS antigen preparation and serologic testing have been described previously (19).

Experimental Infection of Horses

We selected 2 healthy 18- and 20-year-old mares from the Cornell University College of Veterinary Medicine teaching and research herd that were negative by PCR for EqPV-H, TDAV, and NPHV. We inoculated the horses 4 months apart with 2 lots of tetanus antitoxin and 2 lots of tetanus antitoxin in the vial that had been administered to the horse was treated prophylactically with tetanus antitoxin (manufactured in Colorado, USA) 65 days before the onset of clinical signs of liver failure. After the horse died of Theiler’s disease in January 2014, serum and liver samples were collected and frozen or kept on ice before being shipped from the clinic of origin to Cornell University (Ithaca, NY, USA) for viral diagnostic testing. The remaining antitoxin in the vial that had been administered to the horse was shipped on ice for virologic testing. Aliquots of lots of commercial tetanus antitoxin to be used in the experimental inoculation were tested by PCR for EqPV-H, TDAV, NPHV, and EPgV as described previously (9,12,13).
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We pooled both lots for inoculation so that each horse received the identical inoculum. In each experimental horse, we administered 5.0 mL of the pooled tetanus antitoxin intravenously and 5.0 mL subcutaneously in the neck. We collected blood samples for biochemical analysis in the experimental inoculation study from the jugular vein into 7-mL sodium heparin tubes (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) and promptly submitted them to the New York State Animal Health Diagnostic Center at Cornell University for biochemical tests indicative of hepatic disease: aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), γ-glutamyltransferase (GGT), total bile acids, and bilirubin. Tests were performed using a Hitachi Mod P 800 (Roche Diagnostics, Indianapolis, IN, USA).

We performed liver biopsies after sedation with 5 mg detomidine administered intravenously; local anesthetic (2% lidocaine) was injected subcutaneously at the planned insertion site of the percutaneous biopsy instrument (Tru-Cut Biopsy Needle; Travenol Laboratories, Deerfield, IL, USA). We determined the location of the biopsy needle insertion by ultrasonographic visualization of the liver. We placed samples in formalin for histopathologic studies and submitted fresh liver tissue to the same laboratory for quantitative PCR. The case–control and experimental inoculation studies were approved in full by the Cornell University Animal Research Committee (IACUC no. 2014-0024).

Results

Identification of EqPV-H in Horse with Theiler's Disease

Serum and liver samples from the deceased horse obtained postmortem and from the administered tetanus antitoxin...
tested negative for the 3 recently identified horse flaviviruses, NPHV, TDAV, and EPgV (29). We used an unbiased amplification and high-throughput sequencing approach to identify known and new viruses in the antitoxin and liver sample (19,23,30). Bioinformatics of sequence data revealed a 4.5-kb base assembled sequence in the horse liver sample that showed distant yet significant protein similarity with known bovine and porcine parvoviruses. Thereafter, the in silico assembled sequence data were used to design primers for amplifying the complete genome of the new parvovirus, tentatively named EqPV-H. Considering the presence of high virus titer in the liver sample and the precedent of finding virus epimemes in tissue for related parvoviruses (20), we used an inverse PCR–based approach to acquire the complete virus genome. The inverse PCR confirmed that EqPV-H exists as epimemes in the liver tissue.

The complete epimeme of EqPV-H comprises 5,308 nt and is predicted to code 2 large open reading frames whose proteins are related to the NS proteins and the structural proteins (VPs) of known animal parvoviruses (Figures 1, 2). If the first nucleotide of NS protein is considered to be genome position 1, the NS protein is coded by nucleotide positions 1–1,779, followed by an intergenic region of 21 nt, and the VPs are coded by nucleotide positions 1,801–4,722. An intergenic region of 583 nt connects the end of VP coding region to the NS coding region in the episomal genome form of EqPV-H. The genomic termini of parvoviruses play an important role in virus replication and translation. Similar to known animal parvoviruses, the DNA folding programs predicted 1 long hairpin and other small hairpins in the intergenic region of EqPV-H (Figure 2). Attempts to propagate the virus in cell culture using the serum and liver samples of infected horses have been unsuccessful.

EqPV-H genome organization and genetic relatedness to known viruses suggest its classification as a prototype of new species in the genus Copiparvovirus (Figure 1, panel A). Other members of the genus Copiparvovirus include parvoviruses that infect pigs, cows, and sea lions and a recently identified virus found in horse cerebrospinal fluid (CSF), horse parvovirus–CSF (31). Since the NS proteins of parvoviruses are relatively more conserved than the VP, we used the NS protein of EqPV-H for its phylogenetic analysis. Results indicate that EqPV-H is distinct, yet most closely related to different species of copiparvoviruses (Figure 1, panel A). The EqPV-H is more closely related to pig and cow copiparvovirus than to the only other horse copiparvovirus, indicating different evolutionary origins of these 2 horse viruses. This NS gene–based phylogenetic analysis was further confirmed using VP gene–based phylogenetic analysis (data not shown).

Infection Prevalence, Disease Association, and Molecular Epidemiology

To determine the infection prevalence of EqPV-H, we tested 100 horse serum samples of convenience submitted to the New York State Animal Health Diagnostic Center at Cornell University for nonclinical reasons. PCRs targeting the NS and VP region of EqPV-H identified 13 of 100 horses positive for EqPV-H viremia. We also tested these samples for EqPV-H IgG using partial VP1 as antigen in the LIPS assay. We determined that all 13 viremic horses had IgG. In addition, 2 nonviremic horses were seropositive, indicating clearance of EqPV-H viremia. We then tested the 13 virus-positive samples biochemically for evidence of liver disease using GGT as a marker; all results were within normal range. Genetic analysis of EqPV-H variants found in all the studied serum samples indicated a very low level of genetic diversity (<2% nt differences in NS and VP sequences) among isolates.

Experimental Inoculation of Commercial Tetanus Antitoxin

To confirm transmission of EqPV-H by tetanus antitoxin, we inoculated 2 clinically normal mares with 10 mL of tetanus antitoxin positive for EqPV-H. The 2 horses were confirmed PCR negative for EqPV-H, TDAV, and NPHV nucleic acids and LIPS negative for EqPV-H antibody. At weekly intervals after inoculation, we tested both
experimentally inoculated horses for NPHV, TDAV, and EqPV-H nucleic acids by quantitative PCR, for EqPV-H antibodies by LIPS, and for biochemical evidence of liver disease (Figure 3). Both horses remained PCR negative for EqPV-H nucleic acids on weekly sampling until 47 and 48 days postinoculation (dpi), at which time both horses became PCR positive for EqPV-H. Consecutive weekly samples demonstrated increasing viremia in both horses; viremia peaked at 81 and 96 dpi. Thereafter, viremia gradually decreased, but EqPV-H was still detected in the serum of both horses at study termination (123 and 125 dpi). Antibodies against EqPV-H capsid protein were first detected at 88 and 89 dpi in the 2 infected horses. Both horses remained PCR negative for NPHV and TDAV at study termination.

In horse 1, serum biochemical evidence of liver disease was first observed at 82 dpi, when all test parameters except bile acids and total bilirubin showed marked increases (Figure 3). Samples were then tested daily or every other day; bile acids and bilirubin were increased at 84 dpi (Figure 3). At 87 dpi, horse 1 became icteric and depressed and developed orange urine (bilirubinuria). Abnormalities in biochemical parameters increased further until 90 dpi, after which values started to return to normal. Values were normal by 98 dpi except for GGT, which returned to normal range at 114 dpi. Clinical icterus and discolored urine were not observed after 94 dpi. A liver biopsy sample obtained at 82 dpi showed lymphocytic lobular atrophy (Figure 4, panel A) and necrotic hepatocytes (Figure 4, panel B).

Horse 2 first showed abnormal serum biochemistry values at 96 dpi; AST, SDH, GGT, bile acids, and total bilirubin increased gradually until 105 dpi, when all biochemical values began decreasing and were normal by 118 dpi, except for GGT, which remained mildly elevated at the conclusion of the study (123 dpi). A liver biopsy sample obtained at 100 dpi had lymphocytes surrounding clusters of necrotic hepatocytes and lymphocytic satellitosis (Figure 4, panel C). Portal tracts were also infiltrated with small numbers of lymphocytes that breached the limiting plate (Figure 4, panel D).

**Discussion**

The previously unidentified horse parvovirus EqPV-H we describe represents the prototype of a new virus species of genus *Copiparvovirus*. Although a new parvovirus in the genus *Copiparvovirus* was recently found in a CSF sample of a horse with neurologic signs, EqPV-H is ge-

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**Figure 3.** Kinetics of viremia and liver enzymes and time of seroconversion in 2 horses experimentally inoculated with equine parvovirus hepatitis. Gray line indicates horse 1; black line indicates horse 2. A) AST; B) SDH; C) GGT; D) total bilirubin; E) bile acids; F) DNA copies. Asterisk indicates time of seroconversion. AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; SDH, sorbitol dehydrogenase.
netically very distinct from that virus (31). EqPV-H is genetically more similar to other ungulate copiparvoviruses than the horse parvovirus–CSF. Paroviruses are ubiquitous and are proposed to have a wide range of effects on their hosts, ranging from severe disease to nonpathogenic infections (32). The pathogenesis of paroviruses is generally associated with their predilection for actively dividing cells and different paroviruses have different organ tropism (32). Parovirus hepatitis in other species is rare, although parovirus B19 can be associated with acute hepatitis in humans after transfusion of contaminated blood products (33,34).

Although the overall incidence of clinically recognized serum hepatitis in adult horses receiving tetanus antitoxin is low, tetanus antitoxin has been the most commonly reported blood product associated with the disease in the United States for the past 50 years (3,4,6–8). Commercial tetanus antitoxin is heat treated (60°C for 1 h) to inactivate virus, and phenol and thimerosal are added as preservatives. Such treatments could leave detectable viral nucleic acids that would be nontransmissible. However, the 2 successful transmissions of EqPV-H to experimentally inoculated horses confirmed that EqPV-H can be transmitted from heat-treated commercially available tetanus antitoxin. Although this form of heat treatment is known to inactivate heat-labile viruses, such as lentiviruses (35), in blood products, paroviruses and especially animal paroviruses are very resistant to heat inactivation and solvent detergent treatments (35–38).

The incubation period for onset of biochemical disease after experimental inoculation of PCR-positive tetanus antitoxin to the 2 horses was longer (82 and 94 days) than most reported clinical cases of equine serum hepatitis. Clinical cases typically occur 6–10 weeks after blood product inoculation, but cases have been reported as long as 14 weeks after blood product administration (5,7). Complete recovery of both horses in the experimental study was not surprising because rapid (3–7 days) recovery occurs in some horses with Theiler’s disease (5,8). Microscopic findings in clinically affected horses with serum hepatitis consistently include widespread centrolobular to midzonal hepatocellular necrosis with hemorrhage; portal areas have mild inflammatory infiltrate, primarily monocytes and lymphocytes, and moderate bile duct proliferation (39,40). Liver enzymes, including SDH and
AST, are increased several fold, and GGT is increased but often not to the same magnitude as the hepatocellular enzymes (7,8). We therefore believe that the incubation period after tetanus antitoxin administration, combined with serum chemistry and histopathologic finding in the 2 experimentally infected horses, is compatible with prior reports on equine serum hepatitis (3–8,11).

The virus and serologic survey findings of EqPV-H viremia in 13% of horses without biochemical evidence of liver disease suggests that most horses that become infected with EqPV-H do not develop clinical disease. This finding would be compatible with epidemiologic data on Theiler’s disease outbreaks in which clinical hepatitis develops in only 1.4%–2.2% of horses receiving equine blood products (1,2). One limitation of our study was that we could not determine the exact chronicity of infection in the horses in the serologic study and if these horses had biochemical evidence of liver disease at some prior point during infection. Although our limited study indicates low genetic diversity among EqPV-H isolated from different horses, follow-up studies that include horses living in different geographic areas are necessary to define the true prevalence and genetic diversity of EqPV-H. Why some horses develop severe and often fatal disease after EqPV-H infection and others do not also remains unknown and requires further investigation.

Several findings in this study suggest that EqPV-H infections in horses can often be persistent. In the serologic/virus prevalence study, only 2 horses with EqPV-H antibody were virus negative, and the 13 viremic horses all had antibody. In addition, both experimentally infected horses were virus positive after 123 days, despite having high antibody levels. We have no data on the ability of antibody specific for EqPV-H to neutralize the virus or on the role the antibody might play in maintaining infection. Finally, retrospective testing of administered antitoxin and serum samples of recipient horses indicated presence of EqPV-H in all samples that tested positive for TDAV infection in our previous study (9). We believe that because the previous study used RNA-only viral metagenomics, the EqPV-H (a DNA virus) remained elusive.

In summary, information from this study suggests that EqPV-H can cause serum hepatitis (Theiler’s disease) in horses. EqPV-H in horse serum or plasma products should be of concern.

Acknowledgments
We acknowledge Nicholas A. Hollinshead and Nancy Zylich for Figure 4 preparation.

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About the Author
Dr. Divers is the endowed Steffen Professor of Veterinary Medicine at Cornell University, Ithaca. His primary research interests are equine infectious diseases and disorders of the equine liver.

References


Address for correspondence: Amit Kapoor, Ohio State University, Department of Pediatrics, Center for Vaccines and Immunity, Research Institute at Nationwide Children’s Hospital, 700 Children’s Dr, Columbus, OH 43205, USA; email: Kapoor.102@osu.edu
Development of a Pediatric Ebola Predictive Score, Sierra Leone

Felicity Fitzgerald, Kevin Wing, Asad Naveed, Musa Gbessay, J.C.G. Ross, Francesco Checchi, Daniel Youkee, Mohamed Boie Jalloh, David E. Baion, Ayeshatu Mustapha, Hawanatu Jah, Sandra Lako, Shefali Oza, Sabah Boufkhed, Reynold Feury, Julia Bielicki, Elizabeth Williamson, Diana M. Gibb, Nigel Klein, Foday Sahr, Shunmay Yeung

We compared children who were positive for Ebola virus disease (EVD) with those who were negative to derive a pediatric EVD predictive (PEP) score. We collected data on all children <13 years of age admitted to 11 Ebola holding units in Sierra Leone during August 2014–March 2015 and performed multivariable logistic regression. Among 1,054 children, 309 (29%) were EVD positive and 697 (66%) EVD negative, with 48 (5%) missing. Contact history, conjunctivitis, and age were the strongest predictive predictors for EVD. The PEP score had an area under receiver operating characteristics curve of 0.80. A PEP score of 7/10 was 92% specific and 44% sensitive; 3/10 was 30% specific, 94% sensitive. The PEP score could correctly classify 79%–90% of children and could be used to facilitate triage into risk categories, depending on the sensitivity or specificity required.

The Ebola virus disease (EVD) outbreak in West Africa claimed >11,000 lives with nearly 30,000 cases.1 During the outbreak in Sierra Leone, patients arriving at healthcare facilities were screened for EVD using World Health Organization (WHO) case definitions. Those fulfilling the case definition for suspected EVD were admitted to Ebola holding units (EHUs) to have blood taken for EVD testing and receive medical care until test results were available (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/2/17-1018-Techapp1.pdf). Testing was usually performed offsite, with a turnaround time for results of ≈48 hours.2 During admission, however, EVD-negative patients risked exposure to EVD, raising concerns that EHUs could act as amplification sites for infection.3–7 Children, many of whom were unaccompanied, were particularly vulnerable, and, because EHUs were overstretched, supervision to minimize the risk of cross-infection was challenging.4–8

An accurate case definition for suspected EVD is critical for future outbreaks. Insufficient sensitivity of case definitions results in EVD-positive patients not being isolated, risking onward transmission in the community. There is an inherent tension between the public health priority to maximize the sensitivity of the case definition (minimizing onward transmission risk) and the individual patient’s perspective. The trade-off made by lower specificity means that many EVD-negative patients are kept waiting in EHUs for test results, risking nosocomial infection and delaying treatment for their true underlying condition. Case definitions should be flexible because priorities may change as outbreaks progress. In the 2014–2015 epidemic, the proportion of patients testing positive decreased over time: in October 2014, 77% of those admitted to a Freetown EHU tested positive, versus 1% in April 2015.9

In Sierra Leone, 2 case definitions were used for suspected EVD (9). Until November 2014, most EHUs used a WHO case definition that was the same for both adults and children, defining anyone who had ≥3 symptoms consistent with EVD and fever, or who had fever and had contact with a person with EVD, as having a suspected case (early-2014 case definition). Beginning in December 2014, the WHO

1Preliminary results from this study were presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases, Amsterdam, Netherlands, April 9–12, 2016; and the 34th Annual Meeting of the European Society for Pediatric Infectious Diseases, Brighton, UK, May 10–14, 2016.

2These authors contributed equally to this article.
case definition was modified to be age dependent (late-2014 case definition) (Figure 1; online Technical Appendix Table 1). Under this definition, children only required fever and either 1 symptom (in children <5 years of age), 2 symptoms (in children 5–12 years of age), or ≥3 symptoms (in children >12 years of age) (4). This definition increased the likelihood of admitting EVD-negative children. Furthermore, in overstretched EHUs, children may have been admitted without meeting the criteria for suspected EVD, regardless of definition. In a mixed-age West African cohort, 9% of those admitted did not fulfill the early-2014 case definition (3).

We aimed to develop a predictive score that could be used to tailor the pediatric case definition for suspected EVD according to the clinical and epidemiologic setting. The goal was to potentially limit unnecessary admissions to EHUs for EVD-negative children without reducing sensitivity.

Methods

Data Sources

We collected data on all children <13 years of age admitted to 11 EHUs in Sierra Leone (August 2014–March 2015) and built training and validation datasets. We performed multivariable logistic regression on the training dataset to generate a pediatric Ebola predictive (PEP) score, which we tested on the validation dataset. The age cutoff matched the WHO case definition distinguishing between children and adolescents, anticipating that adolescents would have an adult disease phenotype. Settings and data collection methods have been described previously (4,10). We visited each EHU to extract data from paper clinical records, case investigation forms, and site admission books and to interview staff. We cross-referenced data with the Western Area Ebola Response Centre (WAERC) database and 4 further sources, and single-entered data into a password-protected database (Epi Info version 7.1.4; US Centers for Disease Control and Prevention, Atlanta, GA, USA) (online Technical Appendix). We removed personal identifiers before analysis and developed a schema for record matching across databases (online Technical Appendix). We obtained ethics approval for this study from the Sierra Leone Ethics and Scientific Review Committee and the London School of Hygiene and Tropical Medicine Ethics committee (reference 8924).

Statistical Analysis

We used Stata version 14.0 (StataCorp LLC, College Station, TX, USA) to perform analyses and limited analysis to children with EVD laboratory test result data. Variables were sex, age, contact history (yes/no), presence of 16 symptoms at EHU admission (yes/no), and days from symptom onset to EHU visit (4). We included age as a binary variable (<2 years and ≥2 years), given the higher burden of febrile illnesses that appear similar to EVD (e.g., malaria) in younger children. We considered data to be missing from the analysis if no value had been entered in the source documents (i.e., neither yes nor no).

Descriptive analysis of the cohort comprised the number of children with data available for each variable and the prevalence of signs and symptoms by laboratory-confirmed EVD status. We estimated the proportion of children (for whom we had sufficient data) who met the late-2014 WHO case definition.

Predictive Model Building and Validation and Development of Risk Score

We split the data randomly into 2 datasets with equivalent proportions of laboratory-confirmed EVD-positive children: a training dataset for predictive score building, and single-entered data into a password-protected database (Epi Info version 7.1.4; US Centers for Disease Control and Prevention, Atlanta, GA, USA) (online Technical Appendix). We removed personal identifiers before analysis and developed a schema for record matching across databases (online Technical Appendix). We obtained ethics approval for this study from the Sierra Leone Ethics and Scientific Review Committee and the London School of Hygiene and Tropical Medicine Ethics committee (reference 8924).

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Predictive Model Building and Validation and Development of Risk Score

We split the data randomly into 2 datasets with equivalent proportions of laboratory-confirmed EVD-positive children: a training dataset for predictive score building,
and a validation dataset to assess score performance (11). Using the training dataset, we calculated crude odds ratios (ORs) of association between potential predictive variables and outcome (laboratory-confirmed EVD status) and created an initial multivariable model including all potential predictive variables. A final training model was obtained by removing variables with p>0.3 from the fully adjusted model in a backward-stepwise fashion. The variables retained for constructing candidate PEP scores were age, gender, contact history, days from first symptoms to admission, and whether all symptoms were systematically documented (online Technical Appendix).

We created the PEP score by assigning integer scores to variables in the validation dataset on the basis of their regression coefficients in the training dataset model (score = 1 for coefficients <1, score = 2 for coefficients ≥1) (12). We calculated each child’s overall PEP score by adding together the integer scores for the variables present, which resulted in possible PEP scores of 0–10. To identify the most clinically useful PEP score, we computed the sensitivity, specificity, positive predictive value, negative predictive value, and percentage of children correctly classified (compared with the standard of laboratory confirmation of EVD) of each candidate PEP score. Fully calculating the validity of the WHO case definition would require data on false negatives (those turned away at screening who had EVD), but these data were not available. We compared the PEP score with the WHO case definition as accurately as the available data permitted for completeness (online Technical Appendix).

To explore the potential effects of PEP scores on the number of correct and incorrect admissions at different times in the epidemic, we applied 2 PEP scores with different levels of sensitivity and specificity to 2 hypothetical populations of children: early in the epidemic when the proportion of suspected cases testing positive in Western EHUs was 77% (high background prevalence, October 2014); and later in the epidemic when the proportion was 4% (low background prevalence, March 2015). We used these hypothetical background prevalences with the sensitivity and specificity for each score to calculate number of true positives and negatives and false positives and negatives obtained by applying each score (online Technical Appendix Tables 2–5) (5). We used multiple imputation by chained equations to account for missing data in the analysis of training and validation datasets (online Technical Appendix) (13).

Results
Of 1,054 children admitted with suspected cases to 11 EHUs during August 14, 2014–March 31, 2015, no result was available for 48 (5%) (online Technical Appendix). Of the remaining 1,006 children, 309 (31%) were EVD positive and 697 (69%) EVD negative. Admissions rose from a median 8 (interquartile range [IQR] 5–11) per week in August–October 2014 to 50 (40–58) per week in February–March 2015, but the proportion of children that were EVD positive decreased from 57% (95% CI 43%–72%) in October 2014 to 6% (95% CI 2%–9%) in February 2015. At Ola During Children’s Hospital (ODCH), the main children’s hospital in Freetown, the onsite EHU received 59% of all EHU admissions, increasing from 12% in August–October 2014 to 82% in February–March 2015.

We documented admission of 211 (21%) unaccompanied children. Data were missing for 297 (30%) of the children. EVD-positive children were more likely to be unaccompanied than those who were EVD negative (p<0.001).

Median patient age was 4 years (IQR 1.3–8.0 years), and 51% of the children were female (Table 1). Contact with EVD was reported for 275 (36%) of 754 children who had data available (75% of 1,006 total). Median time from symptom onset to hospital visit was 2 days (IQR 1–4). Fever data were available for 787 (78%) of children (Table 1), 775 of whom also had data available on the presence of ≥3 other symptoms. For those with data, fatigue/weakness was most frequently reported (97%), followed by fever (94%), anorexia (80%), vomiting (61%), headache (62%), and diarrhea (46%) (Table 1). Bleeding was rare, reported by 3% of the 809/1,006 (80%) of children who had sufficient symptom and contact history data recorded to ascertain if they fulfilled the late-2014 WHO suspected case definition, 31 (4%) were admitted despite not meeting the definition (online Technical Appendix.).

Children who were EVD negative were younger (median age 3 years [IQR 1–7 years] vs. 6 years [IQR 3–10 years]; p<0.001) (Table 1) and less likely to have conjunctivitis (p<0.001) than those who were EVD positive. Rash was more common in EVD-negative children (p<0.001) (Table 1; Figure 2). Similar proportions of both groups received antimicrobial and antimalarial drugs, and whereas both spent a median of 2 days in an EHU (admission to death or transfer/discharge), those with EVD tended to stay longer (p<0.001) (Table 1).

Randomly splitting the cohort of 1,006 children generated training and validation datasets of 504 and 502 (descriptive, crude, and adjusted analysis in online Technical Appendix Table 6). In the training cohort, positive contact (multivariable OR 9.1, 95% CI 4.9–17); age ≥2 years (multivariable OR 2.9, 95% CI 1.4–5.8); and conjunctivitis (multivariable OR 3.8, 95% CI 1.9–7.8) were the strongest positive predictors of EVD. Headache, difficulty breathing, difficulty swallowing, and rash were negative predictors. The final multivariable predictive model included 12 variables: gender; age; positive contact; and presence or absence at hospital visit of fever, diarrhea, conjunctivitis, anorexia, abdominal pain, headache, difficulty breathing,
difficulty swallowing, and rash. We present only analysis of the complete records, based on the similarity of receiver operating characteristics (ROC) curves for imputed and complete records analyses (online Technical Appendix Table 7, Figure 3).

Assigning predictive model values derived from the training dataset to the validation dataset gave a range of PEP scores of 0–10. Plotting the ROC curve as sensitivity (x) against 1 – specificity (y) for all individual child PEP scores (with sensitivity and specificity calculated using the laboratory test as standard) demonstrated that the model had excellent discriminative ability (area under ROC curve = 0.80; Figure 3) (14). The model coefficients, \( p \) values, and assigned integer PEP scores are shown in Table 2 and the sensitivity, specificity, positive and negative predictive values, and percentage correctly classified for all possible PEP scores within the validation dataset in Table 3. A PEP score of 1 was 97% sensitive (95% CI 89%–100%) and 4% specific (95% CI 1%–8%), whereas the maximum PEP of 10 was 5% sensitive (95% CI 1%–13%) and 99% specific (95% CI 96%–100%) (Table 3).

We considered the effect of using different PEP scores at different times during the outbreak. PEP score 3 (sensitivity 44% and specificity 99%) at the high background prevalence time point would have correctly classified 79 patients, with 16 EVD-negative patients admitted unnecessarily and 5 EVD-positive patients being incorrectly not admitted (Table 4; online Technical Appendix Tables 2, 3). Using a PEP score of 7 (sensitivity 44% and specificity 92%) at the low background prevalence time point would have correctly classified 90/100 patients, with 8 unnecessary admissions and 2 true EVD-positive patients

Table 1. Overview of 1,006 children who attended an Ebola holding unit and had EVD test results recorded, by final EVD test result status, Sierra Leone, August 14, 2014–March 31, 2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All children, no. (%)</th>
<th>EVD negative</th>
<th>EVD positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total†</td>
<td>No./no. available</td>
<td>No./no. available</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>512 (51)</td>
<td>348/697</td>
<td>50 (46–54)</td>
</tr>
<tr>
<td>M</td>
<td>494 (49)</td>
<td>349/697</td>
<td>50 (46–54)</td>
</tr>
<tr>
<td><strong>Median age, y (IQR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (3–8)</td>
<td>3 (1–7)</td>
<td>–</td>
<td>6 (3–10)</td>
</tr>
<tr>
<td><strong>Age 0–2 y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>392 (39)</td>
<td>336/697</td>
<td>48 (44–52)</td>
<td>56/309</td>
</tr>
<tr>
<td><strong>Positive contact, n = 754‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>275 (36)</td>
<td>108/541</td>
<td>20 (17–24)</td>
<td>167/213</td>
</tr>
<tr>
<td><strong>Days from symptoms to EHU admission, n = 772</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (1–4)</td>
<td>2 (1–3)</td>
<td>–</td>
<td>3 (2–4)</td>
</tr>
<tr>
<td><strong>Admitted with caregiver, n = 822</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>822 (82)</td>
<td>516/621</td>
<td>83 (80–86)</td>
<td>127/201</td>
</tr>
<tr>
<td><strong>Signs/symptoms§</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever, n = 787</td>
<td>740 (94)</td>
<td>528/566</td>
<td>93 (91–95)</td>
</tr>
<tr>
<td>Fatigue/weakness, n = 587</td>
<td>568 (97)</td>
<td>393/407</td>
<td>97 (94–98)</td>
</tr>
<tr>
<td>Vomiting/nausea, n = 777</td>
<td>472 (61)</td>
<td>345/556</td>
<td>62 (58–66)</td>
</tr>
<tr>
<td>Diarrhea, n = 763</td>
<td>351 (46)</td>
<td>252/548</td>
<td>46 (42–50)</td>
</tr>
<tr>
<td>Conjunctivitis, n = 669</td>
<td>152 (23)</td>
<td>73/463</td>
<td>16 (13–19)</td>
</tr>
<tr>
<td>Anorexia, n = 779</td>
<td>621 (80)</td>
<td>452/560</td>
<td>81 (77–84)</td>
</tr>
<tr>
<td>Abdominal pain, n = 594</td>
<td>269 (45)</td>
<td>155/392</td>
<td>40 (35–45)</td>
</tr>
<tr>
<td>Muscle pain, n = 577</td>
<td>212 (21)</td>
<td>127/377</td>
<td>34 (29–39)</td>
</tr>
<tr>
<td>Joint pain, n = 569</td>
<td>192 (34)</td>
<td>102/368</td>
<td>28 (23–33)</td>
</tr>
<tr>
<td>Headache, n = 598</td>
<td>370 (62)</td>
<td>256/397</td>
<td>65 (60–69)</td>
</tr>
<tr>
<td>Difficulty breathing, n = 738</td>
<td>199 (27)</td>
<td>165/533</td>
<td>32 (28–36)</td>
</tr>
<tr>
<td>Difficulty swallowing, n = 687</td>
<td>177 (26)</td>
<td>130/481</td>
<td>27 (23–31)</td>
</tr>
<tr>
<td>Rash, n = 728</td>
<td>96 (13)</td>
<td>88/522</td>
<td>17 (14–20)</td>
</tr>
<tr>
<td>Cough, n = 587</td>
<td>70 (12)</td>
<td>57/407</td>
<td>14 (11–18)</td>
</tr>
<tr>
<td>Hiccups, n = 723</td>
<td>62 (9)</td>
<td>52/519</td>
<td>10 (8–13)</td>
</tr>
<tr>
<td>Unexplained bleeding, n = 726</td>
<td>22 (3)</td>
<td>19/518</td>
<td>4 (2–6)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of children admitted to an Ebola holding unit (EHU), Sierra Leone, August 14, 2014–March 31, 2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All children, median (IQR)</th>
<th>EVD negative</th>
<th>EVD positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./no. available</td>
<td>No./no. available</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td><strong>Time from EHU admission until death, discharge, or transfer.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median days of EHU stay#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria RDT+, n = 74</td>
<td>33 (45)</td>
<td>31/57</td>
<td>54 (41–68)</td>
</tr>
<tr>
<td>IV treatment</td>
<td>115 (11)</td>
<td>101/697</td>
<td>14 (12–17)</td>
</tr>
</tbody>
</table>

*Values in Table 1 are based on complete records analyses, which omitted children for whom the variable was not available or for whom the denominator was not available (i.e., children who were not present at EHU admission, were admitted before the valid observation time points, had missing data, or were admitted without an RDT result). EVD, Ebola virus disease; EHU, Ebola holding unit; RDT, rapid diagnostic test.

†Total no. children admitted to holding units with test results available.

‡At EHU.

§At time of EHU admission until death, discharge, or transfer.

#Recorded on presentation at EHU.
Pediatric Ebola Predictive Score, Sierra Leone

incorrectly not admitted (Table 4; online Technical Appendix Tables 4, 5). Because we only have the true EVD status of patients who were admitted despite screening negative by WHO case definition (not the much larger number who were WHO case definition negatives and not admitted), the sensitivity and specificity calculated may be unreliable (online Technical Appendix). However, on the basis of the data available, the WHO case definition was estimated to be 98% sensitive and 5% specific (Table 3; online Technical Appendix Tables 8, 9).

Discussion

This large, multicenter study compared symptoms at hospital visit in children <13 years old who were determined to be positive or negative for EVD during the outbreak in West Africa. As with many childhood diseases, EVD symptoms are nonspecific. The WHO indicators, including fever, breathing difficulties, and gastrointestinal symptoms, are common features in many pediatric pathologies. In this outbreak, gastrointestinal symptoms dominated, whereas bleeding, characteristic of previous outbreaks, was rare (3,15–19). This difference meant clinical diagnosis of EVD in the West African outbreak was difficult, which motivated this study. The lack of specificity of both early- and late-2014 WHO case definitions is highlighted by the fact that 69% of the children admitted as suspected EVD cases in this cohort were uninfected; that number increased to 94% in low-prevalence weeks (10).

Although elegant clinical predictive models have been developed for mixed-age cohorts, the focus of our model is children (3,17,18,20–22). The features at presentation that had the strongest association with a positive laboratory test result in this study were positive contact, conjunctivitis (similar to mixed-age cohorts [17,22]), and age ≥2 years. Fever, anorexia, abdominal pain, and diarrhea were weaker predictors of EVD. Certain features in the late-2014 WHO case definition were either not predictive or negative predictors, including bleeding, vomiting/nausea, difficulty breathing or swallowing, muscle or joint pain, headache, or rash (Table 1) (9). These findings emphasize the challenge of
diagnosing EVD against high background rates of malaria and respiratory and gastrointestinal infections in children. The early-2014 WHO case definition demonstrated similar lack of specificity (32%) in 1 retrospective mixed-age cohort (sensitivity 80%) (3), although slightly better figures were documented in 2 smaller mixed-age cohorts (20,23).

The PEP score model described here could provide the basis for modifying pediatric case definitions as an outbreak evolves, or for different pediatric populations (e.g., at triage in an EHU vs. potentially lower-risk routine outpatient consultations). Similar to the mixed-age, malaria-sensitive score proposed by Hartley et al. (17), a patient with a high score would be strongly suspected and a low score weakly suspected of having EVD. In times of high community prevalence, children with a PEP score ≥7 (>92% specificity, 44% sensitivity) could rapidly be transferred to an ETC while awaiting laboratory confirmation, whereas those with a PEP score of 3 (sensitivity 94%, specificity 30%) could await test results in the EHU. This change could hasten access to specialist care for children with EVD and reduce exposure risk for those who are negative.

Assessing the applicability of our PEP score to future Ebola virus epidemics is important. Ideally, the model should be tested against other datasets from West Africa and prospectively in future outbreaks, because different EVD strains are likely to result in different disease manifestations. Indeed, in another pediatric cohort from Kailahun and Bo, Sierra Leone, containing 91 children <5 years of age, fever was absent in 25% (compared with 4% in our study) whereas bleeding was seen in 15% (15). In a large international cohort of 1,371 children <16 years of age with EVD, fever prevalence was 90% and bleeding 10% (24). However, it is possible that future pediatric case numbers may be smaller than those seen in this outbreak, which limits opportunities for prospective validation. We suggest governmental and nongovernmental organizations use this non-outbreak period to discuss with local stakeholders the acceptability of the trade-offs inherent within the PEP score, such as public health versus individual risk. One option would be the rapid setup of a triage facility admitting children with a PEP score ≥3 to await test results and fast-tracking those scoring ≥7 to specialized Ebola treatment. However, this decision is highly context-specific, and there are dangers in being too prescriptive without taking into account factors such as local healthcare-seeking behavior.

A key limitation to our study is that PEP scores are derived from a population of children admitted to EHUs, all of whom should have fulfilled either the early- or late-2014 WHO suspected case definition. We do not have information on those not admitted (who were either truly EVD negative or missed EVD-positive cases). Therefore, we could only use data on the small number of children admitted who did not meet the WHO case definition to calculate its sensitivity and specificity, and these children may not have been representative of children who were negative by the WHO case definition but not admitted. Our calculations of WHO case definition validity are therefore only included for completeness and must be treated with caution. A further limitation is reducing EVD contact to a binary variable; more in-depth information (such as whether the child has had contact with a dead body, or whether the child

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (95% CI) from multivariable model</th>
<th>p value</th>
<th>Integer score value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive contact</td>
<td>2.21 (1.58–2.83)</td>
<td>&lt;0.001</td>
<td>+2</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>1.34 (0.62–2.05)</td>
<td>&lt;0.001</td>
<td>+2</td>
</tr>
<tr>
<td>Age ≥2 y</td>
<td>1.06 (0.37–1.75)</td>
<td>0.003</td>
<td>+2</td>
</tr>
<tr>
<td>Fever</td>
<td>0.99 (–0.66 to 2.63)</td>
<td>0.241</td>
<td>+1</td>
</tr>
<tr>
<td>Anorexia</td>
<td>0.59 (–0.18 to 1.35)</td>
<td>0.133</td>
<td>+1</td>
</tr>
<tr>
<td>Male gender</td>
<td>0.49 (–0.11 to 1.08)</td>
<td>0.111</td>
<td>+1</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0.42 (–0.23 to 1.08)</td>
<td>0.205</td>
<td>+1</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0.40 (–0.21 to 1.01)</td>
<td>0.197</td>
<td>+1</td>
</tr>
<tr>
<td>Difficulty breathing</td>
<td>–0.57 (–1.39 to 0.24)</td>
<td>0.168</td>
<td>–1</td>
</tr>
<tr>
<td>Difficulty swallowing</td>
<td>–0.59 (–1.39 to 0.19)</td>
<td>0.138</td>
<td>–1</td>
</tr>
<tr>
<td>Headache</td>
<td>–0.63 (–1.29 to 0.35)</td>
<td>0.063</td>
<td>–1</td>
</tr>
<tr>
<td>Rash</td>
<td>–1.00 (–2.13 to 0.14)</td>
<td>0.085</td>
<td>–2</td>
</tr>
</tbody>
</table>
is breastfeeding) could give greater discrimination. However, because 37% EVD-positive children were unaccompany-
died at hospital admission, an in-depth contact history
was unlikely to be reliable.

Missing and unreliable data are another limitation, illus-
trating the challenge of epidemiologic studies that an-
alyze data from emergency settings. This study was
retrospective, using data collected as part of outbreak
data gathering rather than as part of a formal prospec-
tive study. We accounted for missing data using multiple
imputation; reassuringly, imputed analysis gave similar
descriptions of EVD/non-EVD cases may be incomplete.

Epidemiologic studies that have serious public health
implications. Several highly sensitive rapid diagnostic
tests (RDT) for EVD underwent preliminary testing to-
ward the end of the West Africa outbreak, although the
numbers of children included in these studies were limited (25, 26). Judicious use of EVD RDTs coupl-
ed with PCR tests to confirm results could have re-
duced the scale of the Sierra Leone outbreak (27). Fur-
ther development of RDTs, and guidance on selecting the
children on whom to use them, is essential for preparing for
and responding to future outbreaks. Incorporating screen-
ing criteria from an evidence-based clinical prediction
model, such as this PEP score model, should contribute to
this process.

In conclusion, this study compares features at hospital
arrival in EVD-negative and EVD-positive children dur-
ing the West African epidemic. We describe a predic-
tive PEP score model that would allow for the selection of ap-
propriate case definitions (prioritizing sensitivity or speci-
ficity) depending on the clinical and epidemiologic setting.
The selected PEP scores had higher positive and negative

<table>
<thead>
<tr>
<th>Score</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
<th>% Correctly classified (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>1 (0–4)</td>
<td>31 (25–38)</td>
<td>100</td>
<td>31 (25–38)</td>
</tr>
<tr>
<td>1</td>
<td>97 (89–100)</td>
<td>4 (1–8)</td>
<td>31 (25–38)</td>
<td>71 (29–96)</td>
<td>32 (26–39)</td>
</tr>
<tr>
<td>2</td>
<td>97 (89–100)</td>
<td>13 (8–20)</td>
<td>33 (27–40)</td>
<td>91 (70–99)</td>
<td>39 (32–46)</td>
</tr>
<tr>
<td>3</td>
<td>94 (85–98)</td>
<td>30 (22–37)</td>
<td>37 (30–45)</td>
<td>91 (79–98)</td>
<td>49 (42–56)</td>
</tr>
<tr>
<td>4</td>
<td>86 (75–93)</td>
<td>49 (40–57)</td>
<td>43 (34–52)</td>
<td>89 (80–95)</td>
<td>60 (53–67)</td>
</tr>
<tr>
<td>5</td>
<td>77 (64–86)</td>
<td>67 (58–74)</td>
<td>51 (40–61)</td>
<td>87 (79–92)</td>
<td>70 (63–76)</td>
</tr>
<tr>
<td>6</td>
<td>58 (45–70)</td>
<td>82 (75–88)</td>
<td>59 (46–71)</td>
<td>81 (74–87)</td>
<td>75 (68–80)</td>
</tr>
<tr>
<td>7</td>
<td>44 (31–57)</td>
<td>92 (86–96)</td>
<td>70 (54–83)</td>
<td>79 (72–85)</td>
<td>77 (71–82)</td>
</tr>
<tr>
<td>8</td>
<td>23 (14–35)</td>
<td>95 (90–98)</td>
<td>68 (45–86)</td>
<td>74 (67–80)</td>
<td>73 (67–79)</td>
</tr>
<tr>
<td>9</td>
<td>11 (5–21)</td>
<td>98 (94–100)</td>
<td>70 (35–93)</td>
<td>71 (64–77)</td>
<td>71 (64–77)</td>
</tr>
<tr>
<td>10</td>
<td>5 (1–13)</td>
<td>99 (96–100)</td>
<td>75 (19–99)</td>
<td>70 (63–76)</td>
<td>70 (63–76)</td>
</tr>
</tbody>
</table>

| WHO case definition† | 98 (95–99) | 5 (3–7) | 30 (27–34) | 84 (66–95) | 33 (29–36) |

Table 3. Validation of PEP score against a standard of laboratory-confirmed Ebola virus disease status, compared with WHO case definition, based on a cohort of children who attended an Ebola holding unit and had EVD test results recorded, Sierra Leone, August 14, 2014–March 31, 2015*.

Table 4. Comparison of 2 different PEP scores on a hypothetical population of 100 suspected EVD patients at different points in EVD outbreak with differing prevalence of EVD.*

<table>
<thead>
<tr>
<th>PEP score</th>
<th>October 2014, 77% of suspected EVD+ cases†</th>
<th>March 2015, 4% of suspected EVD+ cases†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True EVD+, correctly admitted</td>
<td>True EVD+, incorrectly not admitted</td>
</tr>
<tr>
<td>3: 94% sensitivity, 30% specificity</td>
<td>72</td>
<td>7</td>
</tr>
<tr>
<td>7: 44% sensitivity, 92% specificity</td>
<td>34</td>
<td>21</td>
</tr>
</tbody>
</table>

*Laboratory-confirmed EVD status figures from Connaught Hospital (Freetown, Sierra Leone) during the 2014–2015 outbreak. EVD, Ebola virus disease; PEP, pediatric Ebola predictive; †, positive; –, negative.

†True or false EVD+ or EVD– determined by case ascertainment by PEP score. Admission result represents modeled outcome for patients in terms of EVD holding unit.
predictive values than the current WHO case definition. Applying the score in combination with RDTs could be a successful strategy in future outbreaks. External validation of the PEP score will be key to establishing its utility, but because data are scarce, we suggest local stakeholders use this postoutbreak period to reflect how the PEP score might best be used in their context.

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About the Author
Dr. Fitzgerald is an NIHR academic clinical lecturer at the UCL Great Ormond Street Institute for Child Health in pediatric infectious diseases. Her research interests include Ebola virus disease, HIV, molecular microbiology, and antimicrobial resistance.

References


Address for correspondence: Felicity Fitzgerald, UCL Institute of Child Health—Infection, Immunity, Inflammation and Physiological Medicine, 30 Guilford St, London, WC1N 1EH, UK; email: Felicity.fitzgerald@ucl.ac.uk

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- Epidemiology of Epidemic Ebola Virus Disease in Conakry and Surrounding Prefectures, Guinea, 2014–2015
- Hospital Preparations for Viral Hemorrhagic Fever Patients and Experience Gained from the Admission of an Ebola Patient
- Trematode Fluke Procerovum varium as Cause of Ocular Inflammation in Children, South India
- Association between Landscape Factors and Spatial Patterns of Plasmodium knowlesi Infections in Sabah, Malaysia
- Feasibility of Xpert Ebola Assay in Médecins Sans Frontières Ebola Program, Guinea
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- Sustained Transmission of Pertussis in Vaccinated, 1–5-Year-Old Children in a Preschool, Florida, USA
- Molecular Characterization of Invasive Streptococcus dysgalactiae subsp. equisimilis, Japan
- Population Effects of Influenza A(H1N1) Pandemic among Health Plan Members, San Diego, California, USA, October–December 2009
- Epidemiology of Serotype 1 Invasive N pneumococcal Disease, South Africa, 2003–2013
- Dogs and Opossums Positive for Vaccinia Virus during Outbreak Affecting Cattle and Humans, São Paulo State, Brazil
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- African Buffalo Movement and Zoonotic Disease Risk across Transfrontier Conservation Areas, Southern Africa
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- Candidatus Coviella massiliensis Infection
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- Louseborne Relapsing Fever among East African Refugees, Italy, 2015
- Mediterranean Fin Whales (Balaenoptera physalus) Threatened by Dolphin Morbillivirus
- Blastomyces gilchristi as Cause of Fatal Acute Respiratory Distress Syndrome
- Effectiveness of Meningococcal B Vaccine against Endemic Hypervirulent Neisseria meningitidis W Strain, England
- Frequency and Distribution of Rickettsiae, Borreliae, and Ehrlichiae Detected in Human–Parasitizing Ticks, Texas, USA

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https://wwwnc.cdc.gov/eid/articles/issue/22/2/table-of-contents
We used national statistics from 1983–2015 to evaluate trends in mortality caused by infectious diseases in South Korea. Age-standardized mortality from infectious disease decreased from 43.5/100,000 population in 1983 to 16.5/100,000 in 1996, and then increased to 44.6/100,000 in 2015. Tuberculosis was the most common cause of death in 1983 and respiratory tract infections in 2015. We observed a significant decline in infant deaths caused by infectious diseases, but mortality in persons age ≥65 years increased from 135 deaths/100,000 population in 1996 to 307/100,000 in 2015. The relative inequality indices for respiratory tract infections, sepsis, and tuberculosis tended to increase over time. Although substantial progress has been achieved in terms of infant mortality, death rates from infectious disease has not decreased overall. Elderly populations with lower education levels and subgroups susceptible to respiratory infections and sepsis should be the focus of preventive policies.

In many industrialized countries, deaths caused by infectious disease have decreased after introduction of various public health measures, including improvements in hygiene, vaccination, and antimicrobial therapy. However, the epidemiology of infectious diseases is complicated, and such diseases account for a considerable number of deaths globally (1). Emergence and reemergence of infectious diseases substantially affect the health outcomes of various populations (2). Moreover, deaths caused by infectious diseases have disproportionately affected countries and populations of lower socioeconomic status (3).

South Korea has undergone rapid economic growth over the past few decades and over the same period has developed a sustainable healthcare system that is generally accessible (4). The overall mortality rate in this country decreased substantially from 1,203–1,665 deaths/100,000 population in 1983 to 587–638/100,000 in 2012; the decrease was associated with rising average life expectancy, from 67 years in 1983 to 81 years in 2012 (5,6). Despite these changes, persons in South Korea remain vulnerable to infectious diseases that typically impose great burdens on healthcare systems (7). Particularly vulnerable populations are disproportionately affected by endemic infections from tuberculosis (TB) and vectorborne pathogens (8,9). The emergence of pandemic influenza A(H1N1) in 2009 and Middle East respiratory syndrome in 2015 substantially affected not only overall health outcomes but also societal stability (10,11).

Infectious diseases constitute substantial healthcare burdens even in industrialized countries such as South Korea. The epidemiologic patterns of these diseases change over time. Therefore, exploring changes in population health during phases of economic growth is necessary. Here we describe trends in mortality caused by infectious diseases in South Korea during 1983–2015 and aim to identify factors possibly influencing the observed trends.

**Methods**

In South Korea, physicians are legally required to complete death certificates, which are then sent to Statistics Korea and made publicly available through the Korean Statistics Information Service (KOSIS; http://kosis.kr/eng). The death certificate includes information on disease directly leading to death, antecedent causes, and other major conditions contributing to death (12). Once the death certificate is submitted, a professional staff in Statistics Korea identifies a single most relevant underlying cause for each death, in accordance with the World Health Organization definition (13). The coding is reviewed by a committee to finalize the national public data provided to the users. Nationally, ≈90% of all deaths were certified in 1987, and almost 100% of deaths were certified by 2007 (14,15). The causes of death are coded according to the Korean Classification of Diseases, Sixth Revision, which is based on the International Classification of Diseases (ICD), 10th Revision (ICD-10). The Korean Classification of Diseases was first adopted in 1973 and was revised in accordance with the amendments in the ICD scheme. The death data collected before 1995, which were coded in the ICD’s Ninth Revision codes, are converted to ICD-10 codes by Statistics Korea by using a mapping reference table (https://kssc.kostat.go.kr:8443).
We retrieved infectious disease mortality rates for 1983–2015 from the KOSIS database and identified the infectious disease groups listed (Table 1). We calculated age-standardized mortality rates for 1983–2015 by using the World Standard Population as reference (16,17). We analyzed trends by age group (<1, 1–4, 5–14, 15–64, and ≥65 years) and disease (respiratory tract infection, sepsis, TB, intestinal infection, vaccine-preventable disease, central nervous system [CNS] infection, viral hepatitis, HIV-caused disease, and rheumatic heart disease). We used joinpoint regression analysis to identify years associated with significant changes in mortality rates (18). We calculated annual percentage changes (APCs) by using generalized linear models, assuming that a Poisson distribution was in play. To assess the trend over an interval, we computed an average of the APCs from the joinpoint model and used that result as a summary measure. The p value for a 2-sided test for which the true average of the APCs was zero was calculated based on a t distribution (https://surveillance.cancer.gov/help/joinpoint/setting-parameters/method-and-parameters-tab/average-annual-percent-change-aapc).

We obtained population denominators according to age and education level from the Korean Population and Housing Census data, which are obtained at 5-year intervals. The number of deaths according to age and education level (numerators) calculated from raw death certificate data were available from the KOSIS database. We selected information from the years 2000, 2005, 2010, and 2015 to use as population denominators for calculating mortality rates according to education levels. We analyzed changes in age-standardized mortality associated with selected infectious diseases, by education level, among those age ≥65 years. We categorized education levels as middle school or less, high school, and college or higher. We calculated the slope index of inequality (SII) and the relative index of inequality (RII) to assess the contributions made by socioeconomic disparity to infectious disease–caused mortality among persons of various education levels (19).

We used R Studio version 1.0.136 (R Studio, Boston, MA, USA); the Joinpoint Regression Program version 4.1.0; and the Health Disparities Calculator version 1.2.4 (US National Cancer Institute, Bethesda, MD, USA) in our analyses (19,20). All personal information was anonymized; the study, therefore, did not require review by our Institutional Ethics Board.

**Results**

From 1983 through 2015, the number of deaths from infectious diseases exhibited a U-shaped trend, whereas the number of deaths from noninfectious diseases decreased from 594.3 to 496.9/100,000 population (Figure 1). The age-standardized mortality rate from infectious diseases decreased from 43.5/100,000 population in 1983 to 16.5/100,000 in 1996 but then increased to 44.6/100,000 in 2015. In 1983, infectious diseases caused 6.8% (17,376/254,563) of all deaths in South Korea; in 2015, the percentage was 8.3% (22,766/275,895).

TB was the leading cause of infectious disease death in 1983 (7,853 deaths; 23.7/100,000 population) (Table 2). In contrast, in 2015, respiratory tract infections were the most common causes of death from infectious diseases (15,030 deaths; 19.5/100,000 population). Of the top 5 infectious diseases (by number of deaths per 100,000 population), CNS infections and vaccine-preventable diseases (on the 1983 list) were replaced by sepsis and viral hepatitis (on the 2015 list).

We analyzed mortality rates by age group (Figure 2). Infant mortality (i.e., in persons age <1 year) was >100 deaths/100,000 population during 1983–1985, decreased to 38/100,000 in 1994, and fell further to <10/100,000 in 2011. The trends in mortality caused by infectious diseases varied among elderly populations. Among persons age ≥65 years, mortality rates were 206 deaths/100,000 population in 1983, 135/100,000 in 1996, and 307/100,000 in 2015.

We analyzed trends in age-specific infectious disease mortality rates by using joinpoint analysis (Table 3). Among infants <1 year of age, 3 periods of change were evident. The first period (1983–2007) was associated with an APC of −10.86, the second period (2007–2010) with an APC of 14.56, and the third period (2010–2015) with an APC of −18.45. Overall, the average APC for 1983–2015 was −9.41, reflecting a significant decrease from 124.2 to 4.1 deaths/100,000 population. Among persons age ≥65 years, the average APC did not vary significantly during this period (APC 1.44), but the APC increased significantly during 2007–2015 (APC 7.70).

We analyzed age-standardized mortality rates associated with respiratory infections, sepsis, and TB (Figure 3, panel A). TB-related mortality rates decreased during 1983–2015. In 2015, the rate was 3 deaths/100,000 population. During 1983–2015, mortality attributable to respiratory

| Table 1. Selected infectious disease groups and corresponding ICD-10 codes* |
|-------------------|-------------------|
| Group             | ICD-10 codes      |
| All infectious diseases | A00–B99, J09–J18, J86, G00, G03, G04, 100–109, M86 |
| Intestinal infections | A00–A09 |
| Tuberculosis       | A15–A19 |
| Vaccine-preventable diseases† | A33–A37, B05, B26, B83.0 |
| Sepsis            | A40–A41 |
| Viral hepatitis   | B15–B19 |
| HIV-related diseases | B20–B24 |
| Central nervous system infections | G00, G03, G04 |
| Rheumatic heart diseases | 100–109 |
| Respiratory tract infections‡ | J09–J18, J86 |

*ICD-10, International Classification of Diseases, 10th Revision. †Includes diphtheria, tetanus, pertussis, measles, mumps, Japanese encephalitis. ‡Includes influenza, pneumonia, empyema.
tract infections (pneumonia, empyema, and influenza) increased from 15 to 20 deaths/100,000 population, and sepsis mortality increased from 1 to 4/100,000. We also analyzed age-standardized mortality rates for intestinal infections, vaccine-preventable diseases, CNS infections, and viral hepatitis (Figure 3, panel B). From 1983 to 1999, the mortality rates associated with all of these diseases decreased. Since 2000, the mortality rates associated with intestinal infections and viral hepatitis have gradually increased.

Mortality rates varied by infectious disease; mortality from TB, sepsis, and viral hepatitis exhibited the greatest shifts from 1983 to 2015 (Table 4). The overall average APC (from 1983 to 2015) for respiratory tract infections was 1.06 (15.1 to 19.5 deaths/100,000 population). However, a significant average APC decrease occurred during 1983–1992 (APC –8.89), and a significant increase occurred during 2007–2015 (APC 11.47). The greatest decreases were evident in persons with intestinal infections during 1983–1988 (APC –14.30), in persons with CNS infections during 1983–1986 (APC –13.94), and in persons with vaccine-preventable diseases during 1983–1987 (APC –10.97). The most obvious increases in mortality were in persons with respiratory infections during 2007–2015 (APC 11.47) and sepsis during 2003–2015 (APC 6.99).

We stratified mortality rates from infectious diseases by education level for persons ≥65 years of age (Table 5). For most education levels, the number of deaths attributable to respiratory infections and sepsis tended to decrease during 2000–2010 but increased in 2015. Tuberculosis mortality rates decreased among those of higher education levels during 2000–2015. The SII of respiratory tract infection–associated mortality increased from 46.2 in 2000 to 78.9 in 2015, and the SII of TB-associated mortality fell from 42.0 to 15.2. The respiratory tract infection RII score tended to increase over time. In 2000, the RII was 0.6 but increased to 0.8 in 2005, 1.1 in 2010, and 1.5 in 2015. From 2000 to 2015, the RIIs of sepsis increased from 1.0 to 1.8, and RIIs of TB increased from 0.7 to 1.4.

Discussion

Despite the reduction in all-cause mortality evident in South Korea from 1983 to 2015, infectious diseases remain problematic. The mortality trend for such diseases is U-shaped; a significant decrease is evident from 1983 to 1991 (APC

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**Table 2. Leading causes of death from infectious disease, South Korea, 1983–2015**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Infectious disease</th>
<th>No. deaths</th>
<th>Mortality rate*</th>
<th>Infectious disease</th>
<th>No. deaths</th>
<th>Mortality rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tuberculosis</td>
<td>7,853</td>
<td>23.7</td>
<td>Respiratory tract infections</td>
<td>15,030</td>
<td>19.5</td>
</tr>
<tr>
<td>2</td>
<td>Respiratory tract infections</td>
<td>5,286</td>
<td>15.1</td>
<td>Sepsis</td>
<td>3,045</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>Intestinal infections</td>
<td>1,314</td>
<td>4.3</td>
<td>Tuberculosis</td>
<td>2,209</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>CNS infections</td>
<td>1,244</td>
<td>3.1</td>
<td>Intestinal infections</td>
<td>670</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Vaccine-preventable diseases</td>
<td>509</td>
<td>1.1</td>
<td>Viral hepatitis</td>
<td>667</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Age-standardized mortality rate (deaths/100,000 population).
Infectious Disease Mortality, South Korea

–9.73), followed by an increase from 2006 to 2015 (APC 8.97). During the period of decrease, the largest percentage change was evident in death rates associated with intestinal infections (APC –14.30%), followed by death rates associated with CNS infections (APC –13.94), vaccine-preventable diseases (APC –10.97), and TB (APC –9.12). South Korea underwent rapid economic development in the 1980s, which was associated with the implementation of accessible universal healthcare in 1989. In 2000, an aging society was evident in South Korea, when the proportion of the population ≥65 years of age was 7.2% (6); this proportion increased to 10.7% in 2010 and to 12.7% in 2014. Improvements in healthcare access and public hygiene might have acted as the key drivers of the decrease in infectious disease mortality (5). Surprisingly, however, respiratory infection–associated mortality has increased (APC 11.47), as has mortality attributable to sepsis (APC 6.99) and to intestinal infections (APC 5.48). A major societal turning point occurred in 1997; an economic crisis increased income inequality and weakened social cohesion, which affected health (21). The changes in societal structure and the resulting policies have been influenced by efforts to expand flexible labor markets. While workers have been experiencing job insecurity and disadvantages in wages and benefits, the economic crisis has exacerbated health inequalities; recent studies have shown increasing socioeconomic inequalities in self-rated health, suicide, and infant mortality (21). The increasing health inequalities are an important aspect of the recent increase in infectious disease–caused mortality in South Korea. Health inequalities are not only an ethics and social justice issue but also an important public health problem that needs to be addressed appropriately. Addressing health inequalities should be prioritized in public health policy for the prevention and control of infectious diseases in South Korea.

We also found that the reductions in infectious disease–related mortality rates noted in the 1980s were largely attributable to fewer infant deaths and TB-related deaths. The greatest declines in infectious disease–related infant mortality were observed during 1983–2007 (APC –10.86) and 2010–2015 (APC –18.45). In South Korea, large corporations were mandated by law to provide health insurance, commencing in 1977, and such coverage was gradually expanded to include the entire population by 1989 (22). Improved healthcare access has greatly increased vaccination and antibiotic treatment of infectious diseases. Infants are very susceptible to vaccine-preventable diseases; thus, the introduction of publicly available vaccines and

Table 3. Trends in age-specific mortality rates associated with infectious disease, derived using joinpoint analysis, South Korea, 1983–2015*

<table>
<thead>
<tr>
<th>Age group, y</th>
<th>Mortality rate</th>
<th>AAPC, 1983–2015</th>
<th>Trend 1</th>
<th>Trend 2</th>
<th>Trend 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>56.3</td>
<td>1.3</td>
<td>–10.09</td>
<td>–21.30</td>
<td>1992–2015 –7.02</td>
</tr>
<tr>
<td>5–14</td>
<td>23.7</td>
<td>0.7</td>
<td>–8.74</td>
<td>–19.70</td>
<td>1996–2015 –1.72</td>
</tr>
<tr>
<td>&gt;65</td>
<td>205.6</td>
<td>307.0</td>
<td>1.44</td>
<td>–10.77</td>
<td>1986–2007 0.72</td>
</tr>
</tbody>
</table>

*AAPC, average annual percentage change; APC, annual percentage change in age-standardized mortality rate. Mortality rates expressed as deaths/100,000 population. Values in bold indicate where AAPC or APC differed significantly (p<0.05) from zero (by a 2-sided test for which the true AAPC was zero, calculated based on a t distribution; https://surveillance.cancer.gov/help/joinpoint/setting-parameters/method-and-parameters-tab/average-annual-percent-change-aapc).
expansion of vaccination coverage was effective in preventing infectious disease deaths. In 1991, the vaccination rates for publicly provided childhood vaccines in South Korea were 56%–80%, whereas in 2012, the vaccination rates were 95.9%–100% (23,24). Moreover, although TB is not uncommon in South Korea, our data provide compelling evidence that deaths from TB have significantly declined over the past 23 years. TB control efforts (improved screening and early treatment) clearly have been successful in mitigating mortality (25).

Again, the most notable event during the study period was the 1997 financial crisis in Asia, which had devastating consequences not just on the economy but also the health of undereducated persons (26). Many persons were subjected to layoffs, early retirements, and business failures, which affected major health indices such as healthcare use, rates of chronic and acute disease, and all-cause mortality rates, including that of suicide. Given the limited social security available to the elderly population in South Korea, the impact was more severe in this population, especially among persons with less education. The hospitalization rate decreased during the early stage of the economic crisis. During 1995–1998, rates of chronic disease increased by 27.1% and rates of acute disease by 9.5% (26). All-cause mortality began to increase ≈1 year after the crisis (27). During 1970–2010, overall mortality decreased by 70%–80%; however, only a minimal decline was evident in persons with less education (28). We found that education level affected mortality associated with respiratory tract infections and sepsis. Moreover, the elderly population might have acquired chronic diseases that contribute to the increased incidence and severity of infections. Also, the recent decrease in mortality associated with chronic diseases might have resulted in longer periods of exposure to infectious diseases, increasing the risk for death from certain infections in vulnerable populations (29).

Influenza and pneumococcal pneumonia are the 2 vaccine-preventable respiratory infections. Influenza vaccine has been included in South Korea’s national immunization program since 1997, and coverage among those ≥65 years of age was 79.9% during 2004–2005 (30). However, during this period, coverage rates varied by

Figure 3. Age-standardized infectious disease mortality rates, South Korea, 1983–2015. A) Mortality rates associated with respiratory infections, sepsis, and tuberculosis. B) Mortality rates associated with intestinal infections, vaccine-preventable diseases, central nervous system infections, and viral hepatitis.
sex and lifestyle (31). Influenza vaccination campaigns should focus on underrepresented groups, particularly in the elderly population. The extent of pneumococcal polysaccharide vaccination in the elderly was low (0.8%) in 2005 (32). After the introduction of a publicly funded program in 2013, vaccination coverage increased from 5.0% to 57.9% over a 20-month period (33). Similar to what had been observed in children, the publicly funded program increased vaccination coverage in South Korea. A survey conducted in 2010 showed that state-sponsored vaccination rates in children reached 95.9%–100%, and vaccination rates among the general public were 30.7%–85.4% (24). Nationwide vaccination of underprivileged populations might also help counter unmet medical needs in South Korea, especially in the context of vaccine-preventable respiratory infections.

Changes in population structure and the influences of pathogens on disease development might have affected mortality caused by infectious diseases. The numbers of immunocompromised persons are growing; the number of susceptible persons is increasing as a result of the greater extent of procedural and treatment invasiveness, including that associated with chemotherapy and transplantation (34,35). Nosocomial infections and antimicrobial resistance might increase both the incidence of and death from sepsis, intestinal infections, and pseudomembranous colitis. Few population-level epidemiologic data on sepsis or pseudomembranous colitis are available in South Korea (36). We

Table 4. Trends in age-standardized mortality rates associated with infectious disease (derived using joinpoint analysis), South Korea, 1983–2015*

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mortality rate</th>
<th>AAPC, 1983–2015</th>
<th>Trend 1</th>
<th>Trend 2</th>
<th>Trend 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>All infectious disease</td>
<td>43.5</td>
<td>44.6</td>
<td>0.44</td>
<td>–9.73</td>
<td>1991–2006</td>
</tr>
<tr>
<td>Vaccine-preventable diseases</td>
<td>1.1</td>
<td>0.0</td>
<td>–0.35</td>
<td>–0.35</td>
<td>1983–1999</td>
</tr>
<tr>
<td>Viral hepatitis</td>
<td>0.0</td>
<td>0.0</td>
<td>3.24</td>
<td>2.75</td>
<td>1983–1998</td>
</tr>
<tr>
<td>HIV diseases</td>
<td>0.0</td>
<td>0.0</td>
<td>0.70</td>
<td>–0.01</td>
<td>1983–1998</td>
</tr>
<tr>
<td>Rheumatic heart diseases</td>
<td>0.5</td>
<td>0.2</td>
<td>–0.35</td>
<td>–0.35</td>
<td>1983–1999</td>
</tr>
</tbody>
</table>

* AAPC, average annual percentage change; APC, annual percentage change in age-standardized mortality rate; CNS, central nervous system. Mortality rates expressed as deaths/100,000 population. Values in bold indicate where AAPC or APC differed significantly (p<0.05) from zero (by a 2-sided test for which the true AAPC was zero, calculated based on a t distribution; https://surveillance.cancer.gov/help/joinpoint/setting-parameters/method-and-parameters-tab/average-annual-percent-change-aapc.}

Table 5. Changes in age-standardized mortality rates associated with respiratory tract infections, sepsis, and tuberculosis among adults ≥65 years of age, by education level and inequality index, South Korea, 2000–2015

<table>
<thead>
<tr>
<th>Disease by education level and inequality</th>
<th>2000</th>
<th>2005</th>
<th>2010</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory tract infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle school or less</td>
<td>98.6</td>
<td>90.8</td>
<td>60.2</td>
<td>87.9</td>
</tr>
<tr>
<td>High school</td>
<td>69.4</td>
<td>53.5</td>
<td>28.6</td>
<td>35.9</td>
</tr>
<tr>
<td>College or higher</td>
<td>67.8</td>
<td>55.9</td>
<td>31.1</td>
<td>35.3</td>
</tr>
<tr>
<td>Inequality index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope index of inequality</td>
<td>46.2</td>
<td>52.4</td>
<td>43.7</td>
<td>78.9</td>
</tr>
<tr>
<td>Relative index of inequality</td>
<td>0.6</td>
<td>0.8</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Sepsis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle school or less</td>
<td>33.9</td>
<td>21.7</td>
<td>14.2</td>
<td>17.3</td>
</tr>
<tr>
<td>High school</td>
<td>24.4</td>
<td>12.5</td>
<td>6.1</td>
<td>5.9</td>
</tr>
<tr>
<td>College or higher</td>
<td>17.0</td>
<td>10.2</td>
<td>5.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Inequality index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope index of inequality</td>
<td>25.4</td>
<td>17.3</td>
<td>13.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Relative index of inequality</td>
<td>1.0</td>
<td>1.2</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle school or less</td>
<td>60.0</td>
<td>44.3</td>
<td>15.1</td>
<td>13.9</td>
</tr>
<tr>
<td>High school</td>
<td>45.8</td>
<td>34.6</td>
<td>7.7</td>
<td>6.1</td>
</tr>
<tr>
<td>College or higher</td>
<td>30.1</td>
<td>21.4</td>
<td>5.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Inequality index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope index of inequality</td>
<td>42.0</td>
<td>30.7</td>
<td>16.8</td>
<td>15.2</td>
</tr>
<tr>
<td>Relative index of inequality</td>
<td>0.7</td>
<td>0.7</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>
emphasize, therefore, that public health authorities must note that the trends in deaths caused by sepsis have changed (37). The hepatitis A outbreak in South Korea during the late 2000s reflects an immunity gap in young adults (38). In South Korea, the seroprevalence of hepatitis A has been changing dynamically in adults 20–39 years of age, the population most affected by the outbreak. The increase in viral hepatitis–related deaths in the 2000s suggests that epidemiologic transition cannot be ignored when formulating public health policies.

Our study had several limitations. First, we evaluated only deaths reported on death certificates; these records only partly capture the burden of infectious disease. The need to identify a specific cause of death might encourage miscoding and thus might bias our estimates. However, a previous validation study showed that ≈67% of the deaths from infectious disease were classified accurately (39). Second, the KOSIS database does not provide detailed classification of certain disease groups. For example, perinatal infections (P23 and P35–39) are included among “certain conditions originating in the perinatal period (P00–96),” heart infections (I30 and I33) are included among “other heart diseases (I26–51),” and infections of the kidney and urinary tract (N10–13) are included among “diseases of the genitourinary system: urinary system (N00–39).” Therefore, deaths caused by these disease groups were not included in this analysis. Third, the practical classification of the diseases in this study might not reflect the specific public health measures for control and prevention. For example, influenza and hepatitis A and B are now vaccine-preventable diseases, but influenza is still classified as a respiratory infection, and hepatitis A and B are still classified as viral hepatitis. As another example, the coding of sepsis is sometimes inconsistent and not standardized explicitly. Because sepsis develops mostly as a consequence of other disease processes, establishing a causal relationship with death is difficult, resulting in certain variations in coding. Depending on the etiology, certain subcategories of sepsis, CNS infections, lower respiratory tract infections, and intestinal infections can be prevented by vaccination.

Despite these limitations, our study illustrates the changes over the last 2 decades in the nature of infectious disease deaths in South Korea, a country that has undergone rapid economic and societal transformations during this period. Although substantial progress has been made in infectious disease prevention and treatment, the burden of infectious diseases has not diminished, principally because of changes in population structure and disproportionate mortality rates among less educated persons. Periodic estimations of disease burden in South Korea are required to appropriately transition epidemiologic public health measures. Our findings will be useful in implementation and evaluation of control and preventive strategies. Sepsis and lower respiratory tract infection deserve attention as serious public health issues in the undereducated elderly population in South Korea. We recommend additional efforts toward managed care in this population, which can prevent avoidable death caused by infectious diseases. Moreover, additional studies to better understand how this type of disparity has affected disease mortality are warranted.

In conclusion, the overall mortality rate from infectious diseases in South Korea remained unchanged during 1983–2015. Monitoring of infectious disease mortality data can help identify subjects requiring disease control and prevention. The trends illustrate the continued vulnerability of South Korea residents to infectious diseases. Elderly persons and persons with respiratory infections and sepsis require particular attention in terms of disease control and prevention.

About the Author

Dr. Choe is a pediatric infectious disease fellow at the Warren Alpert Medical School of Brown University. His primary research interests are the methodologies for assessing epidemiology and control of vaccine-preventable diseases.

References

9. Chang KS, Yoo DH, Ju YR, Lee WG, Roh JY, Kim HC, et al. Distribution of malaria vectors and incidence of vivax malaria at Korean army installations near the demilitarized zone, Republic
High levels of macrolide resistance and increasing fluoroquinolone resistance are found in Mycoplasma genitalium in many countries. We evaluated pristinamycin for macrolide-resistant M. genitalium in a sexual health center in Australia. Microbiologic cure was determined by M. genitalium-specific 16S PCR 14–90 days after treatment began. Of 114 persons treated with pristinamycin, infection was cured in 85 (75%). This percentage did not change when pristinamycin was given at daily doses of 2 g or 4 g or at 3 g combined with 200 mg doxycycline. In infections with higher pretreatment bacterial load, treatment was twice as likely to fail for each 1 log_{10} increase in bacterial load. Gastrointestinal side effects occurred in 7% of patients. Pristinamycin at maximum oral dose, or combined with doxycycline, cured 75% of macrolide-resistant M. genitalium infections. Pristinamycin is well-tolerated and remains an option where fluoroquinolones have failed or cannot be used.

Mycoplasma genitalium is a sexually transmitted bacterium and an established cause of urethritis, cervicitis, pelvic inflammatory disease, and obstetric complications (1,2). Azithromycin is frequently used alone or in combination with other antimicrobial drugs to treat these syndromes because of its activity against Chlamydia trachomatis and its long tissue half-life, enabling single-dose administration. European, US, and Australian treatment guidelines recommend azithromycin for treatment of M. genitalium infections (3–6). However a recent meta-analysis revealed the proportion of infections cured by azithromycin fell from 85% (95% CI 82%–88%) before 2009 to 67% (95% CI 57%–77%) during 2009–2013 (7).

M. genitalium lacks a cell wall and is difficult to culture, hindering study of its antimicrobial susceptibilities and resistance mechanisms, but single-nucleotide substitutions in domain V of 23S rRNA do confer resistance to macrolides (1). In 2016, the prevalence of macrolide resistance mutations (MRM) in M. genitalium infections was 40%–60% in studies from Germany, Australia, Canada, and the United States (8–11). Recent work has demonstrated the selection of MRM during single-dose and multidose treatment with azithromycin (11–13).

Moxifloxacin is recommended for treating macrolide-resistant M. genitalium (4–6); however, fluoroquinolone resistance mutations associated with treatment failure recently have been reported in 15% of infected patients in Australia and 47% in Japan (14,15). Macrolide resistance exceeds 50% in M. genitalium–infected patients in Melbourne, and combined fluoroquinolone/macrolide resistance is found in 8.6%, rendering azithromycin and moxifloxacin ineffective in most of these cases (11,16). Moxifloxacin is also costly and not recommended during pregnancy and occasionally causes serious side effects (17).

Pristinamycin comprises 2 synergistic antimicrobial drugs: pristinamycin IA (a macrolide-like streptogramin B–type compound) and IIA (a streptogramin A–type compound) (18,19). Both bind to the 50S subunit of the bacterial ribosome causing bacteriostatic inhibition of protein synthesis, but the combination is bactericidal with a broad antibacterial spectrum that includes Mycoplasma spp. (18). Although mutations in M. genitalium have been associated with resistance to macrolides and fluoroquinolones, experience with pristinamycin for M. genitalium infections is limited, and the influence of mutations in the 23S and ribosomal genes on treatment efficacy are unknown. Mutations in the genes encoding ribosomal proteins L4 and L22 have been associated with in vitro resistance to pristinamycin and telithromycin, respectively, in M. pneumoniae (20) and have been described in cases of azithromycin treatment failure (21,22).
Based on favorable MICs (23) and early success in curing all 6 of 6 patients with dual macrolide and quinolone resistance (12), we evaluated pristinamycin during 2012–2016, initially in patients with M. genitalium infection that azithromycin and moxifloxacin failed to cure and then in patients in whom only azithromycin had failed, at Melbourne Sexual Health Centre (MSHC; Melbourne, VIC, Australia). We report the microbiological outcomes, and factors influencing these outcomes, for M. genitalium infections that were not cured by prior antimicrobial drug regimens and were treated with pristinamycin.

Methods
Patients attending MSHC who have nongonococcal urethritis, pelvic inflammatory disease, cervicitis, or proctitis are routinely tested for M. genitalium, as are their sex partners. From August 2012 through November 2014, patients for whom azithromycin and moxifloxacin failed and for whom no other treatment options were available were treated with pristinamycin at a dose of 1 g 4 times/day for 10 days. Because of promising preliminary results and side effects data, in December 2014, pristinamycin became a second-line treatment after azithromycin failure. Two other pristinamycin regimens were evaluated during the study period: 1 g 2 times/day for 10 days and 1 g 3 times/day in combination with doxycycline. The pristinamycin/doxycycline combination was used based on evidence indicating this combination was effective in treating meticillin-resistant Staphylococcus aureus and theoretical and empirical evidence supporting antimicrobial drug combinations in several infections (24,25).

Patients receiving pristinamycin from MSHC’s pharmacy were prospectively followed by a research nurse. The nurse extracted the following information and recorded it in a database: results of tests of cure, adverse effects, and posttreatment sex with an untreated or inadequately treated partner.

Patients were routinely advised to return for a test of cure 2–4 weeks after treatment. Microbiological cure was defined by a negative test for M. genitalium 14–90 days after start of treatment. Patients reporting posttreatment sex with an untreated partner (where the relationship preceded treatment) were excluded regardless of the result of their test of cure because of their high risk for reinfection. Patients who reported sex with treated partners or new partners were retained in the analysis and coded as being at risk for reinfection so that this information could be analyzed as a risk factor. The Alfred Hospital Ethics Committee approved the study (project 490/16).

Laboratory Methods
We centrifuged urine (1 mL) at 10,000 × g for 10 min, discarded the supernatant, and resuspended the pellet in 0.2 mL of phosphate-buffered saline. Swabs were agitated in 0.4 mL phosphate-buffered saline to release cellular material. We extracted DNA from 0.2 mL of specimen on the MagNa Pure 96 Platform (Roche Applied Science, Mannheim, Germany) and stored it at –30°C. We used an M. genitalium–specific PCR targeting the 16S ribosomal RNA gene for detection and determined load by using a quantitative PCR targeting the MgPa gene (12,26). We performed partial sequencing of the 23S, L4, and L22 genes implicated in macrolide resistance (21) and ParC and GyrA genes indicated for fluoroquinolone resistance (27) on samples before and after treatment with pristinamycin. We sequenced PCR amplicons for each region in both directions by using Sanger sequencing (Australian Genome Research Facility, Melbourne, VIC, Australia) and performed sequence analysis by using the CLC Main Workbench version 7 (CLC Bio, Aarhus, Denmark). To identify mutations/sequence variations, we compared DNA sequences with the reference sequence M. genitalium G37 (GenBank accession no. NC_000908).

Statistical Methods
We calculated proportions cured and 95% CIs using the binomial exact distribution and compared treatment subgroups by using Fisher exact test or the nonparametric trend test, where indicated. Bacterial load was log10 transformed, and the significance of comparisons was assessed by t test, paired when comparing before and after treatment samples from the same patient. We assessed the association between bacterial load and time since treatment by linear regression. We calculated odds ratios (ORs) and 95% CIs for predictors of treatment failure by logistic regression or exact logistic regression as appropriate, using Stata version 13 (StataCorp LLC, College Station, TX, USA).

Results
During August 2, 2012–February 9, 2016, a total of 133 patients were treated with pristinamycin for M. genitalium infection. Patients were excluded from further study for 4 reasons: no test of cure (n = 6), test of cure <14 days from start of treatment (n = 4), test of cure >90 days from start of treatment (n = 5), and sex with untreated partners (n = 4) (Figure 1). The analysis comprised the remaining 114 patients. These patients had been unsuccessfully treated with ≥1 of the following antimicrobial drugs before pristinamycin: single-dose azithromycin (1 g; 34 [30%] patients); azithromycin 1.5 g (500 mg, then 250 mg/d for 4 d; 76 [67%]); moxifloxacin (400 mg/d for 10 d; 19 [17%]); or doxycycline (100 mg 2×/d for 7 d; 19 [17%]). Twenty-five (22%) patients had been treated with ≥2 courses of antimicrobial therapy. Four (4%) received pristinamycin as initial treatment because their partners had a resistant infection.

Of the 114 patients in the analysis, data were available for 99 for analysis of bacterial load (before and after
pristinamycin) and 74 for genetic sequencing of ≥1 region of interest. Median time to test of cure was 30 days (interquartile range 23–41).

Characteristics of the Study Population
Of 114 patients treated with pristinamycin, 65 (57%) were men who have sex with men, 38 (33%) were heterosexual men, and 11 (10%) were women (Table 1). A first-pass urine sample was tested in 83 (73%) of the 114 patients; the remainder had rectal or cervical swab samples tested. The most common diagnosis was nongonococcal urethritis, present in 70 (61%) patients.

Microbiological Cure after Pristinamycin
Of 114 patients treated with any of the 3 pristinamycin regimens, infection was cured in 85 (75% [95% CI 66%–82%]). The proportion cured did not vary among the 3 regimens (p = 0.91) (Table 2). Proportions cured did not vary significantly by site of infection (urethral vs. anorectal), sex, or symptom status. Somewhat more asymptomatic infections were cured (94% [95% CI 70%–100%]) than symptomatic (71% [95% CI 61%–80%]; p = 0.07).

Sixty-eight (60%) patients had data recorded on medication adherence, and 7 (10%) had missed >1 dose. We found no difference in proportions cured between those who missed doses and those reporting 100% adherence (71% vs. 72%; p = 1.0). Data on reinfection risk were available for 92 (81%) patients; 5 (5%) were considered at risk for reinfection, but infections in all 5 were cured (p = 0.3).

Effect of Bacterial Load on Microbiological Cure
Mean pretreatment bacterial loads (log$_{10}$) did not vary significantly among sample types for the 96 samples for which data were available: 2.9 log$_{10}$ from 67 urine samples, 3.1 log$_{10}$ from 26 rectal swab samples, and 2.5 log$_{10}$ from 3 cervical swab samples (p = 0.56 for rectal vs. urine, p = 0.70 for cervical vs. urine) (Figure 2, panel A). We therefore analyzed the effect of bacterial load on treatment outcome in all samples. Mean M. genitalium load in pretreatment samples was 0.92 log$_{10}$ higher in the 26 patients in whom pristinamycin failed (3.6 [95% CI 3.2–4.0] log$_{10}$) than in the 71 in whom infection was cured (2.7 [95% CI 2.4–3.0 log$_{10}$]; p<0.01) (Figure 2, panel B). For each log$_{10}$ increase in bacterial load in pretreatment samples, the OR for treatment failure was 1.9 (95% CI 1.3–2.9; p<0.01) (Table 3). In the 26 cases of pristinamycin failure for which paired samples were available, the mean bacterial load was significantly lower in posttreatment samples (2.3 [95% CI 2.1–2.5] log$_{10}$).

Table 1. Characteristics of patients with Mycoplasma genitalium infections treated with pristinamycin, Melbourne Sexual Health Centre, Melbourne, Victoria, Australia, 2012–2016

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Men who have sex with men, n = 65</th>
<th>Heterosexual men, n = 38</th>
<th>Female, n = 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y (interquartile range)</td>
<td>32.0 (27.4–37.3)</td>
<td>27.9 (24.6–34.6)</td>
<td>26.1 (22.6–28.2)</td>
</tr>
<tr>
<td>Sample, no.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine*</td>
<td>39</td>
<td>38</td>
<td>6</td>
</tr>
<tr>
<td>Rectal swab</td>
<td>26</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cervical swab</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>3</td>
</tr>
<tr>
<td>Diagnosis, no.†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nongonococcal urethritis</td>
<td>37</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Proctitis</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pelvic inflammatory disease</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Other‡</td>
<td>9</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Asymptomatic, no.</td>
<td>11</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*Includes 1 urethral swab sample.
†Clinical diagnosis when first tested for M. genitalium.
‡Comprises urethral gonorrhea (n = 3), anal discharge (n = 3), other anal symptoms (n = 3), female dysuria (n = 2), vaginal discharge (n = 2), vaginal bleeding (n = 2), not recorded (n = 3).
Pristinamycin for *M. genitalium* Infection

1.8–2.7] log_{10} than in pretreatment samples (3.6 [95% CI 3.1–4.0] log_{10}), a mean difference of 1.3 log_{10}; p<0.001), indicating that unsuccessful treatment still reduced bacterial load (Figure 2, panel C). Posttreatment bacterial load did not vary with time to test of cure (p = 0.98). Restricting analyses to urine samples did not change any associations with bacterial load.

**Effect of Resistance Mutations on Microbiological Cure**

Gene sequencing results were available from pretreatment samples of 74 patients. Sixty were successfully sequenced for mutations in the 23S rRNA gene, 38 for mutations in the L22 ribosomal gene, 33 for mutations in the L4 ribosomal gene, and 43 for fluoroquinolone resistance mutations.

**23S Macrolide Resistance Mutations**

Of the 60 samples sequenced for 23S mutations, 6 (10%) had wild-type sequences, 24 (40%) had a known mutation at the 2058 position, and 30 (50%) had a known mutation at the 2059 position (*Escherichia coli* numbering). In all 6 patients without 23S mutations, pristinamycin cured infection, whereas it cured infection in only 36 (67% [95% CI 53%–79%]; p = 0.17) of the 54 patients with 23S mutations. We found no significant difference in proportions cured between those with 2058 and 2059 mutations (58% vs. 73%; p = 0.26) (Table 3). Other mutations were not associated with specific treatment outcomes (Table 4).

**L22 Ribosomal Gene Mutations**

We identified 3 mutations in the 38 infections where the L22 ribosomal gene could be sequenced, but only 1 led to an amino acid change, introducing a stop codon at position Q144 and shortening the protein by 1 amino acid, and none were significantly associated with treatment failure (Table 3). Of the 57 patients for whom sequences were available for L22 mutations and for 23S mutations, L22 mutations were more often co-detected in samples with the 2058 mutation (65%) than in samples with the 2059 mutation (24%) or in 23S wild-type samples (60%) (p<0.01).

**L4 Ribosomal Gene Mutations**

Of 33 isolates sequenced, 9 mutations were found in the L4 ribosomal protein gene (Table 4), but only 3 led to an amino acid change (P32S, E56Q, and N172S).

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**Table 2. Mycoplasma genitalium** infections among 114 patients cured after 10 days of pristinamycin treatment, Melbourne Sexual Health Centre, Melbourne, Victoria, Australia, 2012–2016

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Pristinamycin failure, no. (%)</th>
<th>Cured, no. (% 95% CI)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>29 (25)</td>
<td>85 (75, 66–82)</td>
<td></td>
</tr>
<tr>
<td>Dosage regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pristinamycin 2 g/d</td>
<td>2 (22)</td>
<td>7 (78, 40–97)</td>
<td>0.91</td>
</tr>
<tr>
<td>Pristinamycin 3 g with doxycycline 200 mg/d</td>
<td>14 (26)</td>
<td>40 (74, 60–85)</td>
<td></td>
</tr>
<tr>
<td>Pristinamycin 4 g/d</td>
<td>13 (25)</td>
<td>38 (75, 60–86)</td>
<td></td>
</tr>
<tr>
<td>Site of infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urethral infection, M</td>
<td>22 (29)</td>
<td>55 (71, 60–81)</td>
<td>0.20</td>
</tr>
<tr>
<td>Anorectal infection</td>
<td>4 (14)</td>
<td>24 (86, 67–96)</td>
<td></td>
</tr>
<tr>
<td>Patient sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3 (27)</td>
<td>8 (73, 39–94)</td>
<td>1.0</td>
</tr>
<tr>
<td>M</td>
<td>26 (25)</td>
<td>77 (75, 65–83)</td>
<td></td>
</tr>
<tr>
<td>Patient signs/symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>28 (29)</td>
<td>70 (71, 61–80)</td>
<td>0.07</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>1 (6)</td>
<td>15 (94, 70–100)</td>
<td></td>
</tr>
</tbody>
</table>

*The 3 dosage regimens were compared by nonparametric test for trend. Fisher exact test used for other variables.

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Figure 2. *Mycoplasma genitalium* bacterial loads (log_{10}) and treatment outcomes, Melbourne Sexual Health Centre, Melbourne, Victoria, Australia, 2012–2016. A) *M. genitalium* load compared in urine (n = 67), rectal swab (n = 26), and cervical swab (n = 3) samples. For urine vs. rectal samples, p = 0.56; for urine vs. cervical samples, p = 0.70. B) Comparison of pretreatment *M. genitalium* loads in infections not cured (n = 26) and cured (n = 71) by pristinamycin, p<0.01. C) Comparison of *M. genitalium* loads in pretreatment and posttreatment samples from cases in which pristinamycin failed (n = 26), p<0.001. Box plots indicate 25th percentile (bottom of box), 75th percentile (top of box), median (horizontal line within box), and range (whiskers). Dots represent outlying individual observations. Dots under the error bars indicate individual outliers.
no significant association between any individual or collective L4 ribosomal mutations and treatment outcomes.

We found mutations associated with fluoroquinolone resistance in 8 (19% [95% CI 8%–33%]) of 43 sequenced isolates. As expected, they were not associated with pristinamycin failure (p = 0.61).

Predictors of Pristinamycin Failure

Bacterial load was the only significant factor associated with pristinamycin failure in univariate analysis; adherence, sexuality, or number of prior antimicrobial drugs were not associated with failure (Table 3). Prior prescription of moxifloxacin, doxycycline, or both did not affect outcome. The presence of symptoms was not associated with bacterial load (p = 0.67), and when both symptoms and bacterial load were included in a multivariate analysis, bacterial load was the only significant predictor of failure (adjusted OR 1.9 [95% CI 1.2–2.9]; p<0.01).

Adverse Events

Among the 60 patients treated with pristinamycin without doxycycline, side effects were not common: 3 patients reported diarrhea, 2 reported nausea, and 1 reported headache. Of 54 patients treated with pristinamycin and doxycycline, side effects also were uncommon: 4 reported nausea, vomiting, or abdominal pain, and 2 had candidiasis. Overall, 8 (7% [95% CI 3%–13%]) patients treated with either regimen experienced gastrointestinal side effects.

Table 3. Characteristics associated with pristinamycin failure in *Mycoplasma genitalium* infections, Melbourne Sexual Health Centre, Melbourne, Victoria, Australia, 2012–2016*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cured, no. (%)</th>
<th>Failure, no. (%)</th>
<th>Unadjusted OR for failure (95% CI)</th>
<th>p value</th>
<th>Adjusted OR for failure (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>15 (94)</td>
<td>1 (6)</td>
<td>Reference</td>
<td>0.09</td>
<td>4.1 (0.5–35.9)</td>
<td>0.20</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>70 (71)</td>
<td>28 (29)</td>
<td>6.0 (0.8–47.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherence to treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missed no doses</td>
<td>44 (72)</td>
<td>17 (28)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missed any doses</td>
<td>5 (71)</td>
<td>2 (29)</td>
<td>1.0 (0.2–5.9)</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. antimicrobial drugs before pristinamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1</td>
<td>62 (73)</td>
<td>23 (27)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>20 (80)</td>
<td>5 (20)</td>
<td>0.7 (0.2–2.0)</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sexuality</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men who have sex with men</td>
<td>52 (80)</td>
<td>13 (20)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td>25 (66)</td>
<td>13 (34)</td>
<td>2.1 (0.8–5.1)</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial load, all samples†</td>
<td>NA</td>
<td>NA</td>
<td>1.9 (1.3–2.9)</td>
<td>&lt;0.01</td>
<td>1.9 (1.2–2.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>23S known macrolide resistance mutation‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>6 (100)</td>
<td>0</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation at 2058 or 2059</td>
<td>36 (67)</td>
<td>18 (33)</td>
<td>3.9 (0.52–∞)</td>
<td>0.17§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excluding wild-type cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position 2059</td>
<td>22 (73)</td>
<td>8 (27)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position 2058</td>
<td>14 (58)</td>
<td>10 (42)</td>
<td>2.0 (0.6–6.2)</td>
<td>0.25¶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S G2162T†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>21 (66)</td>
<td>11 (34)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>5 (83)</td>
<td>1 (17)</td>
<td>0.38</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S T2185G‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>23 (74)</td>
<td>8 (26)</td>
<td>3.8 (0.70–21.0)</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3 (43)</td>
<td>4 (57)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S additional G between positions 2212 and 2213‡</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Absent</td>
<td>24 (67)</td>
<td>12 (33)</td>
<td>NA</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2 (100)</td>
<td>0</td>
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</tr>
<tr>
<td>23S G2362A‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>23 (72)</td>
<td>9 (28)</td>
<td>2.6 (0.40–15.1)</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4# A515G N172S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>17 (68)</td>
<td>8 (32)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td>2.1 (0.4–10.7)</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L22# C430T Q144 stop codon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>23 (68)</td>
<td>11 (32)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>2.1 (0.26–16.9)</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The L4 and L22 mutations result in amino acid changes. Two ribosomal L4 gene mutations were not analyzed because only 1 case was identified for each mutation C94T (P32S) and G166C (E56Q). NA, not applicable; OR, odds ratio.
†OR for each log_{10} increase in pretreatment bacterial load in 67 urine samples, 26 rectal swab samples, and 3 cervical swab samples.
‡*Escherichia coli* numbering. 23S mutations at 2058 and 2059 are established causes of macrolide resistance.
§Exact logistic regression.
¶p value for comparison of proportions cured with mutations at 2058 vs 2059, after exclusion of wild-type cases.
#M. genitalium numbering based on reference genome accession number L43967.2.
Discussion

Our investigation found that pristinamycin cured *M. genitalium* infections in 75% of patients who were unsuccessfully treated with azithromycin; in 17% of this population, moxifloxacin had failed to cure infection. The effectiveness of pristinamycin was the same when given at a dose of 1 g 4 times/day as when given as 1 g 3 times/day in combination with doxycycline at 100 mg 2 times/day. Higher pretreatment bacterial load was associated with treatment failure; the odds of failure increased almost 2-fold with each 1 log<sub>10</sub> increase in load. Failed treatment reduced bacterial load by a mean 1.3 log<sub>10</sub>. Almost all of this population had macrolide-resistant infections, precluding assessment of the effect of any 23S ribosomal gene mutation on treatment response, but the site of MRM (2058 vs. 2059) and ribosomal protein gene mutations were not associated with treatment outcomes.

Pristinamycin was well-tolerated with a low rate (7%) of reported side effects, but in this large case series, it did not perform better than moxifloxacin (89% in a recent meta-analysis) for macrolide-resistant *M. genitalium* infection (28). Given the lack of alternatives, pristinamycin remains an option during pregnancy and in other situations where fluoroquinolones have failed or are contraindicated.

Our study has some limitations. Because it was an evaluation in a clinical service and not a trial with controls, the documentation of reinfection risk and treatment adherence is not as complete. Nevertheless, because previous treatments had failed and pristinamycin is not approved to treat *M. genitalium* infections, sexual health physicians at MSHC were asked to be vigilant for these factors, and when documented (60%–80% of records), neither reinfection risk nor treatment adherence proved significant. Further limitations are that the combination of doxycycline with the 3 g/day dose and the few patients who received 2 g/day limit our power to compare the efficacies of different doses.

The only other report of the efficacy of pristinamycin in patients with *M. genitalium* infection was a series of 6 patients at MSHC in whom azithromycin and moxifloxacin treatment had failed (12). These patients were treated with pristinamycin at a dose of 1 g 4 times/day for 10 days at MSHC in 2013, and all 6 infections were cured (100% [97.5% 1-sided CI 54%–100%]). In contrast, infections were cured in 75% of the 51 patients in our study who received this regimen. The initial 6 cures are consistent with 25% therapy failure in our study when the CIs are considered.

Pristinamycin is listed as a third choice in the 2016 European guideline on *M. genitalium*, and our findings do not encourage any stronger recommendation (5). No oral alternatives to pristinamycin are available, but 1 case of *M. genitalium* was cured by 1 week of daily injected spectinomycin, which, if available, is a less convenient option (29).
Given initial treatment successes with pristinamycin and promising in vitro data, we did not expect treatment to fail in 25% of macrolide-resistant *M. genitalium* infections. The reasons for this percentage of failures are not clear and did not appear related to adherence or reinfection, but higher bacterial load was strongly associated with pristinamycin failure. This finding has been observed in studies of the closely related macrolides, which show that higher load infections are more likely to fail treatment and lead to detection of resistance mutations (11,30). Higher load infections might be more likely to harbor heterotypic resistance within a subpopulation of organisms.

Antimicrobial susceptibility testing at the Statens Serum Institute (Copenhagen, Denmark; J.S. Jensen, unpub. data) indicated for both macrolide-resistant (n = 17) and susceptible (n = 23) *M. genitalium* strains, MICs were all susceptible (defined as <1 mg/L) (31). However, these MICs were close to the expected breakpoint, so we used the maximum dose of 1 g 4 times/day for 10 days. Macrolide-resistant strains had significantly higher MIC$_{90}$ (0.50 mg/L) for pristinamycin than susceptible strains (MIC$_{90}$ 0.125 mg/L; p = 0.003) but remained within the susceptible range (32). MRMs are known to confer resistance to the streptogramin B component of pristinamycin (18), and mutations in position 2058 in *M. genitalium* have been associated with a higher MIC against macrolides, such as solithromycin (33), and in *M. pneumoniae* with erythromycin (34). Collectively, our data showing all 6 patients with no 23S MRM were cured, the MIC data, and the finding that infections were cured in 58% of patients with 2058 mutations, compared with 73% with 2059 mutations (p = 0.26), may indicate that 23S mutations influence the efficacy of pristinamycin and that there may be a differential effect of 2058 versus 2059 mutations in *M. genitalium*; however, this possibility requires further study.

Of the L4 and L22 ribosomal gene mutations present, only 1 of each resulted in an amino acid change. The L4 mutations detected were not close to the L4 loop near position 69 (*M. genitalium* numbering, position 66 *E. coli* numbering) known in other bacteria to cause macrolide resistance. By in vitro selection of resistance with subinhibitory concentrations of pristinamycin in the closely related *M. pneumoniae*, variable numbers of G insertions in position G60 (G59 in *M. genitalium*) was the only mechanism identified for pristinamycin resistance. These insertions occurred after a high number of passages suggesting a low potential for selection of resistance with pristinamycin (20). We identified no similar mutations in our dataset. The combination of doxycycline and pristinamycin was also no more effective than pristinamycin monotherapy, despite in vitro data suggesting a small additional effect of the combination, although not true synergy (J.S. Jensen, unpub. data).

We do not have an effective, safe, inexpensive oral drug for treating *M. genitalium* now that macrolide resistance is so prevalent. Fluoroquinolones are potentially toxic and vulnerable to increasing resistance. As our results indicate, pristinamycin cures 75% of macrolide-resistant infections with a course of up to 80 tablets; its price and availability vary around the world, but it may play a role where quinolones are contraindicated or ineffective. Two findings are consistent across several studies: 1) infections with lower bacterial loads are more likely to be cured (even in the presence of resistance) and 2) failed treatment with pristinamycin and azithromycin reduces bacterial load (17). These data highlight the urgent need for further work to determine the activity of new and existing antimicrobial drugs, and combinations of antimicrobial drugs, against this neglected infection.

**Acknowledgment**

We thank Jimmy Twin for his contribution to the laboratory work included in this paper.

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**About the Author**

Dr. Read is a sexual health physician at Melbourne Sexual Health Centre and a research fellow at Monash University, Melbourne. His research interests include *M. genitalium*, urethritis, and HIV prevention.

**References**


Address for correspondence: Tim R.H. Read, Monash University Central Clinical School, Melbourne Sexual Health Centre, 580 Swanston St, Melbourne, VIC 3053, Australia; email: tread@mshc.org.au
We assessed the effect of information sources on Ebola-specific knowledge and behavior during the 2014–2015 Ebola virus disease outbreak in Sierra Leone. We pooled data from 4 population-based knowledge, attitude, and practice surveys (August, October, and December 2014 and July 2015), with a total of 10,604 respondents. We created composite variables for exposures (information sources: electronic, print, new media, government, community) and outcomes (knowledge and misconceptions, protective and risk behavior) and tested associations by using logistic regression within multilevel modeling. Exposure to information sources was associated with higher knowledge and protective behaviors. However, apart from print media, exposure to information sources was also linked to misconceptions and risk behavior, but with weaker associations observed. Knowledge and protective behavior were associated with the outbreak level, most strongly after the peak, whereas risk behavior was seen at all levels of the outbreak. In future outbreaks, close attention should be paid to dissemination of information.

West Africa detected its first case of Ebola virus disease (EVD) in March 2014 within the forest region of Guinea; shortly thereafter, Liberia and Sierra Leone detected cases of the disease (1). More than 28,600 confirmed, probable, and suspected cases of EVD in Guinea, Sierra Leone, and Liberia led to >11,300 deaths by the time the World Health Organization declared the epidemic over (1). Global health security relies on all countries having the capacity to rapidly detect and control public health threats at their source. Accurate and timely risk communication efforts are essential for effective disease control efforts, and therefore for global health security. A key aspect of the Ebola response was to engage and educate the public on preventing transmission and seeking early medical care (2). In Sierra Leone, the Ministry of Health and Sanitation established a social mobilization pillar in June 2014 for the development and coordination of community engagement strategies to contain the spread of EVD (3).

The EVD epidemic dominated headlines around the world (4). Mass and social media are believed to have played a role in disseminating incorrect information, which could have influenced EVD knowledge and health-seeking behavior (5). In Sierra Leone, 88% of persons surveyed reportedly received information about EVD through the radio, which was the preferred means of receiving EVD information (6). The influence of media on disease or health outcomes has previously been studied in different contexts (7–9). In Burkina Faso, a large trial examined the influence of radio campaigns on child mortality (10). Midline results from the trial reported moderate improvement in seeking care for diarrhea-related diseases (11).

Apart from media, information sources such as educational campaigns and community leaders are thought to be essential during an infectious disease outbreak (12). In Sierra Leone, 41% of survey respondents received information about EVD through religious leaders in the initial phase of the EVD outbreak (6). Mathematical models have shown that accurate reporting may slow down the initial phase of an infectious disease outbreak and could lower the number of cases during the peak of an outbreak, but inaccurate reporting could lead to an increase in the number of cases (13). Furthermore, awareness and knowledge during an epidemic are attributable to a wide availability of information, highlighting “the crucial role of mass media and educational campaigns” (14). However, mathematical models are limited in that they represent an ideal, standardized situation and will never be better than the variables included in the model. Knowledge, attitude, and practice (KAP) surveys are an established way of empirically measuring persons’
knowledge and self-reported behavior in relation to a disease (15). A KAP survey administered in Liberia, for instance, found that even though awareness about EVD was high, knowledge was poor (16). In Guinea, 96% of respondents had heard of EVD, but only 36% had comprehensive knowledge (accepting 3 methods of prevention and rejecting 3 common misconceptions) about the disease (17). A study in Nigeria revealed that only half of the respondents knew that EVD was caused by a virus (18). All studies recognize that media and other information sources could play a crucial role in enhancing EVD knowledge (16–18).

In Sierra Leone, the country with the most EVD cases, the roles that different types of information sources played in influencing knowledge and behavior during the EVD outbreak remain unknown. If, and to what extent, knowledge plays a mediating role in changing behavior, especially in an infectious disease outbreak, is debated (19,20). Furthermore, the level of outbreak, or the intensity of an outbreak, is thought to have an effect on knowledge and behavior (21), but this factor has not yet been studied in Sierra Leone. Therefore, we assessed the effects of different information sources on Ebola-specific knowledge and behavior and how the level of the outbreak affected these factors.

Methods

We administered 4 KAP surveys from August 2014 through July 2015 in Sierra Leone (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/2/17-1028-Techapp1.pdf). In all KAP surveys, we applied multistage cluster sampling. KAP 1 was conducted in 9 districts from all 4 regions of Sierra Leone. KAPs 2–4 (October 2014, December 2014, and July 2015) covered all 14 districts in the country. The 2004 Sierra Leone Population and Housing Census List of Enumeration Areas (http://www.sierra-leone.org/Census/ssl_final_results.pdf) served as the sampling frame for random selection of enumeration areas from the districts. Within enumeration areas, we used the random walk method, a form of systematic random sampling, to select households. In each selected household, we conducted 2 interviews: first with the head of the household, and second with either a woman or a young person 15–24 years of age who was randomly selected. All 4 KAP surveys were designed to produce national- and regional-level estimates at a 95% confidence level within a 2.5% margin of error for national estimates and a 3.5% margin of error for regional estimates (online Technical Appendix Table 1).

We captured exposure to a source of information in the KAP surveys with the question: “Through what means/ways did you learn about Ebola?” Categories were not mutually exclusive; data collectors ticked “yes” in all boxes that applied. Furthermore, more response options were included in later versions of the KAP surveys. Five categories reflected the different information sources: electronic media (radio and television); print media (newspapers, brochures, and other print materials); new media (mobile phones, text messages, and internet); government (house visits by health workers); and community (religious and traditional leaders, megaphone public announcements, community meetings, friends, and relatives). We categorized number of exposures into 0–1 source, 2, 3, and 4–5 sources. The sample of respondents exposed to 0 or 5 sources was low (n = 38 for 0 sources and n = 265 for 5 sources). Therefore, we combined 0 with 1 and 5 with 4. The category “level of outbreak” was broken down into before the peak, peak, after peak, and no transmission for each region. We consulted World Health Organization data to create this variable for every region in all 4 KAP surveys (1) (online Technical Appendix Table 2).

We tested Ebola-specific knowledge as both an outcome of exposure to information and a mediating factor in the association between information exposure and behavior (online Technical Appendix Figure 2). We assessed Ebola-specific knowledge in the KAP surveys with 2 open-ended and 5 closed-ended questions, resulting in 2 scores, 1 for knowledge and 1 for misconception (online Technical Appendix Table 3). In the knowledge score, a maximum score of 8 points was possible in KAP 1–4. In the misconception score, 12 points was the maximum in KAP 1 and 13 points in KAP 2–4, due to an additional response option. We dichotomized the scores based on the means (22).

We measured Ebola-specific behavior by 2 open-ended questions and created 2 scores, 1 for protective behavior and 1 for risk behavior. For the protective behavior score, respondents could tally a maximum of 7 in KAP 1; in KAP 2–4, the maximum was 9. The risk behavior score had a maximum of 9 points in KAP 1 and 11 in KAP 2–4. We again dichotomized the scores based on the means.

Working with information from previous KAP survey analyses (17,18), we chose the following covariates as potential confounders: gender (male, female); age (13–20, 21–35, 36–49, ≥50 years); education (no education, primary education, and secondary and above); religion (Islam, Christianity); and region (Northern Province, Eastern Province, Southern Province, Western Area).

Statistical Analyses

To account for the high correlation between cases over time and region, we applied multilevel modeling (23). We pooled all data from the 4 KAP surveys and created clusters that grouped the specific KAP survey and the district. In KAP 1, there were 9 districts, and in KAP 2–4 there were 14 districts, for a total of 51 clusters. We collected
samples proportionally to the district size in the population, and the response rate was 98%. Therefore, the estimators were considered unbiased, and we did not apply the original survey weights. We envisioned the same approach for creating clusters for the analyses regarding the intensity of the outbreak, but because of the high correlation of the level of outbreak variable with the time point of the KAP survey ($r = 0.82$), the cluster level included only districts. We estimated associations with odds ratios (ORs) and their corresponding 95% CIs. To test whether knowledge played a mediating role on the outcome, we performed mediation analyses using the mediated effect model (24). We obtained the $\beta$ coefficients for A and B (online Technical Appendix Figure 2) from the fully adjusted models tested in the multilevel modeling, multiplied them, and calculated SEs. Statistical significance of the mediated effect was determined with the $\chi^2$ distribution within 1 df. We used SPSS version 22 (Armonk, NY, USA) for the analyses and set $\alpha$ to 0.05 for statistical significance.

The Sierra Leone Research and Scientific Review Committee granted ethics permission for all KAP surveys. The KAP 2–4 assessments were further reviewed and approved by the US Centers for Disease Control and Prevention.

**Results**

The overall response rate of the 4 KAP surveys was 98%. The pooled sample of KAP 1–4 consisted of 10,604 respondents. Because of missing values for some variables, we excluded 95 respondents (0.9%), making the total study sample 10,509. The age distribution was similar in the different regions, with those 21–35 years of age representing the largest group (Table 1). The total sample had an even distribution of men and women (49.1% men, 50.9% women). In all regions apart from the Northern Province, most of the sample had at least primary schooling. Two thirds of the respondents were affiliated with Islam (67.3%). EVD messages were received mostly through electronic media (94.1%) and community sources (59.5%) (Table 2); a substantially smaller portion were exposed to print (8.7%) and new media (15.5%). Government information campaigns reached 47.7% of respondents. Most respondents were exposed to $\geq 2$ different types of information sources (72.6%).

**Knowledge and Protective Behavior Scores**

Exposure to any type of information sources was statistically significantly associated with increased knowledge in both the crude and adjusted models (Table 2). Electronic media showed the strongest association in the adjusted model (adjusted OR [aOR] 1.75, 95% CI 1.46–2.09). Strong associations could also be seen between all types of information sources and protective behavior in all adjusted models (Table 3). New media showed the strongest association with protective behavior (aOR 2.15, 95% CI 1.87–2.48). We identified a clear dose-response association between number of sources and knowledge (4–5 sources aOR 3.83, 95% CI 3.17–4.61) and protective behavior (4–5 sources aOR 6.77, 95% CI 5.53–8.28).

**Misconceptions and Risk Behavior**

All sources of information apart from print media were significantly associated with misconceptions; however, the point estimates were substantially lower than in the knowledge models (Table 4). After adjusting for confounders, information from electronic media showed the strongest association with misconceptions (aOR 1.42, 95% CI 1.18–1.70). In the models testing the association between information sources and risk behavior, results for electronic and print media were not significant (Table 5). New media, governmental campaigns, and community sources did have an association, with community sources showing the strongest association with misconceptions (aOR 1.34, 95% CI 1.22–1.47).

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**Table 1.** Demographics of respondents to Ebola knowledge, attitude, and practice surveys, by region, Sierra Leone, 2014–2015

<table>
<thead>
<tr>
<th>Category</th>
<th>Northern Province</th>
<th>Eastern Province</th>
<th>Southern Province</th>
<th>Western Area</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15–20</td>
<td>854 (21.2)</td>
<td>505 (24.4)</td>
<td>440 (23.4)</td>
<td>539 (21.3)</td>
<td>2,338 (22.2)</td>
</tr>
<tr>
<td>21–35</td>
<td>1,392 (34.6)</td>
<td>706 (34.1)</td>
<td>657 (35.0)</td>
<td>930 (36.7)</td>
<td>3,685 (35.1)</td>
</tr>
<tr>
<td>36–49</td>
<td>909 (22.6)</td>
<td>476 (23.0)</td>
<td>416 (22.2)</td>
<td>629 (24.8)</td>
<td>2,430 (23.1)</td>
</tr>
<tr>
<td>$&gt;50$</td>
<td>869 (21.6)</td>
<td>384 (18.5)</td>
<td>365 (19.4)</td>
<td>438 (17.2)</td>
<td>2,056 (19.6)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>2,112 (52.5)</td>
<td>972 (46.9)</td>
<td>898 (47.8)</td>
<td>1,182 (46.6)</td>
<td>5,163 (49.1)</td>
</tr>
<tr>
<td>F</td>
<td>1,912 (47.5)</td>
<td>1,100 (53.1)</td>
<td>980 (52.2)</td>
<td>1,354 (53.4)</td>
<td>5,346 (50.9)</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No education</td>
<td>1,755 (43.6)</td>
<td>758 (36.6)</td>
<td>496 (26.4)</td>
<td>508 (20.0)</td>
<td>3,517 (33.5)</td>
</tr>
<tr>
<td>Primary</td>
<td>733 (18.2)</td>
<td>488 (23.6)</td>
<td>387 (19.5)</td>
<td>371 (14.7)</td>
<td>1,959 (18.6)</td>
</tr>
<tr>
<td>Secondary and above</td>
<td>1,536 (38.2)</td>
<td>825 (39.8)</td>
<td>1,015 (54.1)</td>
<td>1,657 (65.3)</td>
<td>5,033 (47.9)</td>
</tr>
<tr>
<td><strong>Religion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Islam</td>
<td>3,318 (82.5)</td>
<td>1,320 (63.7)</td>
<td>1,104 (58.8)</td>
<td>1,335 (52.6)</td>
<td>7,077 (67.3)</td>
</tr>
<tr>
<td>Christianity</td>
<td>706 (17.5)</td>
<td>751 (36.3)</td>
<td>774 (41.2)</td>
<td>1,201 (47.4)</td>
<td>3,432 (32.7)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>4,024</td>
<td>2,071</td>
<td>1,878</td>
<td>2,538</td>
<td>10,509</td>
</tr>
</tbody>
</table>
Knowledge as Mediator

Knowledge played a mediating role in the association between all different information sources and protective behavior. In the analyses for risk behavior, all information sources apart from print media demonstrated mediation through misconceptions (Table 6). Electronic media had no direct link with risk behavior but still had an effect on risk behavior by influencing misconceptions.

Level of Outbreak

Knowledge was significantly associated with all levels of the outbreak; with higher transmission, more respondents showed higher knowledge, although at no transmission of EVD, the association was weak (Table 7). Protective behavior also increased with the level of outbreak, but at the point of no transmission, the association decreased and was no longer significant (aOR 1.14, 95% CI 0.92–1.42). Misconceptions seemed

### Table 2. Association between information exposure and knowledge among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (% respondents)</th>
<th>Crude OR (95% CI)</th>
<th>p value†</th>
<th>Adjusted OR‡ (95% CI)</th>
<th>p value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source of information</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electronic media</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9,894 (94.1)</td>
<td>1.84 (1.54–2.19)</td>
<td>&lt;0.001</td>
<td>1.75 (1.46–2.09)</td>
<td>&lt;0.001</td>
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<td>No</td>
<td>615 (5.9)</td>
<td>1.0 (Reference)</td>
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<td>1.0 (Reference)</td>
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<tr>
<td>Print media</td>
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<tr>
<td>Yes</td>
<td>918 (8.7)</td>
<td>1.96 (1.67–2.31)</td>
<td>&lt;0.001</td>
<td>1.47 (1.24–1.75)</td>
<td>&lt;0.001</td>
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<tr>
<td>No</td>
<td>9,591 (91.3)</td>
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<td>1.0 (Reference)</td>
<td></td>
</tr>
<tr>
<td>New media</td>
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</tr>
<tr>
<td>Yes</td>
<td>1,627 (15.5)</td>
<td>1.91 (1.68–2.17)</td>
<td>&lt;0.001</td>
<td>1.41 (1.23–1.61)</td>
<td>&lt;0.001</td>
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<td>No</td>
<td>8,882 (84.5)</td>
<td>1.0 (Reference)</td>
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<tr>
<td>Government</td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>5,011 (47.7)</td>
<td>1.77 (1.62–1.94)</td>
<td>&lt;0.001</td>
<td>1.56 (1.42–1.71)</td>
<td>&lt;0.001</td>
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<td>No</td>
<td>5,498 (52.3)</td>
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<td></td>
<td>1.0 (Reference)</td>
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<tr>
<td>Community</td>
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<tr>
<td>Yes</td>
<td>6,248 (59.5)</td>
<td>1.63 (1.49–1.78)</td>
<td>&lt;0.001</td>
<td>1.44 (1.31–1.59)</td>
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<td>4,261 (40.5)</td>
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<td><strong>No. exposures</strong></td>
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<tr>
<td>0–1 source</td>
<td>2,878 (27.4)</td>
<td>1.0 (Reference)</td>
<td></td>
<td>1.0 (Reference)</td>
<td></td>
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<tr>
<td>2 sources</td>
<td>3,408 (32.5)</td>
<td>1.41 (1.26–1.56)</td>
<td>&lt;0.001</td>
<td>1.37 (1.23–1.53)</td>
<td>&lt;0.001</td>
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<tr>
<td>3 sources</td>
<td>3,115 (29.6)</td>
<td>2.18 (1.94–2.45)</td>
<td>&lt;0.001</td>
<td>2.09 (1.86–2.35)</td>
<td>&lt;0.001</td>
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<tr>
<td>4–5 sources</td>
<td>1,108 (10.5)</td>
<td>4.39 (3.65–5.28)</td>
<td>&lt;0.001</td>
<td>3.83 (3.17–4.61)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*OR, odds ratio.
†Wald statistical p value from the multilevel model.
‡Adjusted for region, gender, age, religion, educational level, level of outbreak and all other information exposures.

### Table 3. Association between information exposure and protective behavior among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (% respondents)</th>
<th>Crude OR (95% CI)</th>
<th>p value†</th>
<th>Adjusted OR‡ (95% CI)</th>
<th>p value‡</th>
<th>Adjusted OR§ (95% CI)</th>
<th>p value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source of information</strong></td>
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<tr>
<td>Yes</td>
<td>9,894 (94.1)</td>
<td>2.17 (1.80–2.63)</td>
<td>&lt;0.001</td>
<td>2.14 (1.76–2.59)</td>
<td>&lt;0.001</td>
<td>2.00 (1.65–2.43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No</td>
<td>615 (5.9)</td>
<td>1.0 (Reference)</td>
<td></td>
<td>1.0 (Reference)</td>
<td></td>
<td>1.0 (Reference)</td>
<td></td>
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<tr>
<td>Print media</td>
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</tr>
<tr>
<td>Yes</td>
<td>918 (8.7)</td>
<td>2.43 (2.07–2.87)</td>
<td>&lt;0.001</td>
<td>1.71 (1.44–2.04)</td>
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<td>1.65 (1.38–1.97)</td>
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<td>No</td>
<td>9,591 (91.3)</td>
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<td>1.0 (Reference)</td>
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<td>1.0 (Reference)</td>
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<tr>
<td>New media</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1,627 (15.5)</td>
<td>2.98 (2.61–3.40)</td>
<td>&lt;0.001</td>
<td>2.20 (1.91–2.53)</td>
<td>&lt;0.001</td>
<td>2.15 (1.87–2.48)</td>
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<tr>
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<td>5,011 (47.7)</td>
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<td>1.79 (1.62–1.96)</td>
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<td>Yes</td>
<td>6,248 (59.5)</td>
<td>2.03 (1.85–2.22)</td>
<td>&lt;0.001</td>
<td>1.72 (1.56–1.90)</td>
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</tr>
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<tr>
<td>2 sources</td>
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<td>1.46 (1.30–1.63)</td>
<td>&lt;0.001</td>
<td>1.43 (1.28–1.60)</td>
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<td>1.38 (1.23–1.54)</td>
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<td>3.10 (2.75–3.49)</td>
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<td>3.02 (2.68–3.41)</td>
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<td>2.78 (2.46–3.14)</td>
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</table>

*OR, odds ratio.
†Wald statistical p value from the multilevel model.
‡Adjusted for region, gender, age, religion, educational level, level of outbreak, and all other information exposures.
§Adjusted for region, gender, age, religion, educational level, level of outbreak, and all other information exposures, plus knowledge and misconceptions.
**Table 4. Association between information exposure and misconceptions among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*\(^\ddagger\)**

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (%) respondents</th>
<th>Crude OR (95% CI)</th>
<th>p value‡</th>
<th>Adjusted OR‡ (95% CI)</th>
<th>p value‡</th>
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<tr>
<td>Electronic media</td>
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<tr>
<td>Yes</td>
<td>9,894 (94.1)</td>
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<td>0.005</td>
<td>1.42 (1.18–1.70)</td>
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<tr>
<td>Yes</td>
<td>918 (8.7)</td>
<td>1.03 (0.88–1.21)</td>
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<td>0.98 (0.83–1.15)</td>
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<td>1.17 (1.03–1.34)</td>
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<td>8,882 (84.5)</td>
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<tr>
<td>Government</td>
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<tr>
<td>Yes</td>
<td>5,011 (47.7)</td>
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<td>&lt;0.001</td>
<td>1.26 (1.14–1.38)</td>
<td>&lt;0.001</td>
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<tr>
<td>Yes</td>
<td>6,248 (59.5)</td>
<td>1.46 (1.33–1.60)</td>
<td>&lt;0.001</td>
<td>1.39 (1.26–1.53)</td>
<td>&lt;0.001</td>
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<td>1.0 (Reference)</td>
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</tr>
<tr>
<td><strong>No. exposures</strong></td>
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<td></td>
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</tr>
<tr>
<td>0–1 source</td>
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<td>1.0 (Reference)</td>
<td></td>
</tr>
<tr>
<td>2 sources</td>
<td>3,408 (32.5)</td>
<td>1.04 (0.94–1.17)</td>
<td>0.400</td>
<td>1.07 (0.95–1.19)</td>
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<tr>
<td>3 sources</td>
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<td>1.67 (1.47–1.88)</td>
<td>&lt;0.001</td>
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<td>4–5 sources</td>
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<td>1.73 (1.45–2.05)</td>
<td>&lt;0.001</td>
<td>1.86 (1.56–2.22)</td>
<td>&lt;0.001</td>
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</tbody>
</table>

*OR, odds ratio.  
†Wald statistical p value from the multilevel model.  
‡Adjusted for region, gender, age, religion, educational level, level of outbreak, and all other information exposures.

**Discussion**

The results of this study clearly show that exposure to different types of information sources was associated with increased knowledge and protective behaviors during the EVD epidemic in Sierra Leone. A strong dose-response association was seen between information exposure and knowledge and protective behavior. However, exposure to all information to decline during the peak (aOR 0.76, 95% CI 0.64–0.91), and had no association after the peak (aOR 1.00, 95% CI 0.86–1.16). The stage of no transmission, however, was strongly associated with misconceptions (aOR 1.42, 95% CI 1.15–1.76). For risk behavior, all associations were significant, with the strongest one being at the peak of the outbreak (aOR 1.71, 95% CI 1.43–2.04), with a declining trend over time.

**Table 5. Association between information exposure and risk behavior among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*\(^\ddagger\)**

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (%) respondents</th>
<th>Crude OR (95% CI)</th>
<th>p value‡</th>
<th>Adjusted OR‡ (95% CI)</th>
<th>p value‡</th>
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<td><strong>Type of information</strong></td>
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<tr>
<td>Electronic media</td>
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</tr>
<tr>
<td>Yes</td>
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<td>1.0 (Reference)</td>
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<td>1.23 (1.09–1.39)</td>
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<td>1.0 (Reference)</td>
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<td>1.25 (1.14–1.37)</td>
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<td>&lt;0.001</td>
<td>1.37 (1.25–1.51)</td>
<td>&lt;0.001</td>
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</tr>
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<td>1.0 (Reference)</td>
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</tr>
<tr>
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<td>1.95 (1.66–2.28)</td>
<td>&lt;0.001</td>
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</table>

*OR, odds ratio.  
†Wald statistical p value from the multilevel model.  
‡Adjusted for region, gender, age, religion, educational level, level of outbreak, and all other information exposures.  
§Adjusted for all of the above, plus knowledge and misconceptions.
Risk Communication during 2014–2015 Ebola Outbreak

Table 6. Mediation analyses for knowledge, misconceptions, protective behavior, and risk behavior among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*  

<table>
<thead>
<tr>
<th>Category</th>
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<th>Beta B (SE)</th>
<th>Beta AB (SE)</th>
<th>p value† AB</th>
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<tr>
<td>Type of information</td>
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</tr>
<tr>
<td>Electronic media</td>
<td>0.558 (0.091)</td>
<td>0.662 (0.047)</td>
<td>0.369 (0.066)</td>
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</tr>
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<td>Print media</td>
<td>0.387 (0.088)</td>
<td>0.662 (0.047)</td>
<td>0.256 (0.061)</td>
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</tr>
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<td>New media</td>
<td>0.342 (0.069)</td>
<td>0.662 (0.047)</td>
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</tr>
<tr>
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<td>0.444 (0.048)</td>
<td>0.662 (0.047)</td>
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</tr>
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<td>0.365 (0.049)</td>
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<tr>
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<td>0.317 (0.055)</td>
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<td>0.210 (0.039)</td>
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</tr>
<tr>
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<td>0.489 (0.052)</td>
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<td>0.400 (0.047)</td>
<td>0.204 (0.034)</td>
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<td>Medications for misconceptions and risk behavior</td>
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</tr>
<tr>
<td>Electronic media</td>
<td>0.350 (0.092)</td>
<td>0.400 (0.047)</td>
<td>0.140 (0.040)</td>
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<td>-0.009 (0.034)</td>
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<tr>
<td>0–1 source</td>
<td>0.063 (0.056)</td>
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<td>0.025 (0.023)</td>
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<td>0.400 (0.047)</td>
<td>0.249 (0.046)</td>
<td>&lt;0.001</td>
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</tbody>
</table>

*Beta coefficients of the adjusted ORs (adjusted for region, gender, age, religion, educational level, level of outbreak and all other information sources): A, B refer to the paths in Technical Appendix Figure 2 (https://wwwnc.cdc.gov/EID/article/24/2/17-1028-Techapp1.pdf); AB is the multiplication of A and B. OR, odds ratio.  
†χ² test with 1 df.

Table 7. Association between level of outbreak, knowledge, and behavior among respondents to Ebola knowledge, attitude, and practice surveys, Sierra Leone, 2014–2015*  

<table>
<thead>
<tr>
<th>Level of outbreak</th>
<th>No. (%)</th>
<th>Crude OR (95% CI)</th>
<th>p value†</th>
<th>Adjusted OR 1‡ (95% CI)</th>
<th>p value†</th>
<th>Adjusted OR 2§ (95% CI)</th>
<th>p value†</th>
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<tr>
<td>Before peak</td>
<td>1,095 (10.4)</td>
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<td>1.0 (Reference)</td>
<td>0.277</td>
<td>1.0 (Reference)</td>
<td>0.277</td>
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</tr>
<tr>
<td>Peak</td>
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<td>1.46 (1.23–1.72)</td>
<td>&lt;0.001</td>
<td>1.26 (1.06–1.50)</td>
<td>0.008</td>
<td>1.31 (1.10–1.56)</td>
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<tr>
<td>After peak</td>
<td>6,995 (66.6)</td>
<td>2.06 (1.80–2.36)</td>
<td>&lt;0.001</td>
<td>1.68 (1.46–1.93)</td>
<td>&lt;0.001</td>
<td>1.83 (1.59–2.11)</td>
<td>&lt;0.001</td>
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<td>No transmission</td>
<td>1,020 (9.7)</td>
<td>1.73 (1.42–2.10)</td>
<td>&lt;0.001</td>
<td>1.38 (1.13–1.69)</td>
<td>0.002</td>
<td>1.51 (1.23–1.86)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Association between level of outbreak and protective behavior</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before peak</td>
<td>1,095 (10.4)</td>
<td>1.0 (Reference)</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
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</tr>
<tr>
<td>Peak</td>
<td>1,399 (13.3)</td>
<td>1.35 (1.14–1.61)</td>
<td>0.001</td>
<td>1.18 (0.98–1.41)</td>
<td>0.077</td>
<td>1.20 (0.98–1.43)</td>
<td>0.054</td>
</tr>
<tr>
<td>After peak</td>
<td>6,995 (66.6)</td>
<td>1.67 (1.45–1.92)</td>
<td>&lt;0.001</td>
<td>1.38 (1.19–1.60)</td>
<td>&lt;0.001</td>
<td>1.46 (1.26–1.69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No transmission</td>
<td>1,020 (9.7)</td>
<td>1.50 (1.23–1.82)</td>
<td>&lt;0.001</td>
<td>1.08 (0.87–1.34)</td>
<td>0.470</td>
<td>1.14 (0.92–1.42)</td>
<td>0.229</td>
</tr>
<tr>
<td>Association between level of outbreak and misconceptions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before peak</td>
<td>1,095 (10.4)</td>
<td>1.0 (Reference)</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>1,399 (13.3)</td>
<td>0.78 (0.66–0.93)</td>
<td>0.004</td>
<td>0.77 (0.65–0.92)</td>
<td>0.004</td>
<td>0.76 (0.64–0.91)</td>
<td>0.003</td>
</tr>
<tr>
<td>After peak</td>
<td>6,995 (66.6)</td>
<td>1.13 (0.98–1.30)</td>
<td>0.004</td>
<td>1.13 (0.89–1.29)</td>
<td>0.004</td>
<td>1.00 (0.86–1.16)</td>
<td>0.994</td>
</tr>
<tr>
<td>No transmission</td>
<td>1,020 (9.7)</td>
<td>1.64 (1.33–2.02)</td>
<td>&lt;0.001</td>
<td>1.48 (1.20–1.83)</td>
<td>&lt;0.001</td>
<td>1.42 (1.15–1.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Association between level of outbreak and risk behavior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before peak</td>
<td>1,095 (10.4)</td>
<td>1.0 (Reference)</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
<td>1.0 (Reference)</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>1,399 (13.3)</td>
<td>1.75 (1.44–2.10)</td>
<td>&lt;0.001</td>
<td>1.72 (1.44–2.05)</td>
<td>&lt;0.001</td>
<td>1.71 (1.43–2.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>After peak</td>
<td>6,995 (66.6)</td>
<td>1.38 (1.14–1.67)</td>
<td>&lt;0.001</td>
<td>1.52 (1.31–1.75)</td>
<td>&lt;0.001</td>
<td>1.48 (1.28–1.71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No transmission</td>
<td>1,020 (9.7)</td>
<td>1.50 (1.23–1.82)</td>
<td>&lt;0.001</td>
<td>1.33 (1.09–1.62)</td>
<td>0.005</td>
<td>1.28 (1.05–1.56)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*OR, odds ratio.  
†Wald statistical p value from the multilevel model.  
‡Adjusted for all information exposures.  
§Adjusted for all information exposures, gender, region, age, religion, and educational level.
The results reinforce the importance of radio in Sierra Leone; electronic media (which consists mainly of radio) had the strongest association with knowledge and protective behavior but also with misconceptions. Even though electronic media were not directly linked with risk behavior, there was a mediating effect on risk behavior through misconceptions. In 2013, Sierra Leone had a literacy rate of only 46% (25), which makes radio the preferred and most accessible source of information for a large part of the population (78% have access to radio) (26). Other studies have also reported the importance of radio in promoting knowledge in low-income settings (11,27,28). Nonetheless, the influence of radio on promoting protective behavior in these studies was not as strong (11) or did not have an association at all (28). The setting of our study, an infectious disease outbreak with high death rates, might have provided more urgency and a different risk perception and behavior change pathway than other studies (20). Furthermore, as part of the EVD response, radio was used as a channel to disseminate messages from community and religious leaders (3). An additive effect of different information sources (such as community and government) coming together in radio could explain the strong associations.

The finding that both knowledge and misconceptions and both protective and risk behavior were linked to most of the information sources could be explained by the presence of contradicting messages during the outbreak. Previous studies have reported a high prevalence of misconceptions, which have led to rejection of interventions during the EVD epidemic (12,29,30). Furthermore, correct and incorrect explanations of a disease might coexist in a community. In Guinea, for instance, 83% of respondents believed that a virus caused EVD, but at the same time, 36% believed that higher powers were involved in causing the outbreak (17). This finding could explain, in part, some of the results in our study, in which community sources were most strongly associated with risk behavior. Community sources were employed mostly in areas with high EVD transmission. Selection bias could, therefore, be an explanation of the observed associations. Furthermore, the temporal relationship between community sources and the reported risk behaviors cannot be ascertained.

Print media showed a different pattern from other information sources in the models. Exposure to print media was associated with both knowledge and protective behavior but not with misconceptions and risk behavior. A reason for this finding might be that those who read newspapers and brochures differ from the general population of Sierra Leone in that they are literate and have the means to buy a newspaper. Only 11% of the population reads newspapers on a weekly basis (26). Educational level was taken into account in the analyses, but residual confounding cannot be excluded.

We found a clear dose-response association for knowledge and protective behavior. Other studies highlight this finding as well; exposure to multiple information sources increases the chances of demonstrating higher knowledge and protective behavior (31–34).

Previous studies show conflicting results as to whether knowledge is needed for behavioral change (19). From the results of our study, it seems that behavioral change can be achieved through directly influencing behavior as well as through a mediation effect of improvement in knowledge. Even though having knowledge appears to be beneficial for adopting protective behaviors, misconceptions mediate the effect of risk behavior. This connection was also seen in reality during the EVD outbreak in West Africa, where misconceptions about the virus led to sometimes violent resistance to public health measures (35).

In the analysis of the association between level of outbreak and knowledge and behavior, we found that risk behavior was prevalent during the entire course of the outbreak. In Sierra Leone, concerns were expressed about the response system, such as fears of calling the national hotline and of chlorine spraying (36). Ebola-specific information decreased in intensity after the outbreak, which might have resulted in loss of protective behavior and an increase in misconceptions. It can be assumed that repetition of messages is crucial.

In general, information dissemination during the Ebola epidemic was not unidirectional. For instance, radio channels encouraged listeners to call in and share questions. Communication channels should therefore be placed in a broader context, acknowledging the complex interactions among societal, community, and individual features, which in turn affect knowledge and behavior (37).

Major strengths of this study are the random sampling method, the large sample size, and the timing of the data collection. Even though the KAP survey instrument was not validated because of the urgency of the setting, KAPs are an established way of gathering data (15), and some of the adapted items have been validated in other contexts (38). In the surveys, some questions were asked in an open-ended manner, reducing the likelihood of bias because respondents would name only answers they actually knew. The response rate was high, which also reduced the risk of bias. Clear dose-response associations were found, strengthening the hypothesis that exposure to information sources can influence Ebola-specific knowledge and behavior. Further strengths include the possibility to adjust for potential confounders.

Limitations of the study include the fact that the data came from cross-sectional studies and that, therefore, temporality cannot be established. Consequently, directions of the found associations have to be interpreted with caution, as reverse causality cannot be ruled out. Performing mediation analyses with cross-sectional data can seem controversial because there is no way to properly measure if whether the exposure and mediator happen before the outcome.
However, because EVD was a new disease in this region of Africa, Ebola-specific knowledge was low at the start of the outbreak (29). Furthermore, various information sources were used to disseminate EVD messages at the beginning of the epidemic. It can therefore be assumed that both the exposure and the mediator came before the outcome.

Another limitation is the inherent high correlation between time and district in an infectious disease outbreak, which was addressed by applying multilevel modeling. A further drawback is that, in contrast to KAP 2–4, KAP 1 contained only 9 out of 14 districts. The results were adjusted for region and district, somewhat mitigating this limitation. Furthermore, as previous studies have shown, there is a risk that self-reported behavior might not reflect actual behavior, but in the given outbreak situation, the survey was deemed to be the most feasible method (39). Closed-ended questions could have introduced a bias for the risk behavior outcome, owing to the risk of socially desirable answers. To minimize this factor, respondents were encouraged to give honest opinions.

Misclassification of the exposure to information sources could have influenced the results. The number and types of interventions during the EVD epidemic might have made it difficult for respondents to distinguish between information exposures. For instance, lay health workers and community mobilizers might have been viewed as health workers (which was classified as government exposure), overestimating the exposure to that information source (and because of the nondifferential nature of this potential bias, underestimating the results). Last, because exposures were not mutually exclusive during the data collection, a respondent could have been exposed to more than one source of information. To address this factor, for subsequent analyses, mutually exclusive media categories were created.

In conclusion, the results of this study show the importance of information sources in influencing knowledge and behavior—in both positive and negative directions—and could be used for communication strategies in emergency preparedness and disease outbreaks in low-income settings. Our findings underscore the value of risk communication for rapid disease control efforts and, therefore, for global health security.

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About the Author

Ms. Winters is a research analyst in epidemiology, health economics, and outcomes research and has a background in journalism. She is a prospective PhD student, affiliated with the Global and Sexual Health research group at Karolinska Institutet in Stockholm, Sweden.

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Address for correspondence: Maike Winters, Karolinska Institutet—Department of Public Health Sciences, Tomtebodavägen 18a, Stockholm 17177, Sweden; email: maike.winters@hotmail.com
We compiled records on macacine herpesvirus 1 (McHV-1) seroprevalence and, during 2015–2016, collected saliva and fecal samples from the free-ranging rhesus macaques of Silver Springs State Park, a popular public park in central Florida, USA, to determine viral DNA shedding and perform sequencing. Phylogenetic analysis of the US5 and US5-US6 intragenic sequence from free-ranging and laboratory McHV-1 variants did not reveal genomic differences. In animals captured during 2000–2012, average annual seroprevalence was 25% ± 9 (mean ± SD). We found 4%–14% (95% CI 2%–29%) of macaques passively sampled during the fall 2015 mating season shed McHV-1 DNA orally. We did not observe viral shedding during the spring or summer or from fecal samples. We conclude that these macaques can shed McHV-1, putting humans at risk for exposure to this potentially fatal pathogen. Management plans should be put in place to limit transmission of McHV-1 from these macaques.

Nonhuman primates (NHPs) can harbor multiple zoonotic pathogens owing to their close phylogenetic relationship and frequent interactions with humans and can be successful ecologic invaders (1). In the United States, ≥10 NHP species have been introduced. Of these, the rhesus macaque (Macaca mulatta) has successfully established populations in Florida and Puerto Rico (2,3).

Rhesus macaques are the most frequently used NHP species in biomedical research (4). In laboratory settings, rhesus macaques are considered an occupational health threat because they harbor macacine herpesvirus 1 (McHV-1, also known as herpes B virus). Although the infection does not produce clinical illness in macaques, ~50% of infections cause fatal encephalitis in humans if left untreated (5). Transmission has typically occurred via exposure to macaque bodily fluids, including bites and scratches, although an instance of human-to-human transmission has been reported (6). Like other alphaherpesviruses, after the initial infection in macaques, McHV-1 becomes latent in the trigeminal ganglia and lumbosacral nerve. During periods of stress or immunosuppression in the macaque, the virus can reactivate into the lytic phase, at which time the virus sheds from oral, nasal, or genital mucosa without signs of clinical illness (7). In the laboratory setting, ~40% of seropositive rhesus macaques had viral DNA in the trigeminal ganglia, indicating these animals were carriers of the virus and capable of reactivation (8).

Outside of the laboratory setting, little is known about the risk for transmission or the incidence of human disease resulting from McHV-1 exposure. No human deaths have been reported from contracting McHV-1 from free-ranging macaques, suggesting the risk for transmission from these animals is low (9); however, immunologic surveillance, reporting, and diagnostic investigations in humans are lacking. Aside from characterizing the incidence in humans, the components of transmission risk from free-ranging macaques to humans have not been well characterized. Prevalence of McHV-1 antibodies in free-ranging macaque populations are highly variable (e.g., 72% in Puerto Rico, 64% in Nepal, and 5.3% in Indonesia) (10–12), and only 1 publication reports the prevalence of viral DNA shedding among captively held wild macaques (long-tailed macaques, M. fascicularis; 39%) (13). In that study, viral shedding rates of wild macaques were likely inflated because animals were held in captivity for ≤72 hours, which could have induced a stress response and therefore viral shedding. Comparisons of the genetic composition of viruses circulating in free-ranging and captive macaques are also lacking yet necessary to understand if differences in virulence or pathogenicity are evident. Estimating the frequency of virus shedding and the genetic composition of the virus is a first step toward understanding the public health risk.
In the US state of Florida, the rhesus macaque is an invasive species with a high reproductive capacity, the ability to spread in geographic distribution, and the potential to threaten native fauna with extinction (14). Macaques were introduced into Silver Springs State Park in Florida in the 1930s in an effort to increase tourism (15,16). The population was founded with ≈12 animals from an unknown source in 2 separate introduction events. The population grew to a peak of ≈400 animals in the 1980s and spread into adjacent forests along the Ocklawaha River (15). At the beginning of our investigation (2015), ≈175 rhesus macaques lived in Silver Springs State Park (3).

McHV-1 antibody production was first detected in this population in 1992 (17). Of 29 trapped animals, 12 (41%) were found to be seropositive (17). During 1984–2012, ≈1,000 animals were removed intermittently by permit-holding private trappers, but the practice of animal trapping has ended because of public controversy (3). As of December 12, 2017, no population management plans were in place.

The purpose of our study was to describe the seroprevalence of McHV-1 by using macaque samples collected by trappers during 2000–2012 in Silver Springs State Park and along the Ocklawaha River in central Florida. We further aimed to determine if McHV-1 viral DNA was being shed by free-ranging rhesus macaques passively sampled in Silver Springs State Park during 2015–2016 and to compare antigenic regions of the virus among genotypes recovered from laboratory and free-ranging animals.

Materials and Methods

Serology
During 2000–2012, private trappers collected blood samples from the femoral vein of 317 rhesus macaques along the Silver and Ocklawaha Rivers in Marion County, Florida, across 5 trapping sessions. The trappers sent samples to a commercial laboratory (BioReliance Corporation, Rockville, MD, USA) to evaluate serologic status using an McHV-1 antigen-based ELISA. We considered samples with an optical density ≥40 positive for McHV-1 antibodies. We reported age, as determined by body mass and dentition, for 213 animals. For the 213 animals for which serologic and age data were available, we used a logistic regression model to determine if age was related to serostatus using R version 3.3.3 (https://www.r-project.org/).

Saliva and Fecal Sample Collection
Human visitors to the park are most likely to be exposed to McHV-1 through contact with saliva from macaque bites and scratches (18) or from contact with virus shed through urine and feces (5); therefore, during 2015–2016, we collected saliva and fecal samples from 2 social groups of rhesus macaques in the park that were most habituated to humans (19). We collected samples during 3 seasons considered stressful for animals: in the fall of 2015 during the breeding season, in the spring of 2016 during gestation, and in the summer of 2016 during lactation. Stress has been shown to reactivate the McHV-1 virus and promote virus shedding in rhesus macaques (20), but whether seasonal stress in free-ranging animals is sufficient to induce virus shedding was not known. We collected saliva samples (n = 121) by giving 1.25-inch oral swabs (Salimetrics, State College, PA, USA) soaked in sucrose solution to the macaques. We monitored the animals as they obtained, chewed, and discarded swabs. When possible, we collected the swabs immediately after they were discarded and recorded the age and sex of the animal that handled the swab. Because multiple animals in a group were present when field personnel distributed swabs, determining which macaque deposited saliva on the oral swab was not always possible. Therefore, we used observational data to estimate a minimum and maximum number of animals sampled. We defined the minimum number of animals sampled as the number of unique animals we observed chewing on cotton swabs presented to the social group within a season. We defined the maximum number of animals sampled as the total number of cotton swabs chewed on by macaques minus the number of samples that we knew were duplicates from a single animal. For both serologic and viral shedding data, we estimated CIs for single group prevalence using Wilson approximation of the exact limits for a binomial distribution (21).

We collected fresh feces (n = 21) in the field using sterile plastic spatulas during the spring and summer of 2016. In addition, we collected fecal samples from 2 macaques during necropsies conducted by Florida Fish and Wildlife Conservation Commission veterinarians. One sample came from a 1-year-old male animal found dead in the park in March 2016 and the other from a subadult female animal struck by a car on a highway adjacent to Silver Springs State Park in July 2016. We applied a virus-inactivating buffer containing guanidine (Buffer VXL; QIAGEN, Valencia, CA, USA) to saliva swab samples and fecal samples and placed them on ice packs during collection; immediately after finishing collection, we stored samples at –80°C. In addition to these samples, 10 soil samples were collected in the field as negative controls; these samples were handled, stored, and extracted in parallel to the oral and fecal specimens obtained from live animals.

DNA Purification and Genetic Analyses
We extracted genomic DNA from 121 saliva, 23 fecal, and 10 soil samples with commercial QIAGEN kits (QIAdx cador Pathogen Mini Kit for saliva and soil, QIAdx Stool Mini Kit for feces) using the manufacturer’s protocols. To detect McHV-1 viral DNA, we performed real-time PCR (rPCR) targeting a 124-bp fragment of the glycoprotein G (gG) gene, shown to be sensitive and specific to McHV-1,
as previously described (22) using primers gGBV-323F and gGBV-446R and hydrolysis probe gGBV-403T. We purified amplicons of the US5 gene, and part of the intergenic region between the 3′ end of the US5 gene and the US6 gene. We purified amplicons of the appropriate size from each conventional PCR assay using the QIAquick PCR Purification Kit (QIAGEN) and submitted them to the Interdisciplinary Core for Biotechnology Research at the University of Florida (Gainesville, Florida, USA) for bidirectional sequencing by Sanger methods. We analyzed chromatographs using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) or FinchTV (http://en.bio-soft.net/dna/FinchTV.html) and used MEGA version 7 (http://www.megasoftware.net/) to trim unreadable ends and form consensus sequences between forward and reverse primers. We performed a phylogenetic analysis of the amino acid sequence in MEGA. We aligned using the MUSCLE algorithm and constructed phylogenetic trees using the maximum-likelihood method and the Poisson model with 1,000 bootstrap replicates (25).

Results

Serology

Of the 317 rhesus macaque blood samples, 84 were seropositive for McHV-1 (Table 2), for an annual average (± SD) of 25% (± 9%). As predicted for a directly transmitted pathogen and as previously reported for McHV-1 (26–28), exposure to the pathogen, as determined by positive serostatus, increased with age (x) (logit[p(x)] = −4.44 + 1.07x; odds ratio 2.9 [95% CI 1.74–4.83]; p = 0.0001).

Virus Shedding

Three (2.5%) of 121 oral swab specimens tested positive in triplicate for McHV-1 DNA by rPCR (Table 3). In addition, all conventional PCR assays of these samples were positive, and sequences were verified by using Sanger methods. Of the 317 rhesus macaque blood samples, 84 were seropositive for McHV-1 (Table 2), for an annual average (± SD) of 25% (± 9%). As predicted for a directly transmitted pathogen and as previously reported for McHV-1 (26–28), exposure to the pathogen, as determined by positive serostatus, increased with age (x) (logit[p(x)] = −4.44 + 1.07x; odds ratio 2.9 [95% CI 1.74–4.83]; p = 0.0001).

Table 1. PCR oligonucleotide primers and probe used in the detection of McHV-1 viral DNA in samples from rhesus macaques and soil, Silver Springs State Park, Florida, USA, 2000–2012*

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Reference</th>
<th>Sequence, 5′ → 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>gGBV-323F</td>
<td>(21)</td>
<td>TGGCTACTACCGCGTG</td>
</tr>
<tr>
<td>gGBV-446R</td>
<td>(21)</td>
<td>TGGTAAGTGGAGAGTC</td>
</tr>
<tr>
<td>gGBV-403T</td>
<td>(21)</td>
<td>(6-FAM)CGCGCTTCGACAGCATG(BHQ-1)</td>
</tr>
<tr>
<td>DFA</td>
<td>(22)</td>
<td>TCCGTGACACAGCARNYSCGNMATA</td>
</tr>
<tr>
<td>ILK</td>
<td>(22)</td>
<td>GTCTTGGTCACAGNTCAACNCCYTT</td>
</tr>
<tr>
<td>KG1</td>
<td>(22)</td>
<td>TGAATCTCCTGATAGGT</td>
</tr>
<tr>
<td>TGV</td>
<td>(22)</td>
<td>CACAGAGTCGCTTCAGTCAT</td>
</tr>
<tr>
<td>IYG</td>
<td>(22)</td>
<td>CTCCAGCGCCCGAGCAGC</td>
</tr>
<tr>
<td>HB2A</td>
<td>(8)</td>
<td>ATTCGCGCCCGCGCCAGCAGT</td>
</tr>
</tbody>
</table>
| HB2B       | (8)       | TC[AT]C[AC]GCGGTAGGTT |}

*BHQ-1, black hole quencher 1; 6-FAM, 6-fluorescein amidite; gG, glycoprotein G; McHV-1, macaque herpesvirus 1.
In our study, we found 2 genotypes of herpes B virus that varied by a single amino acid change. Both of these genotypes have been found in laboratory populations, suggesting that ≥2 different laboratory-like strains circulate in the park population of macaques. A second observation from our study was that viral shedding appeared to have temporal variation. We only observed animals shedding virus during the breeding season in the fall. This time of year is particularly stressful because male–male aggression is high; consequently, the mortality (30) and dispersal (31) rates of male animals are highest during this time of year. Indeed, 1 of the positive samples that came from an identified animal was from a subadult male. A better understanding of the demographic and temporal trends will be the key to understanding the impact of herpes B virus on invasive rhesus macaques.

### Discussion

We found evidence that invasive rhesus macaques in Silver Springs State Park shed McHV-1 DNA. Serologic results from the animals trapped during 2000–2012 were consistent with other serologic findings for free-ranging macaques. Although the average (± SD) annual seroprevalence was 25% (±9%), this number is likely an underestimate of the actual seroprevalence because trappers in later years targeted primarily younger animals, rather than a set of animals reflective of the demographic composition of the population. Among the animals for which we had age class data, younger animals had a lower seroprevalence of McHV-1 (0% for <1-year-olds) than older animals (mean ± SD 57% ± 10% for 4-year-olds and 75% ± 23% for ≥5-year-olds). These data suggest that a substantial portion of the population are likely carriers for McHV-1 and capable of reactivation and viral shedding.

We detected McHV-1 DNA in macaque samples only during the fall (breeding season) of 2015 and not during the spring (gestation period) or summer (lactation period) of 2016 (Table 3). On the basis of the estimated minimum and maximum number of animals sampled, shedding occurred in a minimum of 7% (95% CI 2%–22%) and a maximum of 30% (95% CI 11%–60%) of the animals in 1 of 2 social groups sampled during this time period. For both of the sampled groups combined, the percentage of animals shedding in the fall was a minimum of 4% (95% CI 1%–14%) and a maximum of 14% (95% CI 5%–35%) of all animals sampled in the fall. We did not find evidence of viral shedding in feces (n = 23), and all environmental controls (n = 10) tested negative for McHV-1.

### Table 2. Seroprevalence of McHV-1 in rhesus macaques, Silver Springs State Park, Florida, USA, 2000–2012*

<table>
<thead>
<tr>
<th>Year sample collected and animal age, y</th>
<th>No. samples</th>
<th>No. seropositive</th>
<th>% Seropositive (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>2</td>
<td>0</td>
<td>0 (0–66)</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>0</td>
<td>0 (0–16)</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>1</td>
<td>11 (2–43)</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>2</td>
<td>18 (5–48)</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>7</td>
<td>64 (35–85)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>28</td>
<td>19</td>
<td>68 (49–82)</td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>3</td>
<td>0</td>
<td>0 (0–56)</td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>0</td>
<td>0 (0–15)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1</td>
<td>20 (4–62)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0 (0–80)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0 (0–80)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>18</td>
<td>10</td>
<td>56 (34–75)</td>
</tr>
<tr>
<td>Unknown</td>
<td>32</td>
<td>18</td>
<td>56 (39–72)</td>
</tr>
</tbody>
</table>

*The annual average seroprevalence was 25% ± 9% (mean ± SD). McHV-1, macaque herpesvirus 1; NA, not applicable.

### Table 3. Shedding of McHV-1 in rhesus macaque saliva samples collected using 121 oral swabs and quantified on the basis of observational data and rPCR positivity for McHV-1 DNA, by social group, by season, Silver Springs State Park, Florida, USA, 2015–2016†

<table>
<thead>
<tr>
<th>Season</th>
<th>Group 1</th>
<th></th>
<th></th>
<th>Group 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum no. sampled</td>
<td>Maximum no. sampled</td>
<td>No. rPCR positive</td>
<td>Minimum no. sampled</td>
<td>Maximum no. sampled</td>
<td>No. rPCR positive</td>
</tr>
<tr>
<td>Fall 2015, breeding season</td>
<td>11</td>
<td>18</td>
<td>0</td>
<td>10</td>
<td>29</td>
<td>3†</td>
</tr>
<tr>
<td>Spring 2016, gestation period</td>
<td>3</td>
<td>13</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Summer 2016, lactation period</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>26</td>
<td>0</td>
</tr>
</tbody>
</table>

*MHV-1, macaque herpesvirus 1; rPCR, real-time PCR.

†These 3 saliva samples represent 2 or 3 unique macaques. We estimated a conservative prevalence of 7% (95% CI 2%–22%) assuming the 3 positive oral swabs came from 2 animals and 9 swabs were sampled. Taking the least conservative approach, we estimated a prevalence of 30% (95% CI 11%–60%) if the 3 swabs came from 3 unique animals and only 10 animals were sampled.
of viral shedding by macaques in the park is needed to quantify the risk of exposure to McHV-1 among humans.

During 1977–1984, the Florida Fish and Wildlife Commission documented at least 23 bites that occurred during human-macaque incidents near or in Silver Springs State Park and resulted in human injury (17), but this rate is likely an underestimate of the number of bites or scratches that occur each year. Boaters on the Silver River frequently feed the macaques (0.68 provisioning events/h) at close distances (<2 m) (32), which creates a high potential for human-macaque encounters and appears to alter the movement of the macaques. Animals spend more time in close proximity to the Silver River on weekends, when boater traffic is highest (32), than on weekdays (19). This human behavior probably exacerbates the public health threat of the rhesus macaque population to park visitors because incidents of negative human-macaque interactions increase when macaques are fed by humans (33). In 2016 and 2017, public areas of the park were closed on multiple occasions because of the aggressive behavior of these macaque groups (personal observation by C.J.A.).

Although the potential for transmission of virus to humans clearly exists in Silver Springs State Park, no human infections or deaths caused by McHV-1 from free-ranging animals have been reported, despite frequent human-macaque interactions among many macaque populations worldwide. All documented cases of human contraction of and death from McHV-1 have been associated with captive animals within laboratory settings. Multiple explanations exist for these paradoxical observations, and none are mutually exclusive. First, multiple strains of the virus might circulate, and the strains present in laboratories might be more pathogenic to humans than those circulating in free-ranging macaques. Second, free-ranging macaques might shed the virus less frequently than captive macaques, which decreases the transmission potential. Third, McHV-1 infection in humans transmitted from free-ranging macaques might be misdiagnosed or underreported (18).

The first explanation for the apparent difference in macaque-human transmission rates does not appear to be true in this system. The gJ gene sequences found in viruses in macaques in Silver Springs State Park were identical to isolates originating from laboratories (Figure), suggesting that strain divergence has not occurred. This gene is partially responsible for virulence by inhibiting apoptosis and regulating cellular processes (34) and could be a potential gene controlling
pathogenicity. However, information from 1 gene enables limited inference; whole-genome sequencing of virus isolates from laboratory and free-ranging animals is needed to understand the role of pathogenicity and the mechanisms responsible for differences among host populations.

The second explanation is that virus is shed more frequently in laboratory animals, thus exposing humans in this setting more frequently, but data to test this hypothesis are sparse. In a study of 1 laboratory population, 9 (75%) of 12 antibody-positive rhesus macaques tested positive via rPCR for viral shedding in mucosa (35). In another study, free-ranging long-tailed macaques were captured, transported, and held in captivity for ≤72 hours before buccal sampling occurred; 154 (39%) of 392 animals tested positive for viral DNA via oropharyngeal and urogenital sampling (13). In animals we tested by rPCR, the viral shedding prevalence of 4%–14% was lower than that reported for either the captively bred population (35) or the free-ranging animals that were held in captivity (13). These observations support the hypothesis of higher shedding rates in captive animals due to the stress associated with high densities, disruption of social structure, and being in an unfamiliar environment (36). However, not all laboratory exposures, including those from needle sticks and mucosal splashes, have resulted in seroconversion or any disease manifestation (37), nor have bites by pet macaques been linked to any herpes B cases in humans (38). More studies are needed to definitively determine the risk for herpes B virus transmission and to more precisely conclude that laboratory animals shed more frequently or have more pathogenic strains.

The third explanation for increased apparent transmission of McHV-1 to humans in a laboratory setting is provided by a difference in probability of detection among human populations (laboratory workers versus the general public). The etiology of encephalitis, which is the manifestation of McHV-1 infection in humans, is undetermined for 30%–60% of encephalitis cases in the United States because of the rapid and nonspecific onset of disease; the transient nature of the viremia; and the lack of rapid, specific diagnostic tests (39). Diagnosis of viral encephalitis is likely even lower in much of Asia, where human-macaque interactions are common and surveillance is lacking. Thus, it is plausible that McHV-1 transmission to humans from free-ranging macaques has been underdetected and underreported. Because robust surveillance is lacking, conducting comparative studies on the viruses from laboratory and free-ranging animals is imperative to better determine genomic, epigenetic, and epidemiologic factors associated with transmission and pathogenicity and the role host stress plays in disease outcomes in humans.

Given the current information available, we must consider the presence of the population of invasive rhesus macaques in Florida to be a public health concern. We have shown evidence of viral shedding of McHV-1 in free-ranging macaques at the popular public park, Silver Springs State Park. Although shedding rates appear lower than in captive settings, the potential for human-macaque contact in this park is high. Thoroughly characterizing and comparing the whole genomes of McHV-1 isolates is crucial to deciphering the relationship between pathogenic laboratory strains and strains circulating in the free-ranging macaques in Silver Springs State Park. As of December 12, 2017, no evidence of human transmission from free-ranging macaques exists. However, this pathogen should be considered a low-incidence, high-consequence risk, and adequate public health measures should be taken (40).

Acknowledgments
We thank Bambi Clemson and Mark Cunningham for conducting necropsies on macaques. We thank Michael Summers for research assistance in the field and 2 anonymous reviewers for comments that improved the manuscript.

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About the Author
Dr. Wisely is an associate professor at the University of Florida, Gainesville, where she studies the ecology of wildlife diseases at the human-wildlife and livestock-wildlife interface. She is particularly interested in how land use and land cover change interacts with host ecology to influence the transmission of pathogens.

References


37. Freifeld AG, Hall, Gainesville, FL 32611-0430, USA; email: wisely@ufl.edu
Co-circulation of Influenza A H5, H7, and H9 Viruses and Co-infected Poultry in Live Bird Markets, Cambodia


Low-longitudinal surveillance of 2 live bird markets in Cambodia revealed year-round, high co-circulation of H5, H7, and H9 influenza viruses. We detected influenza A viruses in 51.3% of ducks and 39.6% of chickens, and co-infections, mainly by H5 and H9 viruses, in 0.8% of ducks and 4.5% of chickens.

A large variety of avian influenza viruses (AIVs) circulate in live bird markets (LBMs) in countries where highly pathogenic influenza A(H5N1) viruses are endemic. The low pathogenicity AIVs A(H7N9) and A(H9N2) are also potential threats for global public health, related to the ability of these viruses to cause human infections in people in close contact with infected poultry. The segmented genomes of influenza A viruses indicate that co-infections can result in progeny with mixed genomes. Therefore, co-circulation of a large diversity of AIVs is a risk for emergence of novel reassortant viruses affecting animals, humans, or both.

Cambodia is a developing country in Southeast Asia that has a population of >15 million, of which 73% are dependent on agriculture for their livelihood. The country is estimated to have a poultry population of ~18 million chickens and ~8 million ducks; most are raised in small backyard flocks. Poultry are generally transported on trucks and motorbikes into centralized LBMs, where they are slaughtered after being sold to customers. These LBMs have been established as critical for persistence, amplification, and dissemination of AIVs.

Surveillance studies in LBMs in Cambodia have revealed some of the highest AIV detection rates in poultry globally. As of November 2017, a total of 56 human cases (including 37 deaths) and 49 poultry outbreaks of influenza A(H5N1) have been recorded in Cambodia. However, little is known about other AIV subtypes at risk for pandemic emergence, mainly H7 and H9. We investigated the circulation of potentially highly pathogenic AIV subtypes (H5, H7, and H9), that have known public health risks in Cambodia LBMs during 2015.

The Study
We administered a longitudinal survey in 2 LBMs in the highly populated southeast region of Cambodia during February–December (weeks 7–53), 2015. Market 1 is a large LBM in central Phnom Penh that serves as a hub for poultry commerce in the southeast region. Market 2 is a smaller provincial market in Takeo Province. Weekly, we collected pooled oropharyngeal and cloacal swabs from 4 chickens and 4 ducks, randomly selected, in each LBM. We also collected 50-mL samples of carcass wash water (CWW; large buckets of water that are used to wash freshly slaughtered poultry) weekly from each LBM. We transported samples at 4°C, immediately aliquoted them, and stored them at –80°C until testing.

In the Virology Unit laboratory of Institut Pasteur, Cambodia, we concentrated viruses in the CWW samples as previously described. We extracted viral RNA from CWW and swab samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s instructions. We then tested extracts for influenza A (M-gene) and subtypes H5 (primer sets H5a and H5b), N1, H7, and H9 by using quantitative RT-PCR (qRT-PCR) or conventional RT-PCR. The H5, H7, and H9 gene targets were all tested by using 2 separate assays to reduce the chances of false-negative results caused by the presence of single-nucleotide polymorphisms. Except for 1 of the H7 qRT-PCR and the H9 conventional RT-PCR, the assays were sourced from the International Reagent Resource (https://www.internationalreagentresource.org/Home.aspx) (12,13). For the qRT-PCR assays, cycle threshold values <38 were considered positive.

Author affiliations: Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, Australia (P.F. Horwood); Institute Pasteur, Phnom Penh, Cambodia (P.F. Horwood, S.V. Horm, A. Suttie, S. Thet, P. Y, S. Rith, S. Ly, A. Tarantola, E.A. Karlsson, P. Dussart); Federation University Australia, Churchill, Victoria (A. Suttie); National Animal Health and Production Research Institute, Cambodian Ministry of Agriculture, Forestry and Fisheries, Phnom Penh (S. Sorn, D. Holl, S. Tum)

DOI: https://doi.org/10.3201/eid2402.171360
Overall, we collected 940 samples, including 376 chicken swab samples, 376 duck swab samples, and 188 CWW samples. Testing identified AIV RNA year-round from 39.6% of chickens, 51.3% of ducks, and 93.1% of CWW samples (Table 1). We detected H5 in 16.8% of chickens, 20.5% of ducks, and 76.6% of CWW samples; H7 in 0.5% of chickens, 1.1% of ducks, and 2.1% of CWW samples; and H9 10.9% of chickens, 1.1% of ducks, and 2.1% of CWW samples (Table 1). We detected H5 in 16.8% of chickens, particularly from market 1.

Co-infections were more frequent among chickens (4.5%) than ducks (0.8%), and high viral loads (determined by qRT-PCR cycle threshold values <30) of co-infecting viruses were detected in many of the chicken samples (Table 2). Most co-infections detected were H5 and H9 viruses in chickens, particularly from market 1.

The H5 subtype (37.2% of birds) was detected much more frequently than N1 (16.5%) by using the subtype-specific qRT-PCR assays. Previous studies have suggested that the sensitivities of M and H5 qRT-PCRs are higher than that for N1 (1). Isolation and sequence analysis of samples that were H5 positive and N1 negative revealed no evidence for circulation of non-N1 H5 strains. Similarly, further investigations were undertaken to confirm that none of the H7 viruses were H7N9 or HPAI strains (data not shown). The circulation of Eurasian-lineage LPAI H7 viruses in the region has been established through surveillance in South Korea (14).

The peak in AIV and H5N1 circulation occurred during February–April, with a secondary peak during November–December (Figure). Surveillance was not extended into January 2016; however, we assume that the peak

\[\text{Table 1. Positivity rate for avian influenza viruses in live bird markets, Cambodia, February–December 2015}^*\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Market 1, Phnom Penh, no. (%)</th>
<th>Market 2, Takeo, no. (%)</th>
<th>Combined, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken, n = 188</td>
<td>Duck, n = 188</td>
<td>CWW, n = 94</td>
</tr>
<tr>
<td></td>
<td>Influenza A</td>
<td>H5</td>
<td>N1</td>
</tr>
<tr>
<td>47</td>
<td>89 (47.3)</td>
<td>105 (55.9)</td>
<td>86 (91.5)</td>
</tr>
<tr>
<td>54</td>
<td>256 (56.6)</td>
<td>53 (28.2)</td>
<td>71 (75.5)</td>
</tr>
<tr>
<td>134</td>
<td>7 (3.9)</td>
<td>24 (12.8)</td>
<td>48 (51.1)</td>
</tr>
<tr>
<td>181</td>
<td>1 (0.5)</td>
<td>2 (1.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>28</td>
<td>28 (14.9)</td>
<td>3 (1.6)</td>
<td>54 (57.4)</td>
</tr>
</tbody>
</table>

*By quantitative reverse transcription PCR. n values indicate number of samples. CWW, carcass wash water.

\[\text{Table 2. Avian influenza co-infections detected in poultry from live bird markets in Cambodia, February–December 2015}^*\]

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Market/week</th>
<th>Bird</th>
<th>Cycle threshold values by quantitative RT-PCR</th>
<th>H9 RT-PCR</th>
<th>Co-infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-104</td>
<td>M1/W12</td>
<td>D</td>
<td>24.13 32.73 30.74 32.90 – – 23.39 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-150</td>
<td>M1/W14</td>
<td>C</td>
<td>34.25 27.01 39.76 – – 34.47 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-167</td>
<td>M1/W15</td>
<td>C</td>
<td>30.81 36.60 36.36 – – 30.84 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-169</td>
<td>M1/W15</td>
<td>C</td>
<td>27.63 37.93 35.08 – – 27.58 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-170</td>
<td>M1/W15</td>
<td>D</td>
<td>22.79 33.52 32.44 34.79 – – 25.65 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-227</td>
<td>M1/W18</td>
<td>C</td>
<td>28.90 35.77 29.81 30.17 – – 30.60 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-228</td>
<td>M1/W18</td>
<td>C</td>
<td>23.93 29.54 23.63 24.60 – – 29.84 + H5N1/H9</td>
<td></td>
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<tr>
<td>Z-230</td>
<td>M1/W18</td>
<td>C</td>
<td>25.61 – 39.71 38.54 – – 22.65 + H5N1/H9</td>
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<tr>
<td>Z-267</td>
<td>M1/W20</td>
<td>C</td>
<td>22.70 28.69 26.80 30.18 – – 27.66 + H5N1/H9</td>
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<td></td>
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<tr>
<td>Z-269</td>
<td>M1/W20</td>
<td>D</td>
<td>21.04 – – – – 27.00 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-430</td>
<td>M1/W28</td>
<td>C</td>
<td>23.79 – – – – 24.78 24.98 + H7/H9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z-466</td>
<td>M1/W30</td>
<td>D</td>
<td>32.56 40.93 – – – 34.72 36.00 + H7/H9</td>
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<tr>
<td>Z-568</td>
<td>M1/W35</td>
<td>D</td>
<td>32.40 – 41.34 – – 37.39 + H5N1/H9</td>
<td></td>
<td></td>
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<tr>
<td>Z-748</td>
<td>M1/W44</td>
<td>C</td>
<td>21.05 – 35.44 37.75 – – 27.17 + H5N1/H9</td>
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<td></td>
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<tr>
<td>Z-858</td>
<td>M2/W49</td>
<td>C</td>
<td>18.41 40.23 – 24.02 – – 27.67 + H5N1/H9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*C, chicken; D, duck; RT-PCR, reverse transcription PCR; +, positive; –, negative.
‡All samples with an M gene quantitative RT-PCR cycle threshold value <38 were inoculated into embryonated chicken eggs to confirm avian influenza infection.
§Van Borm et al. (13).
¶World Health Organization (12).
*Equivocal H5 or N1 results (defined by cycle threshold value >38) were subsequently confirmed by isolation of influenza A(H5N1) in embryonated chicken eggs (data not shown).
period extends from November through April, coinciding with the dry season in Cambodia. Similar seasonality of AIV circulation was observed in a longitudinal LBM study in 2013, in which February was also the month with the highest prevalence of AIV-infected poultry (1). Knowledge of this peak period of circulation is needed so that animal and human health authorities can target interventions to reduce AIV spread and human exposure. We detected H9 circulation in chickens year-round, with no discernible seasonality. H7 seasonality could not be determined because of the small number of viruses detected.

Conclusions
Throughout the study, we detected high levels of AIVs in 2 LBMs in southeastern Cambodia. In 2013, our research team conducted a similar longitudinal AIV surveillance study that included the same 2 LBMs (referred to as M1, Phnom Penh, and M4, Takeo, in the 2013 study [7]). In that study, we detected AIVs in 32% of ducks, 18% of chickens, and 75% of CWW samples. This new study found a substantial increase in circulation of AIVs: 51% of ducks, 40% of chickens, and 93% of CWW samples. However, no corresponding increase in cases of H5N1 infection among humans was detected in Cambodia during 2015, possibly related to the replacement of the H5N1 clade 1.1.2 reassortant virus with an H5N1 clade 2.3.2.1c virus in early 2014.

The detection of co-infections in 4.5% of chickens during this study was cause for concern. Reassortment between H5N1 and other AIVs could produce novel viruses that have potential to cause epizootics or pandemic emergence. Reassortment with the internal genes of H9N2 viruses has been linked to the emergence of numerous AIVs that raise public health concern, such as H5N1, H7N9, and H10N8 (15). In this study, only H5, H7, and H9 subtypes were screened, because strains of these viruses (particularly H5N1, H7N9, and H9N2) are of leading global health concern. We would expect an even higher detection rate of co-infections if all AIV subtypes were tested.

In summary, we have documented a substantial increase in the prevalence of AIVs in Cambodian LBMs from 2011 (7) to 2013 (1) to 2015. We also have established that co-infections between AIVs commonly occur in the LBM environment and there is potential for emergence of novel viruses through reassortment. Interventions should be considered to decrease the prevalence of AIVs in LBMs to reduce the risk for emergence of novel viruses.

Acknowledgments
We thank the vendors from the participating live bird markets for their cooperation with our study team. We also thank the technical team from the Virology Unit at the Institute Pasteur in Cambodia and the field teams from the National Animal Health
Co-circulation of Influenza A Viruses, Cambodia

This study was funded by the US Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response (grant No. IDSEP 140020-01-00; http://www.asideproject.org/).

About the Author
Dr. Horwood conducted this study as the deputy head of the Virology Unit at the Institut Pasteur in Cambodia. He is now a senior research fellow at the Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, Australia. His research interests include molecular characterization and epidemiology of emerging tropical infectious diseases.

References

Address for correspondence: Paul F. Horwood, Australian Institute of Tropical Health and Medicine, James Cook University, PO Box 6811, Cairns, QLD 4870, Australia; email: paul.horwood@jcu.edu.au
Effects of Culling on *Leptospira interrogans* Carriage by Rats

Michael J. Lee, Kaylee A. Byers, Christina M. Donovan, Julie J. Bidulka, Craig Stephen, David M. Patrick, Chelsea G. Himsworth

We found that lethal, urban rat control is associated with a significant increase in the odds that surviving rats carry *Leptospira interrogans*. Our results suggest that human interventions have the potential to affect and even increase the prevalence of zoonotic pathogens within rat populations.

Norway rats (*Rattus norvegicus*) are a reservoir for *Leptospira interrogans*, the etiologic agent of the zoonotic disease leptospirosis (1). Leptospirosis affects ≈1 million persons worldwide annually and can result in kidney failure or pulmonary hemorrhage (1,2). Increasing urbanization has driven the emergence of leptospirosis in cities globally (3). Within cities, areas of poverty experience a confluence of environmental and socioeconomic factors that heighten the risk for ratborne *L. interrogans* transmission (3).

The ecology of rats and the epidemiology of *L. interrogans* within their populations are intimately connected (4). Previous research on other reservoir species suggests that anthropogenic disturbances may alter reservoir ecology, resulting in new transmission patterns (5,6). Because lethal control is a common technique used to address rat populations (7,8), we aimed to determine whether culling affects *L. interrogans* carriage by urban Norway rats.

The Study

This study, conducted in an inner-city neighborhood of Vancouver, British Columbia, Canada, during June 2016–January 2017, compared the prevalence of *L. interrogans* in rat populations before and after a kill-trapping intervention. Each study site (12 total) comprised 3 contiguous city blocks and was designated as a control site or an intervention site (Figure 1). In control sites, no kill-trapping occurred; in intervention sites, kill-trapping occurred only in the central blocks, and the 2 adjacent blocks were designated as nonkill flanking blocks. We divided trapping in each intervention site into 3 time periods: before, during, and after the intervention (Figure 2). Before and after the intervention, rats were trapped, processed, and released. During processing, rats were marked with an ear tag, and morphometric information was recorded (Table 1). Urine was obtained from these rats and tested for *L. interrogans* by real-time PCR. During the intervention, we euthanized trapped rats; in control sites and flanking blocks, capture-release continued, and rats were not euthanized. The University of British Columbia’s Animal Care Committee (A14-0265) approved all procedures (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/2/17-1371-Techapp1.pdf).

We used mixed-effects multivariable logistic regression to estimate the effect of the intervention on the odds that rats carried *L. interrogans*, while controlling for clustering by city block (4). The outcome was the *L. interrogans* PCR status (negative or positive) of individual rats. The predictor variable categorized rats by block and period of capture: 0, rats caught before the intervention; 1, rats caught after the intervention in control blocks; 2, rats caught after the intervention in nonkill flanking blocks; and 3, rats caught after the intervention in intervention blocks. Although we did not undertake the intervention in control sites, we considered the third 2-week trapping period independently from the other trapping periods in control sites to detect any temporal changes in *L. interrogans* prevalence not associated with the intervention. We excluded the 7 rats captured both before and after the intervention to avoid double-counting individual rats. For rats recaptured within the same period as their first capture (either before or after the intervention), we averaged weight and length across captures. We also excluded 1 rat missing data for covariates under consideration.

We used a hypothesis-testing model-building approach to estimate the effect of the intervention while controlling for covariates (Table 1). We kept covariates, selected on the basis of their potential to confound the relationship between the intervention and *L. interrogans* carriage, in the model if they changed the estimated relationship between the predictor and outcome variables by ≥10%. Because
length and weight were collinear, we used the covariate with the largest effect on the relationship between the predictor and outcome. We dichotomized weight around its median because it was not linear with the log-odds of the outcome. For statistical analyses, we used RStudio (Boston, MA, USA).

Of the 438 rats trapped, we included 430 in the modeling process (Table 1). Sixty-four (14.9%; 95% CI 11.7%–18.7%) rats were PCR-positive for *L. interrogans*. Of 131 rats recaptured, 5 were *L. interrogans* positive at their first capture and recapture; no positive rats changed pathogen status within a trapping period.

Rats caught in intervention blocks after an intervention had 9.55 times the odds of carrying *L. interrogans* than did rats trapped before an intervention, while adjusting for weight and wound presence variables (Table 2). We found no significant changes in either flanking blocks or control blocks. In this model, 52.6% of the total model variance...
was due to the random effect of the block (11). Rerunning the final model including animals that were caught both before and after the intervention did not substantially affect the results (effect of the intervention in intervention blocks; adjusted odds ratio 8.88, 95% CI 1.68–68.08).

Conclusions

This study showed that kill-trapping was associated with increased odds that rats carried *L. interrogans* in the city blocks where trapping occurred. We did not observe this effect in control blocks or nonkill flanking-blocks.

Increased intraspecific transmission of *L. interrogans* resulting from kill-trapping is a plausible explanation for the observed effect. Previous research suggests that rat-to-rat transmission of *L. interrogans* is associated with social structures in rat colonies (4). Given that culling is ineffective at removing entire rat populations (7,8,12), kill-trapping may have disrupted social structures and promoted new interactions that facilitated transmission among remaining rats. For example, culling may have removed dominant rats (13), subsequently increasing aggressive interactions among the remaining rats as they established a

### Table 1. Distributions of covariates by rat-trapping period and *Leptospira interrogans* real-time PCR status, Vancouver, British Columbia, Canada, June 2016–January 2017

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Total</th>
<th>PCR status before intervention</th>
<th>PCR status after intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Season, no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer, Jun–Aug</td>
<td>115 (27)</td>
<td>83 (37)</td>
<td>15 (38)</td>
</tr>
<tr>
<td>Fall, Sep–Nov</td>
<td>203 (47)</td>
<td>143 (63)</td>
<td>24 (62)</td>
</tr>
<tr>
<td>Winter, Dec–Feb</td>
<td>112 (26)</td>
<td>0†</td>
<td>0†</td>
</tr>
<tr>
<td><strong>Sex, no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>205 (48)</td>
<td>107 (47)</td>
<td>16 (41)</td>
</tr>
<tr>
<td>M</td>
<td>225 (52)</td>
<td>119 (53)</td>
<td>23 (59)</td>
</tr>
<tr>
<td><strong>Sexual maturity, no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>178 (41)</td>
<td>117 (52)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Mature</td>
<td>252 (59)</td>
<td>109 (48)</td>
<td>38 (97)</td>
</tr>
<tr>
<td><strong>Continuous median length, cm (IQR)</strong></td>
<td>31 (26–39)</td>
<td>29 (25–37)</td>
<td>41 (36–43)</td>
</tr>
<tr>
<td><strong>Wounds, no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>316 (73)</td>
<td>173 (77)</td>
<td>11 (28)</td>
</tr>
<tr>
<td>Yes</td>
<td>114 (27)</td>
<td>53 (23)</td>
<td>28 (72)</td>
</tr>
<tr>
<td><strong>Weight, g, no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;122</td>
<td>212 (49)</td>
<td>129 (57)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>&gt;122</td>
<td>218 (51)</td>
<td>97 (43)</td>
<td>38 (97)</td>
</tr>
</tbody>
</table>

† IQR, interquartile range.
‡ No periods before the intervention period were conducted during winter.

Table 2. Results of model building in a study of the effects of culling on *Leptospira interrogans* carriage by rats, Vancouver, British Columbia, Canada, June 2016–January 2017

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Unadjusted odds ratio* (95% CI)</th>
<th>Adjusted odds ratio† (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Season</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>Reference</td>
<td>–‡</td>
<td>–</td>
</tr>
<tr>
<td>Fall</td>
<td>0.44 (0.13–1.39)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Winter</td>
<td>0.87 (0.22–3.24)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Reference</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M</td>
<td>1.28 (0.70–2.37)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Sexual maturity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>Reference</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mature</td>
<td>16.26 (6.28–51.95)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Continuous length, cm</strong></td>
<td>1.25 (1.18–1.35)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Wounds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td>Reference</td>
<td>3.67 (1.73–9.12)</td>
</tr>
<tr>
<td>Yes</td>
<td>1.81 (1.42–2.39)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Weight, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;122</td>
<td>Reference</td>
<td>Reference</td>
<td>9.98 (3.70–31.74)</td>
</tr>
<tr>
<td>&gt;122</td>
<td>17.88 (7.22–53.28)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before intervention, all block types, n = 261</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>After intervention, control blocks, n = 97</td>
<td>0.69 (0.22–2.00)</td>
<td>0.77 (0.22–2.58)</td>
<td>0.68</td>
</tr>
<tr>
<td>After intervention, nonkill flanking blocks, n = 33</td>
<td>1.50 (0.49–4.40)</td>
<td>2.22 (0.85–7.47)</td>
<td>0.19</td>
</tr>
<tr>
<td>After intervention, intervention blocks, n = 39</td>
<td>8.67 (2.02–55.00)</td>
<td>9.55 (1.75–78.31)</td>
<td>0.016</td>
</tr>
</tbody>
</table>

* Bivariable relationships between the indicated covariate and *L. interrogans* status, while controlling for the random effect of the block.
† Results of the final multivariable model in which the effect of each covariate is adjusted for other covariates in the model.
‡ Dashes indicate variables not carried forward into the final multivariable model on the basis of statistical confounding criteria.
new social hierarchy. The positive association between *L. interrogans* status and weight/wound presence (which are correlated with hierarchical dominance) supports this hypothesis because the bacteria may be transmitted through specific aggressive/dominance interactions (4).

We assessed only the effect of culling on a single ratborne pathogen. *L. interrogans* might be particularly susceptible to the effects of culling because of its dependence on rat social structures. Other vectorborne (e.g., flea-borne *Rickettsia* spp. [14]) or environmentally acquired (e.g., methicillin-resistant *Staphylococcus aureus* [15]) rat-associated pathogens might not be as easily influenced by culling. Future studies should determine the duration of effects induced by lethal control because effects on *L. interrogans* prevalence may wane with time. However, given that such methods are ineffective at removing entire rat populations and might therefore be used repeatedly as the population rebounds (7,8,12), short-term effects may be particularly important.

We demonstrated that rat culling has the potential to increase the odds for *L. interrogans* carriage among remaining rats and thus could potentially increase the risk for transmission to humans. Although public health risks resulting from such an increase postintervention might be offset by a decrease in the number of rats, we were unable to quantify the size of the rat population before and after intervention. Practical and ethical considerations make it difficult to empirically demonstrate a direct link between culling and increased pathogen transmission from rats to humans. Rather, after culling, the potential for a person to encounter a rat carrying *L. interrogans* increases if a person encounters a rat, suggesting that the risk for zoonotic transmission increases per rat contact.

The convergence of this study with previous literature documenting that reactive culling is often unsuccessful at removing rat populations (7,8,12) indicates that such methods are ineffective. Instead, ecologically based rodent management, which focuses on reducing resources available to rats (8), should be more widely applied to urban environments.

By integrating our results with other studies on the impacts of culling wild animals to control communicable diseases (5,6), we can conclude that killing animal reservoirs of human pathogens might have unintended consequences on the disease risks. This hypothesis underscores the importance of understanding the ecology of the targeted animal reservoir to design effective control programs.

Acknowledgments

We thank Charles Krebs, Bobby Corrigan, and Michael Whitlock for their suggestions in designing this study. We also thank Geoffrey Knaub and Sophia Kontou for their help during the field trial, as well as Erin Zabek for her assistance throughout this study in acquiring laboratory equipment and supplies. We thank the Vancouver Area Network of Drug Users for their participation in this project.

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About the Author

Mr. Lee is a PhD student in the School of Population and Public Health, University of British Columbia, in Vancouver. His research interests include the changing epidemiology of ratborne and other zoonotic pathogens.

References


Address for correspondence: Michael J. Lee, The University of British Columbia, School of Population and Public Health, 4372 Fraser St, Apt 201, Vancouver, BC V5V 4G3, Canada; email: michaeljosephlee2@gmail.com
Scrub Typhus Outbreak in Chonburi Province, Central Thailand, 2013

Wuttikon Rodkvamtook, Narupon Kuttasingkee, Piyada Linsuwanon, Yutthapong Sudsawat, Allen L. Richards, Maneerat Somsri, Noppadon Sangjun, Chien-Chung Chao, Silas Davidson, Elizabeth Wanca, Jariyanart Gaywee

Investigation of a scrub typhus outbreak in Thailand during September 2013 found that 9.1% of Thai soldiers and 11.1% of residents living in areas surrounding training sites had antibodies against the causative agent, Orientia tsutsugamushi. Sequence analysis of O. tsutsugamushi from rodents and chiggers identified 7 genogroups and 3 genotypes.

Although scrub typhus is one of the leading causes of acute febrile illnesses (AFI) in the Asia-Pacific region, with an estimated 1 million cases annually (1,2), its overall molecular epidemiology remains unclear. Scrub typhus occurs when humans are bitten by parasitic larvae of trombiculid mites (chiggers) harboring the bacterium Orientia tsutsugamushi. Chiggers primarily infest mammals, such as rodents, that dwell in tall grasses and scrub vegetation; thus, activities such as camping and hiking can lead to scrub typhus (3,4). Although scrub typhus is treatable with doxycycline if diagnosed early, the median fatality rate among scrub typhus patients is 6%, and the fatality rate may reach 20% among untreated patients (5,6). No effective vaccine, nor accurate assessment of potential health risks to soldiers conducting training exercises in scrub typhus–endemic areas.

The Study

During September 2013, a group of 110 RTA soldiers conducted operational field training near the Bang Phra Navy Agriculture Center in Si Racha (Figure 1). Two to 3 weeks after training, 10 soldiers experienced sudden onset of high-grade fever with clinical symptoms including intense headache, chill, and retroorbital eye pain. Eight of these soldiers were hospitalized at Fort Nawaminthrarachini Hospital, and the other 2 were admitted to other hospitals in Chonburi. Physical examinations revealed that 37.5% had necrotic lesions similar to eschar. Serum samples were send to AFRIMS for diagnostic tests for the most likely pathogens: PCR for dengue viruses and indirect immunofluorescent assay and dot ELISA for rickettsioses (10,11) (Table 1).

Serologic analysis showed that all patients had positive antibody titers against STG, suggesting active infection. Patients were treated with oral doxycycline, and fever subsided within 24 h without any sequelae. For the 2 soldiers admitted at different hospitals, the serum samples from the acute and convalescent phases were not available for the assay but were collected for follow-up analysis. Serologic analysis suggested that these soldiers had higher titers of STG IgM and IgG, indicating history of O. tsutsugamushi infection.

To determine the exposure of the troops to disease vectors in the training areas, we identified the serostatus of 100 additional RTA soldiers who conducted field training exercises in the same areas and of 27 residents and workers in the agriculture center. All 127 participants were healthy and afebrile at the time of blood collection. Serum tests of the residents and workers revealed that 3/27 had STG IgG, suggesting high exposure to O. tsutsugamushi. According to interviews, 24 soldiers reported experiencing AFI during training. Test results demonstrated that none of these soldiers had O. tsutsugamushi infection during the training period. We concluded that seroprevalence of STG antigens among the troops was 9.1% and among residents, 11.1%.
To control the scrub typhus outbreak, AFRIMS applied an established prevention and control program, the AFRIMS-Nawamintharachini model, which was established in 2003 after a scrub typhus outbreak in Bo Thong District. The model describes controlling scrub typhus outbreaks through a surveillance program to clarify vector distribution and rodent population density. The program included developing diagnostic methods, increasing scrub typhus infection awareness, and providing topical insect repellents to soldiers who conduct activities in locations likely to harbor scrub typhus, as well as observing soldiers for 15 days after completing deployment for indications of AFI. We introduced this program in Si Racha after the 2013 outbreak, and since then, no scrub typhus infection has been reported in postdeployment soldiers who trained in previously defined scrub typhus risk areas.

Because rodents influence mite population density (12,13), we surveyed for chiggers and small rodents in these areas to determine the etiology associated with scrub typhus outbreak and evaluate the effectiveness of the prevention program. We conducted surveys in 3 consecutive years (August 2014, November 2015, and August 2016) by following a previously described protocol (10). We surveyed areas covering a 10-km radius near training sites with small villages and various vegetation characteristics. We collected rodent liver, spleen, and serum specimens and stored them in liquid nitrogen. We removed chiggers from rodents’ ears and preserved them in 70% ethanol. We slide-mounted approximately one fifth of chigger samples from each rodent for species identification and

<table>
<thead>
<tr>
<th>ID</th>
<th>Age, y</th>
<th>Fever</th>
<th>Eschar</th>
<th>Headache</th>
<th>Chill</th>
<th>Eye pain</th>
<th>Symptom onset to collection, d†</th>
<th>Test results for O. tsutsugamushi, IgM/IgG‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IFA 1</td>
<td>2</td>
</tr>
<tr>
<td>P01</td>
<td>25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>P02</td>
<td>22</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>5</td>
<td>200/50</td>
</tr>
<tr>
<td>P03</td>
<td>25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>5</td>
<td>200/200</td>
</tr>
<tr>
<td>P04</td>
<td>23</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>5</td>
<td>200/100</td>
</tr>
<tr>
<td>P05</td>
<td>21</td>
<td>+</td>
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<td>+</td>
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<td>5</td>
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<td>P06</td>
<td>21</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>800/100</td>
</tr>
<tr>
<td>P07</td>
<td>21</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>P08</td>
<td>22</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>P09</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>P10</td>
<td>21</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>12</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ID, patient identification number; IFA, indirect fluorescence antibody assay; ND, not determined; + positive; – negative.
†Time from onset of signs and symptoms to first blood collection.
‡Karp, Kato, and Gilliam genotypes. Tests were performed on September 27 (round 1), October 4 (round 2), and October 7 (round 3). Positivity cutoff titer for initial screening was 1:50 and the diagnostic criteria were established as a >4-fold increase in the titer of IgM or IgG in paired serum samples. If only 1 serum sample was available, titer of 1:50 to <1:400 was determined as recent or previous infection and titer >1:400 as active infection.

Figure 1. Chonburi Province, Thailand, showing scrub typhus outbreak areas in Bo Thong district in 2002 and Sri Racha district in 2013 (gray shading). Inset shows location of Chonburi Province in Thailand.
subjected the remaining chiggers to nucleic acid extraction. We extracted nucleic acid from chiggers and rodent liver and spleen samples using GeneJET Viral DNA/RNA Purification Kit (Invitrogen, Waltham, Massachusetts, USA) and assayed samples for O. tsutsugamushi DNA using 47-kDa quantitative PCR and 56-kDa nested PCR (14). We bidirectionally sequenced partial sequences of 56-kDa genes (First BASE Laboratories, Singapore) and used BioEdit software (http://bioedit.software.informer.com/7.1/) with the ClustalW algorithm to align nucleotide sequences with the sequences of O. tsutsugamushi prototypes and variants retrieved from GenBank. We constructed phylogenetic trees using MEGA software (http://www.megasoftware.net/) (Figure 2).

We captured a total of 45 rodents, accounting for a 7% capture rate. Bandicota (n = 5) and Rattus (n = 30) spp. rats were the most commonly trapped small mammals (77.8%), followed by ground squirrels (Menetes berdmorei, 17.8%) and tree shrews (Tupaia glis, 4.4%) (Table 2). Of the small mammals collected, 71.1% (32/45) tested STG seropositive. Most ectoparasites collected from rodents were chigger mites (88.9%), with Leptotrombidium deliense (72.1%) the predominant species of the 400 individual chiggers identified. A total of 20% of rodent liver and spleen samples and
15.6% of individual chiggers were PCR positive for *O. tsutsugamushi*. Phylogenetic analyses of the 56-kDa genes of 9 rodent and 7 chigger samples suggested that sequences were clustered in 3 genogroups and were closely related to 3 genotypes (Karp-related, Kato-related, and Gilliam-related genotypes) with 92.6%–99.8% nucleotide identity with reference sequences (Figure 2). Variants from the same clusters showed 94%–98% nucleotide identity with each other. Additionally, phylogenetic trees suggested that *O. tsutsugamushi* strains recovered from chiggers differed from those of rodent hosts, indicating high genetic variation of *O. tsutsugamushi* strains circulating in the survey area.

**Conclusions**

High seropositive status for *O. tsutsugamushi* among RTA soldiers and the presence of disease-carrying vectors and rodent hosts trapped in training and surrounding areas indicate that soldiers in these areas are at risk of scrub typhus, with the infection rate estimated at 9%–11%. Military personnel and residents who participate in exercises or travel to these areas in Chonburi should be alerted to the risk for scrub typhus. Appropriate protection, such as proper use of treated uniforms and application of topical insect repellent, are recommended to prevent chigger bites. Environment modification, including vegetation cleanup and trash consolidation, is necessary to control rodent and chigger populations in training location and decrease disease transmission.

**Table 2. Chigger infestation and Orientia tsutsugamushi infection in rodent and shrew species, Chonburi Province, Thailand, 2013***

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>No. captured (% of total)</th>
<th>No. infested with chiggers (% of species total)</th>
<th>No. (%) seropositive for STG by IFA</th>
<th>No. (%) positive for OT by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muridae</td>
<td>Bandicota indica rat</td>
<td>4 (8.9)</td>
<td>4 (100)</td>
<td>4 (100)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B. savilei rat</td>
<td>1 (2.2)</td>
<td>1 (100)</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td></td>
<td>Rattus rattus rat</td>
<td>27 (60)</td>
<td>25 (92.6)</td>
<td>27 (100)</td>
<td>7 (25.9)</td>
</tr>
<tr>
<td></td>
<td>R. exulans rat</td>
<td>3 (6.7)</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Sciurida</td>
<td>Menetes berdmorei squirrel</td>
<td>6 (17.8)</td>
<td>7 (87.5)</td>
<td>ND</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Tupaiidae</td>
<td>Tupaia glis shrew</td>
<td>2 (4.4)</td>
<td>2 (100)</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>45</td>
<td>40 (88.9)</td>
<td>32 (71.1)</td>
<td>10 (22.2)</td>
</tr>
</tbody>
</table>

*IFA, indirect fluorescence antibody assay; ND, not determined; OT, Orientia tsutsugamushi; STG, scrub typhus group of antigens.

**About the Author**

Dr. Rodkvamtook, a colonel in the Royal Thai Army, works as the Deputy Director of the Research Division, RTA-AFRIMS, Bangkok. His research projects include epidemiology of scrub typhus and rickettsiae, chiggers in Thailand, and diagnostic test kit development for rickettsiosis.

**References**


Address for correspondence: Wuttikon Rodkvamtook, Armed Forces Research Institute of Medical Sciences (AFRIMS), Royal Thai Army Component, 315/6 Rajvithi Rd, Rajthevee, Bangkok 10400, Thailand; email: rwuttikon@gmail.com, ltwutti@hotmail.com

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Epidemic Varicella Zoster Virus among University Students, India

Josh Meyers, Muthunarayanan Logaraj, Balaji Ramraj, Padmanesan Narasimhan, C. Raina MacIntyre

We investigated a yearlong varicella zoster virus outbreak in a highly susceptible young adult population at a large university in India. Outbreaks of varicella infection among adults are not well described in the literature. Infection control measures and vaccination policy for this age group and setting are needed.

Infection with varicella zoster virus (VZV; also known as chickenpox virus), a human herpesvirus, occurs worldwide, and most persons living in temperate regions become immune by contracting varicella illness or receiving vaccinations by early adulthood (1,2). However, in tropical climates, as little as half the population is exposed to VZV by adulthood (1). The extent of disease and severity in adults in tropical climates is also greater than for temperate climates (1,3). In India, >30% of persons >15 years of age are susceptible (4). Routine infant vaccination has substantially reduced transmission of wild-type varicella (3); however, VZV vaccine is not a part of the Indian Universal Immunisation Program (3). We describe an outbreak of varicella in 2016 at a large private university in Chennai, India.

The Study
The university at which the outbreak occurred has previously experienced recurrent varicella epidemics that have not been formally investigated. The university had 6,000 staff and 40,000 students (domestic and international) in 28 schools and colleges. The campus includes a 1,200-bed hospital, and residents are housed in >25 dormitory-style hostel blocks, most of which, except block 1, have single-sex occupancy.

We identified all cases among students or staff who received a varicella diagnosis at the university hospital during February 2016–January 2017 by using the hospital’s notifiable disease register. We selected controls by using systematic stratified sampling by hostel block at a ratio of 1:1 and matched according to age, sex, and hostel; we excluded those who self-reported a history of varicella illness.

Hospital clinicians used a clinical case definition to diagnose varicella: an illness with acute onset of diffuse maculopapular vesicular rash without another cause (6). No laboratory tests were done. Severity of disease was defined by the number of lesions (7).

We distributed questionnaires to eligible students and staff and sought written informed consent for participation in the study. Information gathered included demographics; disease characteristics; and risk factors such as region of birth, contact with varicella, living quarters, and study program. Vaccination status was determined by participants’ recall and defined as receipt of any varicella-containing vaccine. We calculated denominators for dormitories from occupied bed data at the start of the 2016–2017 semester and determined rates by state of residence by using the number of enrolled students from each state in India according to university dormitory and admissions records.

We used Microsoft Excel (Microsoft, Redmond, WA, USA) and IBM SPSS 21 (IBM, Armonk, NY, USA) to analyze the data. We used descriptive statistics calculated by using the Pearson $\chi^2$ test for comparison of proportions and the Mann-Whitney U test for comparison of nonparametric variables. A p value of <0.05 was considered statistically significant. Ethics approval was obtained from Sri Ramaswami Memorial University Ethics Committee, Chennai, India (ethics clearance no. 1160/IEC/2017).

During February 2016–January 2017, a total of 110 cases of varicella were diagnosed at the university hospital. Of these, 87 (79%) case-patients were male, 23 (21%) were female, and 100 (91%) were residents in the dormitory blocks of a single college. A peak of 34 cases was reported in November 2016 (Figure 1).

Among the 110 case-patients, 77 completed the questionnaire and were included in further analysis (Table 1). Students born in Odisha had the highest incidence (16 cases/1,000 students); those from Uttar Pradesh had the second-highest incidence (9.8 cases/1,000 students) (Figure 2).

The infection predominantly affected resident students; 72 study participants (94%) resided in a dormitory, whereas the other 6% lived in private housing (Table 1). The attack rate in dormitories was 14 cases/1,000 residents; the rate was lower in women’s dormitories than in men’s (online...
Of the 77 case-patients who completed the questionnaire, 25 (32.5%) reported attending class during their illness; 67 (87%) were isolated at some point, either at home or in the hospital. A rash was reported by all case-patients, fever by 59 (77%), cough by 24 (31%), and malaise by 34 (44%). The number of days absent from work or study was lower for vaccinated than nonvaccinated groups (6.2 days vs. 8.0 days; \( p = 0.046 \)). The number of days from onset of symptoms to hospitalization ranged 0–11 days (median 2 days) (Table 2).

**Conclusions**

Varicella epidemics among adults are not described well in the literature. This study describes a year-long propagated epidemic in a university in India with a background of past epidemics. A large student body with low immunization rates living in dormitories provides fertile ground for transmission of varicella and highlights a potential role for vaccination in young adults in India. Although studies in India and Thailand show that community transmission and household crowding predispose to varicella epidemics (8), transmission in a tertiary education setting has not been described.

### Table 1. Demographics of participants in study of varicella outbreak at a private university in Chennai, India, February 2016–January 2017*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case patients, n = 77</th>
<th>Controls, n = 77†</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex, %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>63 (81.8)</td>
<td>63 (81.8)</td>
<td>NS—matched variable</td>
</tr>
<tr>
<td>F</td>
<td>14 (18.2)</td>
<td>14 (18.2)</td>
<td>NS—matched variable</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>18</td>
<td>18</td>
<td>NS—matched variable</td>
</tr>
<tr>
<td>Range</td>
<td>17–40</td>
<td>17–52</td>
<td></td>
</tr>
<tr>
<td><strong>State of birth, % (SE)</strong></td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Uttar Pradesh</td>
<td>19 (24.7–4.9)</td>
<td>6 (7.8–3.1)</td>
<td></td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>9 (11.7–3.7)</td>
<td>14 (18.2–4.4)</td>
<td></td>
</tr>
<tr>
<td>Andhra Pradesh</td>
<td>8 (10.4–3.5)</td>
<td>9 (11.7–3.7)</td>
<td></td>
</tr>
<tr>
<td>Odisha</td>
<td>6 (7.6–3.1)</td>
<td>1 (1.3–1.3)</td>
<td></td>
</tr>
<tr>
<td>Other/NA</td>
<td>35 (45.5–5.7)</td>
<td>47 (61.0–5.6)</td>
<td></td>
</tr>
<tr>
<td><strong>Degree program,‡ % (SE)</strong></td>
<td></td>
<td></td>
<td>0.95</td>
</tr>
<tr>
<td>Bachelor of Technology</td>
<td>70 (90.9–3.3)</td>
<td>70 (90.9–3.3)</td>
<td></td>
</tr>
<tr>
<td>Bachelor of Medicine, Bachelor of Surgery, Doctor of Medicine and Research</td>
<td>2 (2.6–1.8)</td>
<td>3 (3.9–2.2)</td>
<td></td>
</tr>
<tr>
<td>Master of Technology</td>
<td>1 (1.3–1.3)</td>
<td>1 (1.3–1.3)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>4 (5.2–2.5)</td>
<td>3 (3.8–2.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Student year or staff position, % (SE)</strong></td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>1</td>
<td>63 (81.8–4.4)</td>
<td>65 (84.4–4.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8 (10.4–3.5)</td>
<td>7 (9.1–3.3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 (1.3–1.3)</td>
<td>2 (2.5–1.8)</td>
<td></td>
</tr>
<tr>
<td>Staff</td>
<td>5 (6.5–2.8)</td>
<td>3 (3.8–2.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Campus category, %</strong></td>
<td></td>
<td></td>
<td>NS—matched variable</td>
</tr>
<tr>
<td>Resident</td>
<td>72 (93.5)</td>
<td>70 (90.9)</td>
<td></td>
</tr>
<tr>
<td>Day student</td>
<td>1 (1.3)</td>
<td>4 (5.2)</td>
<td></td>
</tr>
<tr>
<td>Staff</td>
<td>4 (5.2)</td>
<td>3 (3.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Self-reported vaccination for varicella, % (SE)</strong></td>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>Yes</td>
<td>26 (33.8–5.4)</td>
<td>36 (46.8–5.7)</td>
<td></td>
</tr>
<tr>
<td>No/not sure</td>
<td>51 (66.2–5.4)</td>
<td>41 (53.2–5.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Known exposure to varicella during study period, % (SE)</strong></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Yes</td>
<td>26 (33.8–5.4)</td>
<td>14 (18.2–4.4)</td>
<td></td>
</tr>
<tr>
<td>No known exposure</td>
<td>51 (66.2–5.4)</td>
<td>63 (81.8–4.4)</td>
<td></td>
</tr>
</tbody>
</table>

*NS, not stated; NA, not available.
†Controls were matched by age, sex, and place of residence (hostel residents were matched by hostel block).
‡Degree program was suspected as potentially contributing to transmission of the disease by close contact during classes.
A serologic study of undergraduate students in Sri Lanka further suggests high susceptibility in this cohort (9).

With a highly susceptible young adult population living in a densely populated campus setting, adequate infection control strategies must be practiced (6). Approximately 33% of case-patients attended class while ill, and 13% were not kept in isolation postdiagnosis. Furthermore, a median of 2 days from onset of symptoms passed before hospitalization, increasing potential for transmission of infection before isolation. In addition to students being in close contact on campus, adherence to infection control procedures was poor, possibly leading to the increased transmission of VZV, as described by Greenaway et al. (8).

Vaccination can control epidemics but was not used in this setting (4,6,10). Estimates of vaccination of adults in India are ≈20% (3), lower than the 34% self-reported figure in this study, which suggests recall bias by the patients. A serologic study and validation of vaccination history of participants would assist in examining this factor in more detail.

The usual seasonal peak of varicella infections in southern India is January–April (6). The peak incidence during this study was in November and April; the larger November peak represented a marked variation from the usual seasonal pattern. From a policy perspective, it would be necessary to ascertain the frequency and magnitude of university epidemics, geographic hot spots, and the overall burden of disease in India. Immunization in settings such as universities would reduce transmission and is an essential intervention during an acute epidemic (6,11).

Most students affected were teenage boys in their first year of study. The analysis by state of birth showed high incidence of varicella for students born in both Uttar Pradesh and Odisha. Different climate and susceptibility by states should be investigated further to inform disease control efforts in India.

This study has several limitations. We had no confirmatory laboratory data; however, the clinical syndrome of varicella is fairly unique. The study is subject to recall bias on vaccination history, previous exposure, disease
About the Author
Mr. Meyers is a clinical pharmacist and research student at the University of New South Wales. His primary research interest is infectious diseases.

References

Address for correspondence: Josh Meyers, University of New South Wales, 15 Inkerman Ave, Blackwall, NSW 2256, Australia; email: meyers_100@outlook.com

Table 2. Characteristics of 77 patients with varicella in study of varicella outbreak at a private university in Chennai, India, February 2016–January 2017

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms, no. (%) patients</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>59 (76.6)</td>
</tr>
<tr>
<td>Rash</td>
<td>77 (100.0)</td>
</tr>
<tr>
<td>Cough</td>
<td>24 (31.2)</td>
</tr>
<tr>
<td>Malaise</td>
<td>34 (44.2)</td>
</tr>
<tr>
<td>Illness duration, no. (%) patients</td>
<td></td>
</tr>
<tr>
<td>&lt;1 week</td>
<td>11 (14.3)</td>
</tr>
<tr>
<td>1–2 weeks</td>
<td>54 (70.1)</td>
</tr>
<tr>
<td>&gt;2 weeks</td>
<td>12 (15.6)</td>
</tr>
<tr>
<td>No. days off work or study</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>7</td>
</tr>
<tr>
<td>Range</td>
<td>0–18</td>
</tr>
<tr>
<td>Attend class while ill, no. (%) patients</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25 (32.5)</td>
</tr>
<tr>
<td>No</td>
<td>52 (67.5)</td>
</tr>
<tr>
<td>Kept in isolation, no. (%) patients*</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>67 (87.0)</td>
</tr>
<tr>
<td>No</td>
<td>10 (13.0)</td>
</tr>
<tr>
<td>No. days from onset of symptoms to hospital admission†</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
</tr>
<tr>
<td>Range</td>
<td>0–11</td>
</tr>
<tr>
<td>No. case-patients who responded &quot;not sure&quot;</td>
<td>27</td>
</tr>
</tbody>
</table>

*Isolation indicates a patient spent time in an isolation room in the hospital or was in isolation at home at any point during the illness.
†Those who responded “not sure” were excluded from calculation.

characteristics, and respondent bias. Furthermore, only case-patients whose medical records included a positive diagnosis were included in the study. Active case finding was not done, so the true incidence is likely higher. We were unable to ascertain the index case for the epidemic.

This study describes a substantially circulated, year-long adult varicella epidemic at a large university in India. Dormitory students were most affected, but day students and staff were also affected. A substantial proportion of case-patients attended classes while ill, highlighting the need for active case finding and isolation during epidemics. A larger study is required to inform vaccination policy and disease control. Appropriate infection prevention and control strategies as well as use of vaccination during outbreaks should be considered.

This work was supported by Sri Ramaswami Memorial University Hospital and Research Centre, Community Medicine Department, and the School of Public Health and Community Medicine, University of New South Wales.

C.R.M. has received funding or in-kind support for investigator-driven research from Merck, GSK, Pfizer, and Seqirus and has been on vaccine advisory boards for Merck, GSK, and Pfizer.

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Fly Reservoir Associated with Wohlfahrtiimonas Bacteremia in a Human

Jesse H. Bonwitt, Michael Tran, Elizabeth A. Dykstra, Kaye Eckmann, Melissa E. Bell, Michael Leadon, Melissa Sixberry, William A. Glover

Wohlfahrtiimonas species bacteria were isolated from the bloodstream of a patient with septicemia and wound myiasis. Environmental investigations identified a Wohlfahrtiimonas sp. among insects in the Americas and in a previously undescribed vector, the green bottle fly (Lucilia sericata). The isolates possibly represent a new species within the genus Wohlfahrtiimonas.

Wohlfahrtiimonas chitiniclastica is a rarely reported cause of bacterial infection that has been isolated in humans and other mammals from a variety of organs (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/2/17-0913-Techapp1.pdf). In addition, Wohlfahrtiimonas spp. have been isolated from 4 species of nonbiting flies in Asia and Europe (1–4) that can cause myiasis, fly larvae infestation of a host’s tissue. Wound myiasis has been reported in patients infected with W. chitiniclastica and with Ignatzschineria spp., an organism closely related to W. chitiniclastica (online Technical Appendix Table). These findings provide evidence that W. chitiniclastica is transmitted by flies or fly larvae during myiasis. However, no reported attempt has been made to isolate Wohlfahrtiimonas spp. or Ignatzschineria spp. from larvae associated with a patient. We report a case of Wohlfahrtiimonas infection in a man in Washington, USA, and results of environmental investigations.

The Study
The case-patient was a 57-year-old man who developed wet gangrene of the right ankle and myiasis below the waist. Hematology at hospital admission was notable for leukocytosis and a predominance of neutrophils with a high ratio of band neutrophils (online Technical Appendix). Chronic cirrhosis, localized lung atelectasis, and multiorgan failure secondary to septic shock were diagnosed. The patient underwent amputation below the right knee but died 3 days after admission. Blood, urine, and tracheal aspirates collected <8 hours after admission revealed a mixed bacterial infection, including gram-positive cocci and gram-negative rods (online Technical Appendix). Propionibacterium acnes and Staphylococcus hominis spp. hominis were isolated from blood cultures, in addition to an unidentifiable gram-negative rod. No medical history was available; proxy interviews excluded recent travel outside Washington.

We performed presumptive identification of the gram-negative rod with phenotypic studies and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (online Technical Appendix). Amplification and sequencing of the near full-length 16S ribosomal RNA (rRNA) gene was performed, a phylogenetic tree was inferred by using the neighbor-joining method, and the topology was assessed by a bootstrap analysis of 1,000 replicates (online Technical Appendix). We used pulsed-field gel electrophoresis (PFGE) to assess isolate relatedness (online Technical Appendix).

Because larvae found on the patient had been discarded, we collected live and dead insects from the patient’s home and identified them to genus or species level (online Technical Appendix). To remove surface contamination, all live fly larvae and adult specimens were rinsed 5 times with sterile phosphate-buffered saline (PBS), homogenized, and sequentially diluted. We cultured the first rinse, fifth rinse, and diluted homogenates to isolate Wohlfahrtiimonas spp. (online Technical Appendix).

We identified 6 species of flies (online Technical Appendix) and collected live larvae (≈20) from the patient’s house (Table, batch 1). We performed bacterial culture on a pooled sample of half of these larvae (Table, sample 2) and then individually on adult flies that emerged from the other half (Table, samples 3–5). One green bottle fly (Lucilia sericata) (Figure 1) was caught alive in the house in a sterile container and laid eggs inside the container before dying (Table, batch 2). We isolated a Wohlfahrtiimonas sp. from 2 of 6 insect samples on blood agar plates (Table, samples 2–7) but not from any other samples,
including adult flies that emerged from the positive batch of larvae.

The isolates grew on blood agar, yielding colonies with a smooth center and rough edges, and displayed α hemolysis. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry yielded a presumptive result of \( W. chitiniclastica \) (online Technical Appendix). A phylogenetic analysis of the 16S rRNA gene sequence of all isolates (1,462 bp; see Figure 2 for GenBank accession numbers) showed that the most closely related type strains were \( W. chitiniclastica \) DSM 18708\( ^{T} \) (98.3% sequence similarity) and \( W. larvae \) JCM 18424\( ^{T} \) (97.3% sequence similarity) (Figure 2). The PFGE pattern indicated that all isolates from flies and fly larvae were indistinguishable and 74% similar to that of the patient isolate (online Technical Appendix).

Conclusions

Our isolates possibly represent a new species in the genus \( Wohlfahrtiimonas \) based on the percentage sequence similarity with \( W. chitiniclastica \) and \( W. larvae \) type strains (5). We isolated \( Wohlfahrtiimonas \) sp. from insects in the Americas and in a previously undescribed host, the green bottle fly (\( L. sericata \), Diptera: Calliphoridae). Previously, \( Wohlfahrtiimonas \) spp. have been identified in only 4 species of flies in Asia and Europe (\( Wohlfahrtia magnifica \), \( Chrysomya megacephala \), \( Hemetia illucens \), \( Musca domestica \)) (1–4), each representing a different fly family (Diptera: Sarcophagidae, Calliphoridae, Stratiomyidae, and Muscidae, respectively). We isolated a \( Wohlfahrtiimonas \) sp. from a larva hatched from eggs laid by a fly in a sterile container, providing evidence that \( Wohlfahrtiimonas \) can be transmitted vertically.

\( L. sericata \) has been associated with \( W. chitiniclastica \) infection in a patient with myiasis and bacteremia only once, in the United Kingdom (6), but a definitive link could not be established in that case because larvae from the patient had been discarded. The scarcity of reports of \( Wohlfahrtiimonas \) spp. infections might be attributable to the difficulty in laboratory identification (7,8) or because wound myiasis is routinely addressed with broad-spectrum antimicrobial drugs.

Because the pooled larvae (Table, sample 2) emerged as multiple fly species, we are unable to ascertain in which other species \( Wohlfahrtiimonas \) sp. growth occurred. \( Wohlfahrtiimonas \) was not isolated from these adult flies, which

Table. Culture results for \( Wohlfahrtiimonas \) spp. from samples collected from patient with septicemia and wound myiasis and the patient’s home, Washington, USA*

<table>
<thead>
<tr>
<th>Collection batch no.</th>
<th>Sample no.</th>
<th>Specimen</th>
<th>Specimen description</th>
<th>Culture ( Wohlfahrtiimonas ) spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>1</td>
<td>Blood</td>
<td>Isolate sent to public health laboratories from admitting hospital Larvae collected from underneath carpet where patient was found</td>
<td>Aerobic growth on blood agar (isolate 22912) Growth on diluted homogenate on blood agar at 25°C (isolate 22913) No growth</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Fly larvae (unidentified species, n = 20) House fly (( Musca domestica ))</td>
<td>emerged from larvae of sample no. 2</td>
<td>No growth</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>House fly (( Musca domestica ))</td>
<td>Emerged from larvae of sample no. 2</td>
<td>No growth</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>Unidentified species in the family Calliphoridae Calliphora vicina</td>
<td>Emerged from larvae of sample no. 2</td>
<td>No growth</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Green bottle fly and eggs (( Lucilia sericata ))</td>
<td>Emerged from larvae of sample no. 2</td>
<td>No growth</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Green bottle fly caught inside the patient’s home and laid eggs inside a sterile container</td>
<td>Not cultured</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>( Lucilia sericata ) larva</td>
<td>Larva obtained from the egg in batch no. 2</td>
<td>Growth on fifth wash (isolate 22914) and diluted homogenate (isolate 22915) on blood agar at 25°C</td>
</tr>
<tr>
<td>NA</td>
<td>8</td>
<td>Meat and fruit</td>
<td>Fed to flies from samples 3–7 were extracted</td>
<td>No growth</td>
</tr>
</tbody>
</table>

*NA, not applicable

Figure 1. Green bottle fly (\( Lucilia sericata \)), caught inside home of patient with septicemia and wound myiasis in Washington, USA. The fly laid eggs inside a sterile container, and \( Wohlfahrtiimonas \) spp. were isolated from a larva hatched from these eggs. Photo courtesy of T. Whitworth.
might be because the competent host was not present in the batch of larvae left to emerge or because of the association between Wohlfahrtimonas spp. and flies during successive developmental stages. Indeed, previous studies isolated W. chitiniclastica from the gut of larvae and adult flies (1,3,4), and Ignatzschineria spp. are hypothesized to play a role in larval development (9), indicating that these bacteria might belong to fly microbiota. In one study, the relative abundance of Ignatzschineria spp. fluctuated during life stages of L. sericata and was among the dominant bacterial genera during the larval and pupal life stages (10). Bacterial flora further decline during pupation, when reorganization of the intestinal tract leads to extrusion of the gut lining (11). These factors might explain why we did not isolate Wohlfahrtimonas spp. from the adult flies that emerged, or alternatively, our protocol might have been of insufficient diagnostic sensitivity to detect Wohlfahrtimonas spp. among adult flies.

The concurrent isolation of Wohlfahrtimonas sp. from the blood of a patient with myiasis and from fly larvae found at the patient’s home provides further evidence that fly larvae can act as vectors of Wohlfahrtimonas spp. Because PFGE patterns of the isolates obtained from the fly larvae and from the patient’s blood did not match, we cannot definitively identify the fly species that led to his infection.

We isolated Wohlfahrtimonas sp. from a patient’s blood along with other bacteria, precluding us from assessing the pathogenicity of our isolate. However, in 2 previous reports (12,13), W. chitiniclastica was the only bacterium isolated from the blood, indicating its pathogenic potential (online Technical Appendix Table 1).

Most cases of W. chitiniclastica infection have occurred among persons with a history of poor hygiene and exposed wounds (online Technical Appendix Table 1). Green bottle flies are among the most common species associated with myiasis in the United States (14), and risk for infection is expected during warm environmental conditions favorable to their development. In addition, green bottle fly larvae are the most commonly used larvae for maggot debridement therapy (15). Infection with Wohlfahrtimonas spp. should be considered as a potential risk for patients undergoing this therapy.

Acknowledgments
We thank Marisa D’Angeli, Marcia Goldof, and Travis Kushner for providing care to our maggots and flies; Denny Flodin-Hursh and Vance Kawakami for help with the field investigation; Terry Whitworth for assistance with fly identification and photography of L. sericata; Romesh Gautom, Tracie Gardner, and Marcia Goldof for their advice and manuscript review; and Scott Lindquist for all of the above. We are grateful to the staff of the Washington State Public Health Laboratories and the Centers for Disease Control and Prevention (CDC) Special Bacteriology Reference Laboratory for their assistance with laboratory work.

This study was supported by the Washington State Department of Health Public Health Laboratories and CDC.

About the Author
Dr. Bonwitt is a veterinarian and Epidemic Intelligence Service officer assigned to the Washington State Department of Health. His research interests are zoonotic and emerging infectious diseases and qualitative research at the animal–human interface.

References


Address for correspondence: Jesse H. Bonwitt, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E92, Atlanta, GA 30329-4027, USA; or Washington State Department of Health, 1610 NE 150th St, Shoreline, WA 98155, USA; email: jbonwitt@doh.wa.gov

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etymologia revisited

**Tularemia**

[tū-lə-rē-mē-ə]

An infectious, plague-like, zoonotic disease caused by the bacillus *Francisella tularensis*. The agent was named after Tulare County, California, where the agent was first isolated in 1910, and Edward Francis, an officer of the US Public Health Service, who investigated the disease. Dr. Francis first contracted “deer fly fever” from a patient he visited in Utah in the early 1900s. He kept a careful record of his 3-month illness and later discovered that a single attack confers permanent immunity. He was exposed to the bacterium for 16 years and even deliberately reinfected himself 4 times.

Tularemia occurs throughout North America, many parts of Europe, the former Soviet Union, the People’s Republic of China, and Japan, primarily in rabbits, rodents, and humans. The disease is transmitted by the bites of deerflies, fleas, and ticks; by contact with contaminated animals; and by ingestion of contaminated food or water.

Clinical manifestations vary depending on the route of introduction and the virulence of the agent. Most often, an ulcer is exhibited at the site of introduction, together with swelling of the regional lymph nodes and abrupt onset of fever, chills, weakness, headache, backache, and malaise.

Containment of Highly Pathogenic Avian Influenza A(H5N1) Virus, Lebanon, 2016

Zeina E. Farah, Omaya Khatib, Sahar Hamadeh, Khadija Ahmad, Bassel El Bazzal, Pierre Zalloua, Walid Ammar, Nada Ghosn

A preparedness plan for avian influenza A(H5N1) virus infection was activated in Lebanon in 2016 after reported cases in poultry. Exposed persons were given prophylaxis and monitored daily. A total of 185 exposed persons were identified: 180 received prophylaxis, 181 were monitored, and 41 suspected cases were reported. All collected specimens were negative for virus by PCR.

Highly pathogenic avian influenza A(H5N1) virus has caused ≈1,000 human infections since the first case was reported in 1997 (overall case-fatality rate 54%) (1). The number of affected countries increased during 2003–2008, and infections spread from East and Southeast Asia to West Asia and several regions of Africa (2). The highest cumulative number of confirmed human cases was reported in Egypt (3,4). This virus has been detected in poultry and wild birds in >50 countries, and the virus is epizootic in Bangladesh, China, Egypt, India, Indonesia, and Vietnam (5).

Since 2006, several infections with this virus have been detected in areas in the Middle East, including Iran, Jordan, Kuwait, Saudi Arabia, Turkey, the West Bank, the Gaza Strip, and Yemen. In 2015, outbreaks of infection with this virus were reported in the regions of the Gaza Strip, the West Bank, Turkey, and Iran (6). In addition, 6 outbreaks of infection with this virus were reported in Iraq during December 2015–February 2016 (6). Although no cases have been reported in Syria, the Food and Agriculture Organization of the United Nations is concerned about circulation of this virus because of extensive poultry production in Syria and the low biosecurity along its border with Iraq (6).

In Lebanon, no cases of infection with avian influenza A(H5N1) virus were reported in poultry or humans before 2016. On the basis of World Health Organization recommendations, a multisectoral preparedness plan was developed in 2007. The purpose of the plan was early detection and containment of any influenza outbreak caused by this virus and to ensure good coordination between public health sectors and ministries (agriculture, health, interior, and environment), the national influenza center, and laboratories (7).

The Study

On April 20, 2016, the Lebanese Ministry of Agriculture confirmed the presence of avian influenza A(H5N1) virus on 2 poultry farms in Nabi Chit village located in the Bekaa region, adjacent to the border of Lebanon with Syria. This virus caused the deaths of 20,000 domestic birds. Consequently, the preparedness plan was activated after a multisectoral meeting attended by representatives from the Ministry of Agriculture, the Ministry of Public Health, the Ministry of Interior, the Lebanese Army, and the local community.

The Ministry of Agriculture culled all domestic birds within a 3-km radius of infected farms. A total of 60,000 birds were culled from 7 farms (including the 2 infected farms) and 10 households with backyards. All farms were disinfected, and organic remains were disposed safely. Indemnity was provided to farmers by the Higher Relief Council. Epidemiologic investigations indicated that illegal movement of animals was the possible source of infection. The isolated virus (clade 2.3.2.1c) was similar to that detected in wild and domestic birds in Bulgaria, Romania, and Turkey during January–March 2015 (6).

The Ministry of Public Health plan focused on exposure identification, early detection of cases, and awareness. Identification of all exposed persons was crucial for providing prophylaxis and early detection of any human case. An exposed person was defined as any person who was exposed to 1) poultry or their remains or 2) environments contaminated by poultry feces in the area targeted by the Ministry of Agriculture, regardless of the use of personal protective equipment. Lists of target farms and households, as well as field teams working in culling and disinfection, were provided by the Ministry of Agriculture. A field team from the Ministry of Public Health visited the target farms and households and identified exposed persons. Exposed persons were also identified in 2 assigned health centers

Author affiliations: Ministry of Public Health, Bekaa, Lebanon (Z. E. Farah, O. Khatib, S. Hamadeh, K. Ahmad); Ministry of Agriculture, Beirut, Lebanon (B. El Bazzal); National Influenza Center, Beirut (P. Zalloua); Ministry of Public Health, Beirut (W. Ammar, N. Ghosn)

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1Preliminary results from this study were presented at the Mediterranean Programme for Intervention Epidemiology Training Annual Scientific Conference, December 6–8, 2016, Marrakech, Morocco.
in the village and neighborhood. The laboratory team that handled infected specimens and the veterinarian who examined infected poultry were also considered to be exposed persons.

Oseltamivir was used as prophylaxis and orally administered to persons >1 year of age up to 10 days after the last documented exposure (75 mg/d for adults and 35 mg/d for children). Exposed persons were monitored on a daily basis by telephone calls to assess their clinical status and to check for respiratory symptoms. For persons with signs or symptoms, oropharyngeal swab specimens were collected and tested at the National Influenza Center by using real-time reverse transcription PCR.

Three sensitization sessions for health professionals working in hospitals, medical centers, and private clinics were conducted to enhance detection and reporting of any suspected case of infection with influenza A(H5N1) virus. We adopted a highly sensitive H5N1 subtype case-patient definition. We defined a suspected case-patient as an exposed person with fever (temperature >38°C) or respiratory signs/symptoms (cough, dyspnea, coryza, sore throat) after April 20, 2016, who lived or worked in Nabi Chit village. In addition, 3 health education sessions were conducted for the public in collaboration with the local community to increase awareness about avian influenza.

We entered data into Epidata version 3.1 software (http://www.epidata.dk/download.php). We then analyzed data by using EpiDataStat version 2.2.1.171 software (http://www.advanceduninstaller.com/EpiData-Analysis-2_2_1_171-fb-08f19a7f340da85e8b2d85e90e006-application.htm). We then analyzed data by using EpiDataStat version 2.2.1.171 software (http://www.epidata.dk/download.php). We then analyzed data by using EpiDataStat version 2.2.1.171 software (http://www.advanceduninstaller.com/EpiData-Analysis-2_2_1_171-fb-08f19a7f340da85e8b2d85e90e006-application.htm).

We identified 185 exposed persons; 138 were male, 96 were Lebanese, and 88 were Syrian (mean age 30 y) (Table 1). A total of 151 had contact with poultry on farms or near their homes, and 34 were members of response teams. Of 185 exposed persons, 180 (97.3%) received antiviral prophylaxis (86.1% received the adult dose, and 13.9% received the pediatric dose), and 5 did not receive antivirus prophylaxis (4 were <1 year of age, and 1 refused prophylaxis). Duration of antivirus therapy ranged from 10 to 30 days. Of 185 exposed persons, 183 (99.0%) were monitored for ≥7 days, 1 was monitored for 4 days and then was lost to follow up, and 1 refused to participate. A total of 41 persons who received prophylaxis had signs/symptoms. The most commonly reported symptom was coryza (65.9%) (Table 2). Specimens were collected from 39 persons. In addition, 2 hospitalized suspected case-patients were tested. All results were negative for H5N1 subtype virus by real-time reverse transcription PCR.

On May 10, 2016, twenty days after the start of the outbreak in the first village, another focus appeared in the neighboring village of Sarin Tehta. We used the same case definition for suspected cases and detected 8 suspected case-patients; specimens were collected from 7, but no H5N1 cases were detected. The association between the 2 foci remains unclear. Since then, repetitive sampling conducted by the Ministry of Agriculture did not detect any infections in poultry. The 2 outbreaks were reported to the World Health Organization and the World Organisation for Animal Health within 24 h of confirmation (8), and containment was declared in June 2016.

**Conclusions**

Infections with avian influenza A(H5N1) virus in Lebanon in 2016 resulted in the deaths of 20,000 poultry and culling of 60,000 poultry. Although 185 persons were exposed to this virus, no human cases were identified. The reason behind the successful containment of the infection foci was early intervention of the Ministries of Agriculture and Public Health and use of a preparedness plan. This proactive effort enabled efficient coordination in the context of an acute shortage of human resources and rapid dissemination of false information about avian influenza. Because of high risk for new infections with this virus, the next step is to update the plan on the basis of lessons learned during the recent outbreaks.

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### Table 1. Demographic characteristics of 185 persons exposed to highly pathogenic avian influenza A(H5N1) virus, Lebanon, 2016

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>138 (74.6)</td>
</tr>
<tr>
<td>F</td>
<td>47 (25.4)</td>
</tr>
<tr>
<td>Mean age, y (range)</td>
<td>29.8 (2 mo–89 y)</td>
</tr>
<tr>
<td>Nationality</td>
<td></td>
</tr>
<tr>
<td>Lebanese</td>
<td>96 (51.9)</td>
</tr>
<tr>
<td>Syrian</td>
<td>88 (47.6)</td>
</tr>
<tr>
<td>Ethiopian</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>Exposure</td>
<td></td>
</tr>
<tr>
<td>Farms</td>
<td>78 (42.2)</td>
</tr>
<tr>
<td>Households</td>
<td>41 (22.2)</td>
</tr>
<tr>
<td>Neighborhood</td>
<td>32 (17.3)</td>
</tr>
<tr>
<td>Municipality team</td>
<td>18 (9.7)</td>
</tr>
<tr>
<td>Ministry of Agriculture team</td>
<td>14 (7.6)</td>
</tr>
<tr>
<td>Laboratory team</td>
<td>2 (1.1)</td>
</tr>
</tbody>
</table>

*Values are no. (%) unless otherwise indicated.

### Table 2. Reported signs/symptoms of 41 persons with suspected cases of infection with highly pathogenic avian influenza A(H5N1) virus, Lebanon, 2016

<table>
<thead>
<tr>
<th>Sign/symptom</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coryza</td>
<td>27 (65.9)</td>
</tr>
<tr>
<td>Cough</td>
<td>24 (58.5)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>18 (43.9)</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>11 (26.8)</td>
</tr>
<tr>
<td>Fever</td>
<td>7 (17.1)</td>
</tr>
</tbody>
</table>
About the Author
Ms. Farah is an epidemiologist in the Epidemiological Surveillance Program, Ministry of Public Health, Bekaa, Lebanon. Her research interests are surveillance during emergencies and humanitarian crises, zoonotic diseases, and mortality surveillance.

References

Address for correspondence: Zeina E. Farah, Epidemiological Surveillance Program, Ministry of Public Health, Bekaa Office, Old Governmental Hospital, Maalaka, Zahle, Bekaa 0096170187112, 009618809148, Lebanon; email: zfarah.esu@gmail.com
Emergomyces africanus in Soil, South Africa

Ilan S. Schwartz, Barbra Lerm, J. Claire Hoving, Chris Kenyon, William G. Horsnell, W. Joan Basson, Patricia Otieno-Odhiambo, Nelesh P. Govender, Robert Colebunders, Alfred Botha

We detected *Emergomyces africanus*, a thermally dimorphic fungus that causes an HIV-associated systemic mycosis, by PCR in 18 (30%) of 60 soil samples from a wide range of habitats in South Africa. Direct and indirect culture techniques were unsuccessful. Experimental intraperitoneal inoculation of conidia induced murine disease.

The newly described thermally dimorphic fungal genus *Emergomyces* comprises human pathogens that cause systemic mycoses in immunocompromised persons globally (1). Among these fungi, *Emergomyces africanus* (formerly *Emmonsia* sp. [2]) is the species responsible for the most human disease. HIV-associated emergomycosis is the most common endemic mycosis in South Africa and is associated with a high case-fatality ratio (3,4).

Although an environmental reservoir for *Es. africanus* has not been established, soil is presumed to harbor the mycelial phase (2). We tested soils in South Africa for *Es. africanus* by using molecular- and culture-based methods.

The Study

We collected 60 soil samples from various soil habitats around South Africa by convenience sampling; 82% percent of samples came from the Western Cape Province, with the remaining samples from Gauteng (7%), Eastern Cape (7%), KwaZulu-Natal (2%), and Northern Cape (2%) provinces. For each sample, we used sterile, plastic tubes to collect ≈100 mL of topsoil.

We extracted DNA from soil by using the ZR Soil Microbe DNA Miniprep Kit (Zymo Research, Irvine, CA, USA). DNA extraction was successful for 56 soil samples (93%). We subjected extracted genomic DNA (gDNA) to a nested PCR. To amplify the internal transcribed spacer (ITS) region of the ribosomal RNA, we used the universal primers ITS1 and ITS4 in the first reaction (5). We used an Applied Biosystems 2720 Thermal Cycler (Foster City, CA, USA); thermocycling conditions consisted of 95°C for 5 min, 30 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 45 s, and 72°C for 7 min. We subjected PCR products to amplification by using *Es. africanus*-specific primers (forward, 5′-CCTGGTTTGGGGAGAGGGGT-3′; reverse, 5′-CCGGGGGAGCTTGTGCGT-3′), followed by electrophoresis on a 2% agarose gel. We performed amplification as described, except with an annealing temperature of 57°C. PCR mixtures consisted of 10 µL 2× KAPA Taq ReadyMix (KAPA Biosystems, Wilmington, MA, USA); 1 µL of each primer (10 µmol/L; Inqaba Biotechnical Industries, Pretoria, South Africa); and 1 µL of extracted gDNA or ITS PCR product, in a final reaction volume of 20 µL. We sequenced amplified products and compared them using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR could detect as few as 10²–10⁴ conidia/10 g of soil (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/2/17-1351-Techapp1.pdf).

We plotted results of molecular testing and residential postal codes of persons with confirmed infections (Figure 1). We detected *Es. africanus* DNA in 18 (32%) of 56 soil samples representing all types of soil habitats tested (Table).

We used soil dilution plates prepared with Sabouraud agar (40 g/L glucose [Merck, Darmstadt, Germany], 10 g/L peptone [Merck], and 15 g/L agar) supplemented with 0.2 g/L chloramphenicol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to culture *Es. africanus* from 4 randomly selected soil samples. We incubated the resulting spread plates at 26°C, inspecting plates daily for 1 week and then twice weekly for an additional 3 weeks. All culture plates were rapidly overgrown by filamentous fungi other than *Es. africanus*.

To overcome rapid contamination, we used indirect culture methods. First, we used the flotation method adapted from Larsh et al. (7) to separate the conidia from other particles in the soil (online Technical Appendix). We plated the resulting soil suspensions on Sabouraud agar and brain heart infusion (BHI) plates and incubated them at 26°C, conducting daily examinations for fungal colonies.

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resembling *Es. africanus* (1). This preparation also resulted in rapid contamination of all plates.

Thereafter, we passaged soil suspensions through mice to screen out nonpathogenic soil organisms (8; online Technical Appendix). Animal studies were approved by the University of Cape Town’s Animal Ethics Committee (protocol 016–002). We created soil suspensions by using the flotation method and sampling from the bottom third of the column; penicillin G (1,000 IU/mL) and gentamicin (0.1 mg/mL) were included in the solution. We inoculated 1 mL of soil suspension intraperitoneally into each of 4 BALB/c or C57BL6 mice. We euthanized the mice after 2 weeks and plated livers, spleens, or both onto Sabouraud agar plates with and without chloramphenicol, which we then incubated at 30°C and 35°C–37°C. We inspected plates as described previously. Pilot studies demonstrated that this method could detect as few as 10^2 conidia in 10 g of soil (online Technical Appendix). Notably, in a pilot study in

<table>
<thead>
<tr>
<th>Soil habitat</th>
<th>Western Cape</th>
<th>Eastern Cape</th>
<th>Gauteng</th>
<th>KwaZulu-Natal</th>
<th>Northern Cape</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garden</td>
<td>6/30</td>
<td>0/2</td>
<td>1/4</td>
<td>–</td>
<td>–</td>
<td>7/36</td>
</tr>
<tr>
<td>Agricultural</td>
<td>3/5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3/5</td>
</tr>
<tr>
<td>Compost</td>
<td>3/5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3/5</td>
</tr>
<tr>
<td>Disturbed</td>
<td>1/2</td>
<td>0/2</td>
<td>–</td>
<td>0/1</td>
<td>–</td>
<td>2/5</td>
</tr>
<tr>
<td>Fynbos</td>
<td>1/2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/2</td>
</tr>
<tr>
<td>Veld</td>
<td>1/1</td>
<td>0/1</td>
<td>–</td>
<td>–</td>
<td>0/1</td>
<td>1/3</td>
</tr>
<tr>
<td>Rotting tree</td>
<td>1/1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1/3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1/3</td>
</tr>
<tr>
<td>Total</td>
<td>17/49</td>
<td>0/4</td>
<td>1/4</td>
<td>0/1</td>
<td>0/1</td>
<td>18/60</td>
</tr>
</tbody>
</table>

*Data represent number of samples in which *Es. africanus* was detected by nested PCR/total number of samples. Soil habitats: garden, soil from private gardens; agricultural, soil used for farming purposes; compost, soil rich in compost; disturbed, nutrient-poor uncultivated soil subjected to anthropogenic activities; fynbos, soil from a natural indigenous vegetation type endemic to the Cape Floristic region; veld, soil from grassland or uncultivated land; rotting tree, decaying woody debris; unknown, soil from unknown origin. –, sample not taken.

Figure 1. Results of molecular tests for the presence of *Emergomyces africanus* in soil samples in relation to residential locations of 14 patients diagnosed with emergomycosis (6), Cape Peninsula, Western Cape Province, South Africa. Black circles indicate *Es. africanus* detected in soil sample; white circles indicate *Es. africanus* not detected in soil sample; plus signs indicate residential locations of patients with emergomycosis. A larger cross indicates >1 infected patient at that particular location.
which BALB/c and C57BL/6 mice were challenged with graded doses of *Es. africanus* conidia, genetic background of mice influenced host susceptibility to the organism; C57BL/6 mice were more sensitive to infection and had significantly higher mortality and weight loss in response to the high dose of 10⁶ conidia compared with BALB/c mice (Figure 2).

We screened 26 soil samples for the presence of *Es. africanus* by using mouse passage. These samples included all 18 soil samples in which *Es. africanus* was detected by nested PCR, as well 8 soil samples that were PCR-negative. None of these samples, however, led to the isolation of *Es. africanus* through mouse passage.

**Conclusions**

*Es. africanus* is a newly described dimorphic fungal pathogen and causes an important HIV-associated systemic mycosis in South Africa (9). Many aspects of this organism remain unknown, including its ecologic niche. Our findings demonstrate that *Es. africanus* is present in a high proportion of soil samples collected from a range of habitats in South Africa, suggesting that soil might be a natural reservoir for this pathogen.

The isolation of pathogenic fungi from soil is challenging. Soil naturally contains a vast array of bacteria, viruses, fungi, and protozoa, all of which can interfere with or contaminate culturing the organism of interest (8). Since 1932, when Stewart and Meyer first cultured *Coccidioides immitis* from soil (10), flotation and animal passage has been the most robust method to isolate pathogenic fungi from soil. However, animal passage is laborious and expensive, can take months of turnaround time, requires special animal facilities, and results in discomfort and loss of life to laboratory animals, necessitating stringent ethics review (11).

Molecular detection is a valuable tool for establishing the presence of genetic material in environmental samples (11). In addition to high sensitivity, molecular detection has the advantages of being easy to apply, inexpensive, and rapid, and it can be performed in most laboratories. Alternatively, molecular detection lacks specificity because it cannot determine the viability (and hence infectivity) of the detected target (11). In our study, mouse passage of soil samples shown by nested PCR to contain *Es. africanus* genetic material did not result in the isolation of this fungus.

We have demonstrated that experimental infection with *Es. africanus* can produce pathology in mice. Moreover, susceptibility to disease appears to be mouse strain–dependent, with C57BL/6 mice being more susceptible than BALB/c mice.

This study has some limitations. The number of samples, and especially those from outside Western Cape Province, was relatively small, limiting inferences about the geographic range of *Es. africanus* in the environment. Moreover, our method of convenience sampling is prone to sampling bias. Nonetheless, this study is instructive for future ecologic studies, which should use random sampling to refine knowledge of the ecologic niche of this fungus.

In conclusion, this study demonstrates that *Es. africanus* can be frequently detected in a wide range of soils in South Africa. Moreover, our findings support the hypothesis that soil serves as a reservoir for this pathogen.

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About the Author
Dr. Schwartz is an infectious diseases physician and researcher with interests in emerging fungal infections, immunocompromised hosts, and global health. This work comprises part of a doctoral thesis at the Global Health Institute, Faculty of Medical Sciences, University of Antwerp.

References

Address for correspondence: Ilan S. Schwartz, University of Manitoba, 543 Basic Medical Sciences Bldg, 745 Bannatyne Ave, Winnipeg, Manitoba, Canada, R3E 0J9; email: ilan.steven.schwartz@gmail.com
Ceftriaxone-Resistant *Neisseria gonorrhoeae*, Canada, 2017

Brigitte Lefebvre, Irene Martin, Walter Demczuk, Lucie Deshaies, Stéphanie Michaud, Annie-Claude Labbé, Marie-Claude Beaudoin, Jean Longtin

We identified a ceftriaxone-resistant *Neisseria gonorrhoeae* isolate in a patient in Canada. This isolate carried the penA-60 allele, which differs substantially from its closest relative, mosaic penA XXVII (80% nucleotide identity). Epidemiologic and genomic data suggest spread from Asia. Antimicrobial susceptibility surveillance helps prevent spread of highly resistant *N. gonorrhoeae* strains.

Ceftriaxone is one of the last remaining treatments available for gonorrhea and a component of the recommended dual therapy with azithromycin in Canada (1). As of October 15, 2017, only 5 ceftriaxone-resistant *Neisseria gonorrhoeae* isolates had been reported worldwide (MIC range 0.5–2 mg/L) (2–6). The highest ceftriaxone MIC reported in Canada was 0.25 mg/L, representing only 0.45% (49/10,805) of all *N. gonorrhoeae* isolates tested during 2010–2016 (7). We describe ceftriaxone-resistant *N. gonorrhoeae* isolated in Canada.

The Study

An asymptomatic 23-year-old woman had a positive *N. gonorrhoeae* nucleic acid amplification test (NAAT) result (Cobas 4800 CTNG; Roche Diagnostics Canada, Laval, Canada) on January 17, 2017, obtained as part of a screening for sexually transmitted infections (STIs). Upon receiving the result, a physician instructed the patient to follow up with the STI clinic to have proper counseling. She visited on January 24 and obtained a prescription of single-dose cefixime 800 mg and azithromycin 1 g (recommended therapy according to Québec STI Treatment Guidelines) (8). Because the patient was from a low-prevalence population, the healthcare provider decided to perform a genital gonorrhea culture. The culture was positive for *N. gonorrhoeae* (no. GC063564/47707), thus confirming the positive NAAT result.

Because antimicrobial susceptibility testing (Etest, bioMérieux, Marcy l’Etoile, France) demonstrated nonsusceptibility of the isolate to ceftriaxone and cefixime but susceptibility to azithromycin, a second follow-up visit was requested by the practitioner. The second visit occurred February 7, 2017, and the patient was then prescribed empirically a single 2-g dose of azithromycin. Tests of cure by NAAT and cervical culture were performed during this visit and were negative for *N. gonorrhoeae*, indicating a successful initial treatment with cefixime and azithromycin administered 14 days earlier.

The patient reported a month-long sexual relationship 60 days before the STI screening. The partner was assessed by clinical examination and screening tests and treated with cefixime 800 mg and azithromycin 1 g on January 27, 2017. He was asymptomatic and his urinary NAAT screening result was negative for *N. gonorrhoeae*. He did not have sex with men or a sex worker but did report unprotected sexual activity during a trip to China and Thailand in November 2016, before his relationship with the patient in this case study. Information about antimicrobial drug use during his trip to Asia was not available. He was followed up in February 2017, and NAAT test results of his urine and pharyngeal specimens were again both negative for gonorrhea. Public health professionals also contacted the case study patient’s next-to-last partner (5 months earlier), and his screening result was also negative for *N. gonorrhoeae*.

The bacterial isolate from our patient was confirmed to be *N. gonorrhoeae* by API NH (bioMérieux), VITEK (bioMérieux), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (bioMérieux), and whole-genome sequencing. Antimicrobial susceptibilities for isolate GC063564 were confirmed by using the agar dilution method according to the Clinical and Laboratory Standards Institute protocol (9). The strain was resistant to ceftriaxone (MIC 1 mg/L), cefixime (MIC 2 mg/L), ciprofloxacin (MIC 32 mg/L), and tetracycline (MIC 4 mg/L) and susceptible to azithromycin (MIC 0.5 mg/L). The Clinical and Laboratory Standards Institute does not have a resistance breakpoint for cefixime or ceftriaxone but defines susceptibility...
at an MIC $\geq 0.25$ mg/L. The European Committee on Antimicrobial Susceptibility Testing defines cefixime and ceftriaxone resistance at an MIC $>0.125$ mg/L (10), and the World Health Organization defines decreased susceptibility to cefixime as MIC $\geq 0.25$ mg/L and to ceftriaxone as MIC $\geq 0.125$ mg/L (11). Although the defined resistance breakpoint is not consistent among these organizations, a ceftriaxone MIC 1 mg/L has been previously reported as resistance (2,6).

We performed molecular typing in silico using whole-genome sequence data (BioProject PRJNA415047). We sequenced the isolate with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) and used genomic quality, assembly, and annotation pipelines as previously described (12). The multilocus sequence type (ST) of GC063564 was ST1903, and N. gonorrhoeae multiantigen sequence type (MAST) was ST1614. Using a novel antimicrobial genomic sequence analytic tool called NG-STAR (N. gonorrhoeae Sequence Typing for Antimicrobial Resistance) (13), we identified the isolate GC063564 as NG-STAR ST233, which contains a mosaic penA allele, mtrR-35A deletion, porB G120K/A121D, pona L421P, gyrA S91F/D94A, parC S87R, and no 23S rRNA A2059/C2611 mutations. The GC063564 isolate also had an rpsJ V57M mutation, and tetM was not detected.

The molecular antimicrobial resistance profile corresponds to the MICs determined phenotypically (13). The multilocus sequence type (ST1903) and mosaic penA allele (penA-60) of this isolate from Canada were identical to those of the ceftriaxone- and multidrug-resistant N. gonorrhoeae FC428 isolated in 2015 in Japan (14). PenA-60 has a mosaic penicillin-binding protein 2 structure with 2 key mutations (A311V and T483S) that confer ceftriaxone resistance. PenA-60 differs substantially from previously described penA types (5), resembling only 80% of the closest-related mosaic allele penA-XXVII. Bacterial isolates GC063564 and FC428 had identical porB1b NG-MAST alleles but different tpbB alleles (GC063564 had tpbB-33; FC428 had tpbB-21), resulting in different N. gonorrhoeae MAST profiles (ST-1614 for GC063564 and ST-3435 for FC428). The variation in the N. gonorrhoeae MAST types between the 2 isolates collected 2 years apart is not unexpected, considering the highly recombinant nature of the N. gonorrhoeae genome (15).

Conclusions

We identified a ceftriaxone-resistant N. gonorrhoeae isolate in Canada that contained the penA-60 allele formerly reported in Japan in 2015. Epidemiologic information suggests international spread of a penA allele associated with high-level ceftriaxone resistance. Antimicrobial susceptibility surveillance successfully identified this novel isolate introduced into Canada and prompted public health officials to rapidly conduct an investigation to prevent further spread in the community. In an era of multidrug-resistant gonorrhea, ongoing antimicrobial susceptibility surveillance of N. gonorrhoeae is critical to support treatment guidelines, public health intervention, and protection.

About the Author

Dr. Lefèvre is the head of the Antibiotics Resistance Department at the Laboratoire de Santé Publique du Québec, Québec, Canada, and in charge of the provincial surveillance programs, which investigate invasive Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae infections and monitor for resistance of Neisseria gonorrhoeae and carbapenemase-producing enterobacteria. Her research interests include antimicrobial drug resistance of pathogenic agents of public health interest.

References


Address for correspondence: Brigitte Lefebvre, Institut National de Santé Publique du Québec, Montréal, Laboratoire de Santé Publique du Québec, 20045, Chemin Sainte-Marie, Sainte-Anne-de-Bellevue, Québec H9X 3R5, Canada; email: brigitte.lefebvre@inspq.qc.ca

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- Variably Protease-Sensitive Prionopathy, a Unique Prion Variant with Inefficient Transmission Properties
- Geographic Divergence of Bovine and Human Shiga Toxin–Producing Escherichia coli O157:H7 Genotypes, New Zealand
- Bacterial Pathogens Associated with Hidradenitis Suppurativa, France
- Replication and Shedding of MERS-CoV in Upper Respiratory Tract of Inoculated Dromedary Camels
- Transmission Characteristics of Variably Protease-Sensitive Prionopathy
- Seroconversion for Infectious Pathogens among UK Military Personnel Deployed to Afghanistan, 2008–2011
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EMERGING INFECTIOUS DISEASES https://wwwnc.cdc.gov/eid/articles/issue/20/12/table-of-contents
Cysticercosis in Shandong Province, Eastern China

Gongzhen Liu, Yanshen Li, Yong Cui, Bingcheng Huang, Hongfa Wang, Yanping Chen, Jingxuan Kou, Fuyong Wang, Chongxing Zhang, Yong Huang, Yin Li, Meng Wang, Qingkuan Wei, Jin Li, Hui Sun, Kun Yin, Guihua Zhao, Yan Jiang, Xin Mao, Zhenhua Yu, Xin Liu

Author affiliations: Shandong Institute of Parasitic Diseases, Shandong Academy of Medical Sciences, and World Health Organization Collaborating Centre on Vector-Borne Diseases and Food-Borne Parasitic Diseases, Jinan, China (G. Liu, Y. Cui, B. Huang, H. Wang, Y. Chen, J. Kou, F. Wang, C. Zhang, Y. Huang, Q. Wei, J. Li, H. Sun, K. Yin, G. Zhao, Z. Yu, X. Liu); Yantai University, Yantai, China (Yanshen Li, Y. Mao); China Animal Health and Epidemiology Center, Qingdao, China (Yin Li); Qufu Normal University, Jinan, China (M. Wang); The People’s Liberation Army No. 405 Hospital, Yantai (Y. Jiang)

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We analyzed demographic and clinical data and estimated the incidence of cysticercosis in Shandong Province, China, during 1975–2014. Our analyses showed that a cysticercosis-endemic area is present in Shandong Province, especially in its western regions. Improved surveillance and control are needed to address the elevated risk for cysticercosis in this region.

Cysticercosis is an infection of human tissues caused by a tapeworm parasite, *Taenia solium*, commonly found in pork meat. Patients initially see its symptoms in different areas of the human body as cysts (1). Cysticercosis is a major cause of epilepsy in low-income countries and is endemic to countries in Latin America, sub-Saharan Africa, and large regions of Asia, including China and India (2). Although *T. solium* tapeworms had virtually disappeared from industrialized countries, increased immigration from cysticercosis-endemic areas has led to a resurgence of cysticercosis in North America, Europe, and Australia (3,4).

Although cysticercosis is one of the most severe tropical diseases in China, few epidemiologic studies of cysticercosis patients have been performed. We analyzed patients’ demographic and clinical data to estimate the cysticercosis incidence for risk in Shandong Province during 1975–2014.

Shandong Province encompasses 91 counties and 17 major cities. We obtained cysticercosis data from Shandong Institute of Parasitic Diseases, the only professional institution for systematic diagnosis and treatment for cysticercosis in Shandong Province during the study period. Any cysticercosis patient identified in the 17 major cities in Shandong Province was sent to and registered at Shandong Institute of Parasitic Diseases for therapy. A confirmed case was considered on the basis of several criteria that included the following (5): 1) surgically removed nodules identified as *Cysticercus cellulosae* by tableting, an incubation test, or histopathologic examination; 2) serum or cerebrospinal fluid positive by immunologic examination; 3) patient history of travel to or residence in a disease-endemic area and a history of tapeworms or contact with tapeworm-infected patients; 4) positive results by computed tomography or magnetic resonance imaging for neurocysticercosis or for B-mode ultrasound for cutaneous muscular or ophthalmic cysticercosis; and 5) diagnosis of cysticercosis supported by clinical symptoms, which could include subcutaneous or muscular nodules, headache, dizziness, epilepsy, or visual disturbance. All cysticercosis cases were recorded in medical records each year. Moreover, the source population for our data represented the total population of Shandong Province.

We calculated the 40-year incidence rate by dividing the number of newly diagnosed cases during the examined time period by the province’s midperiod population (i.e., the 1995 population). In total, 1,952 cysticercosis case-patients were identified. The crude 40-year incidence rate was, therefore, 22.4 (95% CI 21.4–23.4) cases per 1 million population.

We further calculated incidence rates by age, sex, and residence. Of the 1,952 case-patients, 1,288 (66%) were male and 664 (34%) were female, and more patients lived in rural areas (69.0%) than in urban areas (31.0%). Study data indicated a higher incidence rate for male (29.1 [95% CI 27.5–30.7] cases/1 million population) than female (15.5 [95% CI 14.4–16.7]) patients and for rural residence (27.9 [95% CI 25.7–30.1]) than for urban residence (20.6 [95% CI 19.5–21.7]). For age, we observed the highest incidence rate for the 30–39-year age group (37.2 [95% CI 34.1–40.3] cases/1 million population), followed by the 40–49-year age group (32.7 [95% CI 29.5–35.9]) and the 20–29-year age group (26.6 [95% CI 24.0–29.1]). The <1–9-year age group had the lowest incidence risk (6.7 [95% CI 5.4–8.1]) (Table).


Other studies have found similarly elevated rates of cysticercosis in Shandong Province (6–8). Our data highlight several distribution features of cysticercosis in this province, including an increased incidence among men, consistent with findings of a previous report (8). However, our data showed elevated incidence in different age groups.
and regions than the study by Chen et al., in which the 10–29-year age group and middle regions of the province showed the highest incidence rates (6).

Our study has a few limitations. First, the long, asymptomatic latent period of cysticercosis affects diagnostic efficiency and age-specific incidence estimates. Second, our data were incomplete because of some missing information for cases we identified. Third, independent confirmation might affect incidence estimates from early in the study period. However, our multidiagnostic approach substantially reduced misdiagnosis rates and increased the efficiency of diagnosing cysticercosis (9).

In summary, our analyses show that Shandong Province has been a cysticercosis-endemic area for many years. Improved surveillance and control are needed to address the elevated risk for cysticercosis in western regions of this province.

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About the Author
Dr. Gongzheng Liu is an assistant researcher in the field of pathogen biology, Shandong Institute of Parasitic Diseases, Shandong Academy of Medical Sciences, World Health Organization Collaborating Centre on Vector-Borne Diseases and Food-Borne Parasitic Diseases, Jining, China. His current research interest is the role of invasive parasites and interactions with host cells.

References

Address for correspondence: Zhenhua Yu or Xin Liu, Shandong Institute of Parasitic Diseases, Shandong Academy of Medical Sciences, World Health Organization Collaborating Centre on Vector-Borne Diseases and Food-Borne Parasitic Diseases, Jining 272033, China; email: sfyzzh123@163.com or liux3276@163.com

Rickettsia africae and Novel Rickettsial Strain in Amblyomma spp. Ticks, Nicaragua, 2013

Helena Vogel, Janet Foley, Christine V. Fiorello
Author affiliation: University of California, Davis, California, USA
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We report molecular detection of Rickettsia africae in Amblyomma ovale ticks from Nicaragua and a novel rickettsial strain in an A. triste tick. Of 146 ticks from dogs, 16.4% were Rickettsia PCR positive. The presence of Rickettsia spp. in human-biting ticks in Nicaragua may pose a public health concern.

Obligately intracellular Rickettsia spp., typically transmitted by ticks, cause a multitude of mild to severe rickettsial diseases in humans and other animals. Novel Rickettsia species have been identified through molecular
We collected ticks in 2013 from villages at similar latitude and longitude measured by using global positioning system (GPS): Arang Dak (14.51583, −84.99944), Amak (14.06542, −85.142233), and Raiti (14.59464, −85.02772) (Table). Arang Dak is the smallest of the 3 villages and closest to the densest part of the rainforest; Raiti is the largest and most developed village of the 3 and is situated on a heavily traveled route through the reserve. We obtained owner consent before physical examination and sampling of ticks from dogs and stored ticks in 70% ethanol. In the laboratory, we identified ticks for sex, life stage, and sampling of ticks from dogs and stored ticks in 70% ethanol. In the laboratory, we identified ticks for sex, life stage, and species by using a key (3) and screened tick DNA for Rickettsia spp. by real-time PCR (4). Rickettsia-positive samples were further tested by conventional PCR targeting the outer membrane protein A gene (ompA) (5). We also amplified the rpmB and 17kDa genes of the rickettsia in the Amblyomma triste ticks we recovered (4). We sequenced each amplicon by using the forward primer at University of California Davis Sequencing (Davis, CA, USA) and compared sequences to those in the GenBank database by using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov).

Of 146 ticks from 40 dogs, 126 (86%) were A. ovale, 12 were A. sculptum, and 7 were A. triste. We detected rickettsial DNA in 24 (16.4%, 95% CI 11.0%–23.7%) of the 146 ticks: 18 A. ovale, 5 A. sculptum, and 1 A. triste. We deposited rickettsial sequences from these ticks into GenBank (accession no. KX530472, KX576685, and KX576686).

By location, the PCR prevalence was 25.5% (95% CI 15.1%–39.3%) in Raiti, 16.0% (95% CI 5.25%–36.9%) in Amak, and 9.09% (95% CI 3.75%–19.4%) in Arang Dak. These differences were statistically significant (p = 0.05 by Fisher exact test). The finding of highest prevalence in the most populated community is consistent with peridomestic animals maintaining the infection, and the rainforest and remote wildlife not being significant sources.

For the 576-bp ompA sequence, all from A. ovale ticks were identical and were 99.6% homologous with sequences from GenBank identified as R. africae. R. africae has not been reported in A. ovale ticks or in North, Central, or South America. R. africae causes a mild rickettsiosis known as African tick-bite fever and was first described in a patient in the Western Hemisphere in 1998 (1). R. africae has been detected in A. variegatum ticks by using PCR and in humans in Guadeloupe by using serology (6) and more recently in A. loculosum ticks from New Caledonia (7). In Brazil, adult A. ovale ticks bite humans most frequently and are present from the borders of Mexico to those of Argentina (8). A. ovale is a common human-biting tick in Central and South America and poses a public health concern.

Sequences of ompA in 2 of 5 PCR-positive A. sculptum matched 99.6% to Candidatus R. amblyommi in GenBank (ompA of the other samples did not amplify, likely because they were relatively weak on real-time PCR). Candidatus R. amblyommi is common among Amblyomma spp. ticks in the New World and was reported in A. sculptum ticks in Brazil (9). Candidatus R. amblyommi has unknown pathogenicity but has been implicated in rickettsiosis cases in humans (9).

The ompA amplicon from A. triste ticks matched Rickettsia sp. ARAGAOI; sequencing of the rpmB and 17kDa genes was unsuccessful. This rickettsial species was originally described in marsupials in Brazil (9). Further monitoring of tick vectors in this remote area is needed to characterize local risk and detect possibly emerging vector-borne disease.

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About the Author
Miss Vogel is a veterinary student at University of California Davis School of Veterinary Medicine and works as a laboratory intern in J.F.’s laboratory. Her research interest is the ecology of emerging vectorborne diseases.

References

Address for correspondence: Janet Foley, University of California, Department of Medicine and Epidemiology, 1320 Tupper Hall, Davis, CA 95616, USA; email: jefoley@ucdavis.edu

Amebaborne *Attilina massiliensis* Keratitis, France

Alexandre Battaini, Bernard La Scola, Gaëlle Ho Wang Yin, Louis Hoffart, Michel Drancourt

Author affiliations: Aix Marseille Université, Marseille, France (A. Battaini, B. La Scola, M. Drancourt); Aix-Marseille University–APHM, Hôpital de la Timone, Marseille (G.H.W. Yin, L. Hoffart)

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We report a case of *Acanthamoeba castellani* keratitis in a person who wore contact lenses. The amebae hosted an ameba-resistant bacterial symbiont, provisionally named “*Attilina massiliensis,*” a yet undescribed α-Proteobacterium.

A mebal keratitis is an aggressive ocular infection that can lead to blindness (1). It is usually associated with wearing soft contact lenses; Dart et al. documented that in countries with a high prevalence of contact lens wear, 85%–88% of *Acanthamoeba* keratitis cases occurred in contact lens users (1). These amebae host ameba-resistant bacteria, and increase their pathogenicity to the host (2). Ameba hosting intra-amebal microorganisms have been rarely documented in cases originating in contaminated contact lenses (3) and never in mixed keratitis. We report a case of mixed ameba–amebal-resistant bacterial keratitis.

A 17-year-old woman who wore contact lenses consulted the ophthalmology department of the clinic associated with Hôpital de la Timone, Marseille, France, in July 2016, after experiencing 1 month of keratoconjunctivitis symptoms related to an undocumented clinical diagnosis of herpes virus keratitis of the left eye. The patient had been prescribed a 1-week treatment with valacyclovir (3×/d) and a corneal dressing. Examination of the left eye showed 4/10 visual acuity; the right eye was normal. Slit-lamp examination showed a central radial keratoneuritis, central corneal edema, central diffuse infiltrate, and a punctate superficial keratitis with no predescemetic precipitates and no satellite lesions (Figure). The patient was admitted to the hospital and was administered hourly topical treatments of polyhexamethylene biguanide eye drops, hexamidine, and 1% atropine. The patient, whose diagnosis was early-stage *Acanthamoeba keratitis* infection, was discharged after 5 days of treatment; a corneal swab sample at discharge was negative for herpes virus, varicella zoster virus, adenovirus, enterovirus, cytomegalovirus, and *Chlamydia trachomatis.* Follow-up 7 days later yielded reduced symptoms. We followed up on the patient biweekly and slowly tapered drugs over 4 months; the previously negative
pathogen tests remained negative. However, culture yielded *Corynebacterium ureicelerivorans*, identified on the basis of a 98.7% partial rpoB gene sequence similarity with the reference sequence (GenBank accession no FJ392018.1), *Acanthamoeba castellani*, identified on the basis of a 99% 18S rRNA gene sequence similarity with reference genotype T4 (GenBank accession no. U07416.1). Further culture of the amebal isolate in sterile peptone-yeast-extract-glucose broth (Culture-Top, Courtaboeuf, France) by using both optic microscopy and electron microscopy (Figure) yielded an intra-amebal Holosporaceae bacterium observed in the cytoplasm of the ameba. This symbiont, a yet undescribed α-Proteobacterium of the family of Holosporaceae that had been provisionally named “*Attilina massiliensis,*” was identified on the basis of a 100% 16S rRNA gene similarity with the reference sample (GenBank HM138368). After subculture, this “*A. massiliensis*” isolate was shown to be flagellated and highly mobile. Moreover, it was shown to lyse the *A. castellani* Neff strain (ATCC 30010), an observation suggesting motility-linked pathogenicity. Culturing the lens storage case yielded *Serratia liquefaciens* and *Stenotrophomonas maltophilia*, identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (4).

In this patient, mixed *C. ureicelerivorans*, *A. castellani*, and “*A. massiliensis*” keratitis was firmly documented by the isolation and culture of the pathogens identified by using appropriate controls and validated protocols. The fact that we had never documented such an infection in our laboratory excludes mere false-positive results caused by contamination. *C. ureicelerivorans* has mainly been reported to cause septicemia, not ophthalmologic infections (5,6). Its sources and potential relationships with waterborne amebae are unknown. *Acanthamoeba* spp. amebae cause severe keratitis, which may cause visual loss (1). *Acanthamoeba* spp. amebae are ubiquitous in tap water (7). Tap water could be a source of contamination of contact lenses through the wearer’s handwashing habit before lens manipulation (8). Wide varieties of amebae have been documented in contaminated contact lenses, eventually leading to amebal keratitis outbreaks (9).

Culturing an *A. castellani* ameba isolated from a diseased cornea yielded “*A. massiliensis,*” which we isolated once 8 years ago from an *Acanthamoeba polyphaga* ameba collected from a contact lens storage case that belonged to a patient unrelated to the case-patient we report here. The potential for this emerging ameba-resistant bacterium to cause keratitis remains to be analyzed, but we observed that this mobile symbiont lysed the reference amebal strain, demonstrating its cytopathogenicity. Also, ameba-resistant organisms do comprise acknowledged opportunistic pathogens (2), and corneal toxicity was previously reported as significantly higher for *Acanthamoeba*-hosting endosymbionts (10). This investigation illustrates that amebae present in cases of keratitis may shelter organisms that should be provisionally regarded as potential opportunistic pathogens under these circumstances.

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Influenza D Virus in Cattle, Ireland

Orla Flynn, Clare Gallagher, Jean Mooney, Claire Irvine, Mariette Ducatez, Ben Hause, Guy McGrath, Eoin Ryan

Author affiliations: Department of Agriculture, Food, and Marine Laboratory Services, Celbridge, Ireland (O. Flynn, C. Gallagher, J. Mooney, C. Irvine, E. Ryan); École Nationale Vétérinaire de Toulouse, Toulouse, France (M. Ducatez); Kansas State University College of Veterinary Medicine, Manhattan, Kansas, USA (B. Hause); University College Dublin, Dublin, Ireland (G. McGrath)

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We detected influenza D virus in 18 nasal swab samples from cattle in Ireland that were clinically diagnosed with respiratory disease. Specimens were obtained from archived samples received for routine diagnosis during 2014–2016. Sequencing showed that virus from Ireland clustered with virus sequences obtained in Europe within the D/swine/OK/1334/2011 clade.

Influenza D virus is a recently characterized addition to the family Orthomyxoviridae. This virus was originally detected in pigs in the United States (1); however, cattle are now believed to be the main reservoir species (2). Evidence suggests that this virus plays a role in bovine respiratory disease, although experimentally, it caused only mild disease by itself (3). Influenza D virus has been found to be associated with respiratory disease in feedlot cattle (4).

The zoonotic potential of influenza D virus remains unclear; this virus can replicate in ferrets (a model for human influenza infection), and a seroprevalence of 91% was found in persons working closely with cattle (5). However, a study of 3,300 human respiratory samples from Scotland did not detect any influenza D virus–positive samples (6). This virus has been detected in bovine samples in several other countries, including France (7), Italy (8), Japan, and China (9).

Cattle are a major part of the economy in Ireland, where there are ≈7 million (10). To determine whether influenza D virus was present in cattle in Ireland and to investigate epidemiologic factors that might be related to this virus, we conducted a cross-sectional study by using 320 nasal swab specimens from cattle with respiratory disease that were submitted to the Central Veterinary Research Laboratory (Celbridge, Ireland) for routine bovine virus pathogen testing during 2014–2016.

We tested swab specimens by using real-time PCR for influenza D virus as described (1). We selected samples...
with a cycle threshold (Ct) ≤25 for further molecular characterization by using 3 primer sets (7) that are specific for the 7 virus gene segments. We performed cDNA synthesis by using qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) and PCR amplification by using AccuStart II PCR ToughMix (Quantabio). We processed PCR products by using Illustra ExoProStar 1-Step (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s instructions before Sanger sequencing. We analyzed sequence data by using DNASTAR Lasergene 12 SeqMan Pro (DNASTAR, Madison, WI, USA) and performed sequence alignment by using ClustalW in MEGA 5.01 (http://www.megasoftware.net/). We constructed phylogenetic trees by using the maximum-likelihood method in MEGA 5.01.

Herd information for 2015 was available for 84 herds of origin of these nasal swab specimens. Data were obtained from the Animal Identification and Movement System database of the Department of Agriculture, Food, and Marine of Ireland (https://www.agriculture.gov.ie/animalhealthwelfare/animalidentificationmovement/cattle/irishbovineanimalidentificationsystem-overview/). We performed univariate statistical analysis by using Stata/SE14.1 (StatCorp LLC, College Station, TX, USA). Herd factors investigated for a possible association with influenza D virus herd status were herd size, numbers of stillbirths, dairy cows in herd, beef cows in herd, inward movements from markets, inward movements to farm, and carcasses moved to knackeries.

A total of 18/320 samples were positive for influenza D virus by PCR. Of the 18 positive samples, 13 were also positive by PCR for 1 or 2 other viral pathogens (bovine herpesvirus 1, parainfluenza 3 virus, bovine coronavirus, bovine respiratory syncytial virus, bovine viral diarrhea virus). Seven of the influenza D virus–positive samples were from calves, 2 from weanlings, and 1 from a cow; other specimens were not described by animal age. Nine of the influenza D virus–positive samples had a Ct ≤25 and were selected for sequencing. We obtained partial sequences for 5 samples and deposited the sequences in GenBank (accession nos. KY992090–KY992103).

Phylogenetic analysis (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/2/17-0759-Techapp1.pdf) showed that the 5 influenza D virus isolates from Ireland clustered with viruses from Europe in the D/swine/OK/1334/2011 clade. We also determined the distribution of positive and negative samples at county level in Ireland (online Technical Appendix Figure 2).

Herd information was available for 10 of the influenza D virus–positive herds and for 74 comparison herds (for which the nasal swab specimens were negative for influenza D virus but which had clinical respiratory disease outbreaks). We found no associations between herd characteristics and influenza D virus status; this finding was determined by evaluating mean values with 95% CIs for infected herds and noninfected herds.

This study confirms the emergence of influenza D virus in Ireland. Presence of the virus in nasal swab specimens submitted from routine respiratory disease cases supports the hypothesis that this virus plays a role in the bovine respiratory disease complex. Analysis of herds for infected cattle did not show any epidemiologic differences between influenza D virus infection and infection with other common respiratory viral pathogens. This finding is consistent with the hypothesis that influenza D virus might have limited effect by itself but can potentiate effects of other respiratory pathogens in causing respiratory disease (3,4).

Detection of 2 virus lineages in Ireland clustering with viruses isolated in Europe within the D/swine/OK/1334/2011 clade raises the issue of how influenza D virus might spread internationally. Surveillance efforts could be targeted for data on trade of live cattle, which is extensive within Europe. Further research is planned to investigate the seroprevalence of influenza D virus in cattle in Ireland and to determine the effect of this virus in a cattle farming context in this country.

About the Author
Ms. Flynn is a scientist at the Central Veterinary Research Laboratory, Department of Agriculture, Food, and Marine Laboratory Services, Celbridge, Ireland. Her research interests include avian and mammalian pathogens affecting farmed animals.

References
Novel Streptococcus suis Sequence Type 834 among Humans, Madagascar

Mihaja Rabarahona, Saïda Rasoanandrasana, Vonintsoa Lalaina Rahajamanana, Felana Ranaivo-Rabekotokany, Volatiana Andriananarivo, Betra Angelot Rakotomalala, Mamý Jean de Dieu Randria, Luc Rakotovao, Corinne Marois-Créhan, Véronique Toqueville, Fabrice Touzain, Mala Rakoto-Andrianarivo

Author affiliations: Hôpital Universitaire Joseph Rasetra, Befelatana, Antananarivo, Madagascar (M. Raberahona, S. Rasoanandrasana, F. Ranaivo-Rabekotokany, V. Andriananarivo, M.J.D. Randria, L. Rakotovao); Hôpital Universitaire Mère-Enfants Tsalalalana, Antananarivo (V.L. Rahajamanana); Centre d’Infectiologie Charles Mérieux, Antananarivo (F.A. Rakotomalala, M. Rakoto-Andrianarivo); Agence Nationale de Sécurité Sanitaire de l’Alimentation, de l’Environnement et du Travail, Ploufragan, France (C. Marois-Créhan, V. Toqueville, F. Touzain); Université Européenne Bretagne-Loire, Rennes, France (C. Marois-Créhan, V. Toqueville, F. Touzain)

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Two cases of meningitis caused by Streptococcus suis occurred in Madagascar, 1 in 2015 and 1 in 2016. We report the characterization of the novel sequence type, 834, which carried the mpp+/sly+/lep+ virulence marker and a mutation G→T at position 174, leading to a substitution mutS1 to mutS284.

Streptococcus suis is a common pathogen among pigs that can be transmitted to humans, in whom it causes invasive infection. In recent years, cases among humans have been reported worldwide, and a large outbreak occurred in China in 2005 (1). In some areas, S. suis appeared as the most common etiology of adult meningitis (2). Data from Africa were scarce until the recent report of 15 cases in Togo (3). We report 2 cases of S. suis meningitis in Antananarivo, Madagascar.

In March 2015, a 24-year-old man (patient 1) was admitted to the Infectious Diseases Unit of Befelatana Hospital (Antananarivo) seeking treatment for fever, headache, and unilateral sixth nerve palsy. One year later, in March 2016, a 60-year-old woman (patient 2) was admitted to the same unit with meningitis and sudden hearing loss. Patient 1 worked in a slaughterhouse and patient 2 as a cook; both were frequently exposed to pork meat. Both patients were febrile (temperature ≥39.9°C) and confused and had a score of ≤13 out of 15 on the Glasgow Coma Scale. Laboratory results for lumbar puncture showed turbid cerebrospinal fluid (CSF) with increased cell numbers (446 cells/µL), 56% neutrophils for patient 1; 1,180 cells/µL, 86% neutrophils for patient 2 [reference range <10/µL]; high protein levels (1.4 g/L for patient 1, 2.34 g/L for patient 2 [reference range 0.15–0.45 g/L]); and low glucose levels (0.28 mmol/L for patient 1; 0.73 mmol/L for patient 2 [reference range 2.7–4.2 mmol/L]). CSF Gram stain results showed gram-positive diplococci resembling Streptococcus pneumoniae. CSF samples inoculated onto chocolate agar showed microbial growth that was further found to contain optochin-resistant α-hemolytic streptococci. In-house real-time PCR on CSF and colonies, targeting the specific gene for S. pneumoniae, were negative. The colonies were finally identified as S. suis by using ApiStrep20 (bioMérieux, Marcy l’Étoile, France) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with a log (score) value of spectra >2.3 (cutoff ≥2.0). Both patients achieved overall recovery after a complete course of intravenous ampicillin (patient 1) or ceftriaxone (patient 2), but a mild hearing deficit acquired during the infection remained for both patients.

We retrieved only strain Ss1223, isolated from patient 2, for further investigation. By using slide agglutination with type-specific hyperimmune serum and specific multiplex PCR (4), we identified the Ss1223 strain as S. suis serotype 2. We performed whole-genome sequencing by
using an Ion Torrent Proton sequencer (Thermo Fischer Scientific, Waltham, MA, USA). After cleaning data with Trimmomatic 0.36 (5), we align reads on of S735 *S. suis* sequence type ST1 European reference strain (GenBank accession no. NC_018526.1) by using the maximal exact matches algorithm of Burrows-Wheeler Aligner software version 0.7.15-r1140 (https://omictools.com/burrows-wheeler-aligner-tool), downsampled to fit an estimated coverage depth of 80× before assembly. SPAdes 3.8.1 (6) assembly was deposited in GenBank (accession no. NHOL00000000). By applying the previously described pipeline (7), we confirmed the Ss1223 strain as serotype 2 and a novel sequence type (ST), 834, bearing the common occurring virulence markers of *S. suis* among persons who handle pork meat, *mrp*/sly+/epf+. The *epf* gene contains a single-nucleotide polymorphism and 2 insertions/deletions. Moreover, ST834 carries the mutation G→T at position 174, leading to a substitution *mutS1* to *mutS284* (http://pubmlst.org/ssuis/), an element involved in the mismatch repair mechanism that contributes to maintaining the overall fidelity of DNA replication. These findings indicate that ST834 is closely related to ST1, which is among the most prevalent and virulent *S. suis* clones worldwide.

We report emergence of human *S. suis* infection in Madagascar and describe the epidemiology of *S. suis* in Africa, in addition to research conducted in Togo recently (3,8). Both case-patients were at risk for infection because of their professional occupations (9). The diagnosis would have been missed if there had been no in-depth discussion between clinicians and biologists and if thorough laboratory investigation by using conventional biochemical methods has not been done. Because *S. suis* infection is not commonly known as an etiology for meningitis through population-based surveillance, Togo, 2010–2014. Emerg Infect Dis. 2016;22:1262–4. http://dx.doi.org/10.3201/eid2207.151511


Address for correspondence: Mala Rakoto-Andrianarivelo, Centre d’Infectiologie Charles Mérieux, Université Ankatso, BP 4299, Antananarivo 101, Madagascar; email: mala@cicm-madagascar.com
Cronobacter sakazakii Infection from Expressed Breast Milk, Australia

Rowena McMullan, Vidthiya Menon, Alicia G. Beukers, Slade O. Jensen, Sebastiaan J. van Hal, Rebecca Davis

Author affiliations: Royal Prince Alfred Hospital Women and Babies, Sydney, New South Wales, Australia (R. McMullan); Royal Prince Alfred Hospital, Sydney (V. Menon, A.G. Beukers, S. van Hal, R. Davis); Western Sydney University, Sydney (S.O. Jensen)

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Cronobacter sakazakii neonatal infections are often epidemiologically linked to the consumption of contaminated powdered infant formula. We describe a case resulting from consumption of contaminated expressed breast milk, as confirmed by whole-genome sequencing. This case highlights potential risks associated with storage and acquisition of expressed breast milk.

Cronobacter sakazakii neonatal infections can cause severe systemic infection and meningitis, resulting in mortality rates as high as 42% (1). C. sakazakii infections have been epidemiologically linked with contaminated powdered infant formula (PIF), whereas reports of Cronobacter infection in infants exclusively fed breast milk are rare (1). In 2016, a case of clinical meningitis was reported in an infant who had consumed expressed breast milk (EBM) contaminated with C. sakazakii (2). The source of contamination was unknown; however, pulsed-field gel electrophoresis revealed indistinguishable isolates from a contaminated breast pump and EBM. We report a similar case of an infant with onset of C. sakazakii clinical meningitis after consumption of contaminated EBM. We confirmed the source of the infection by using whole-genome sequencing (WGS).

In 2015, a 30-year-old woman underwent preterm labor at 27 weeks and 5 days and delivered a male infant. Cultures of infant blood specimens collected soon after birth were negative for bacteria and fungi. From day 2 of life, the infant received probiotics (Infloran; Laboratorio Farmaceutico, Mede, Italy) and was fed exclusively with EBM administered through an orogastric feeding tube. On day 10 of life, the infant’s health suddenly deteriorated, requiring intubation and ventilation. Blood cultures grew mucoid yellow colonies that we identified as C. sakazakii by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Breman, Germany). Despite appropriate antimicrobial treatment with meropenem, the infant had onset of status epilepticus, pulmonary hemorrhage, and acute renal failure. After discussion with his parents, care was redirected to palliation, and the infant died at 11 days of age.

Samples of EBM stored on the neonatal unit at 4°C were sent for culture. Two milk samples expressed during the mother’s 7-day inpatient stay were cultured and grew skin flora. Three samples expressed during the 6 days after discharge grew C. sakazakii. After leaving the hospital, the mother expressed breast milk by using a handheld breast pump that had not been sterilized before use. EBM was brought to the unit and stored in the same manner as EBM expressed while in hospital.

We conducted WGS on isolates of C. sakazakii cultured from EBM and the infant’s blood (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/2/17-1411-Techappl1.pdf). The EBM and infant isolates were identical, with 6 single-nucleotide polymorphisms between them, confirming that the infant was exposed to the pathogen through consumption of EBM (Figure).

C. sakazakii has been shown to colonize equipment used to prepare and administer milk formula (3). The risks associated with consumption of PIF and Cronobacter infection in infants are well understood. Consequently, much effort has gone into providing safe instructions and guidelines for preparation and storage of PIF to prevent such infections, including appropriate cleaning and sterilization procedures and storage conditions for this heat-resistant organism. C. sakazakii have been shown to survive and grow in human breast milk at temperatures of 10°C, 23°C, and 37°C (4) after introduction of the organism from an external source. Therefore, in the case of our neonate patient, the handheld breast pump probably was colonized with C. sakazakii, leading to contamination of the EBM (especially because EBM cultures while in hospital were negative for Cronobacter) and subsequent infection.

Per hospital practice at the time of this case, mothers who were inpatients and expressing breast milk were advised to perform hand hygiene before using or cleaning the hospital breast milk pump kits. The kits were washed in hot soapy water, rinsed and dried after use, and sterilized every 24 hours. After discharge from the hospital, mothers were to use their own reusable kits and breast pumps and were given the same cleaning advice about the kits. In this case, it appears that although verbal and written advice was given initially, no follow-up discussion occurred, and a pump was used without sterilization of the kit. Subsequently, several changes have been instituted, including processes to ensure daily discussion with mothers about breastfeeding and breast milk hygiene, especially given that parents of preterm infants are often in an
unexpected and highly stressful situation, when information retention is difficult. Women are also advised to rent or buy a breast pump rather than borrow a pump.

Unfortunately, the risks associated with EBM are not well recognized. This fact is becoming increasingly important because globally an increasing number of premature infants are cared for on neonatal units and require EBM until feeding is established. This case and others of Cronobacter isolation from EBM or contaminated expressing equipment suggest that consumption of contaminated EBM might be more common than initially thought, highlighting the importance of education to new parents who will be expressing breast milk for their infants. Recommendations by the US Centers for Disease Control and Prevention include correct sanitation procedures to clean breast pumps, safe storage techniques between breast pump use, and safe storage of EBM (5). If infants are unable to feed directly at the breast, reducing exposure of EBM to environmental organisms through appropriate care of equipment is essential to maintain the safety of this vital source of nutrition.

About the Author
Dr. McMullan is a neonatologist at the Royal Prince Alfred Women and Babies Hospital. Her primary research interest includes central line–associated infections in neonates.

References

Address for correspondence: Alicia G. Beukers, Department of Microbiology and Infectious Diseases, Royal Prince Alfred Hospital, Building 65, 50 Missenden Rd, Camperdown, NSW 2050, Australia; email: alicia.beukers@health.nsw.gov.au

Figure. Maximum-likelihood phylogeny of Cronobacter isolates cultured from the blood of an infant (Ck0001) and the mother’s expressed breast milk (Ck0002) with C. sakazakii Sp291 as reference. Shaded circle highlights the clustering of sequence type 4 isolates. Scale bar indicates nucleotide substitutions per site. Methods for culturing isolates described in online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/2/17-1411-Techapp1.pdf). ST, sequence type.
Cerebral Syphilitic Gumma in Immunocompetent Man, Japan

Tatsuya Kodama, Hidenori Sato, Morichika Osa, Yuji Fujikura, Akihiko Kawana

Author affiliations: National Defense Medical College, Saitama, Japan (T. Kodama, M. Osa, Y. Fujikura, A. Kawana); IMSUT Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan (H. Sato)

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Although cerebral syphilitic gummas are generally considered to be rare manifestations of tertiary syphilis, many reports exist of early cerebral syphilitic gumma. Our finding of cerebral syphilitic gumma in an HIV-negative man within 5 months after syphilis infection suggests that this condition should be considered in syphilis patients who have neurologic symptoms.

In September 2017, a 36-year-old man sought care at the National Defense Medical College Hospital (Saitama, Japan) because of hearing loss in his right ear and right-sided facial weakness, which had been worsening for 2 weeks. Other than heterosexual intercourse with a commercial sex worker 5 months earlier and a broken right fibula 3 months earlier, his medical history was unremarkable. At a routine medical examination 5 months earlier, serum rapid plasma reagin (RPR) and treponema pallidum hemagglutination (TPHA) were negative. However, TPHA was positive (1:145.3) at a preoperative workup for his right fibula fracture.

At hospital admission, his temperature was 36.4°C; pulse and respiratory rates were normal. Physical examination revealed right-side facial paralysis. Ophthalmologic findings were normal. Meningeal signs were absent, and deep sensation was intact. No skin lesions were apparent. Audiograms revealed severe-to-profound right sensorineural hearing loss. Brain computed tomography scan showed no abnormalities. Magnetic resonance imaging (MRI) showed a nodule-like lesion in the left temporal lobe, which was enhanced on T1-weighted imaging (Figure, panel A). Enhancement was also found within the cisternal segment of the vestibulocochlear nerve complex and the facial nerve on gadolinium-enhanced T1-weighted imaging (Figure, panel B). The mass-like lesion was hyperintense on T2-weighted images and fluid-attenuated inversion recovery images (Figure, panel C).

At admission, RPR titers were 1:5.9 (reference 1:<1) and TPHA titers were 1:327.1 (reference 1:<80). An HIV test was negative, and the patient did not have any other history or laboratory findings suggesting immunosuppression. Blood culture was negative. Cerebrospinal fluid (CSF) analysis showed 142 cells/µL (reference <5 cells/µL), of which 96% were lymphocytes; glucose level 60 mg/dL (reference range 45–80 mg/dL); serum glucose level 96 mg/dL [reference range 70–110 mg/dL)]; and a total protein level of 64 mg/dL (reference 15–45 mg/dL). CSF RPR titer was 1:2.4 and treponema pallidum latex agglutination titer was 1:53.4. Fluorescent treponemal antibody absorption was 2+ positive. CSF culture was negative.

We suspected that the right facial nerve palsy and the hearing loss were due to neurosyphilis causing cranial nerve (CN) VII and CN VIII dysfunction and that the lesion on the left temporal lobe was a cerebral syphilitic gumma. However, we could not exclude primary central nervous system lymphoma or a brain tumor, such as glioma, and bacterial brain abscess.

Considering the possibility of bacterial brain abscess, we used ceftriaxone for treatment (I), which is also a
recommended alternative regimen for treating neurosyphilis under the 2015 UK national guidelines (2). A few hours after the initial dose of ceftriaxone (2 g), the patient developed a Jarisch–Herxheimer reaction consisting of left temporal headache and nausea, but the symptoms resolved spontaneously in half a day. We found no evidence of bacterial hematogenous spread and bacterial parameningeal foci, which could have caused bacterial brain abscess. Therefore, we administered ceftriaxone (2 g/d for 2 weeks) as treatment for neurosyphilis. Thereafter, the right facial nerve palsy improved markedly, and the hearing loss improved gradually.

An MRI performed 2 weeks after treatment started indicated enhancement in the lesion on T2-weighted image and fluid-attenuated inversion recovery images had disappeared (Figure, panel D). The hyperintensity of the cranial nerve had also resolved. Therefore, we concluded that the lesion had indeed been a cerebral syphilitic gumma. Repeat studies of CSF RPR and serologic RPR 6 months after completion of therapy were both 1:<1. There have been no signs of recurrence as of 1 year after therapy.

The classic diagnosis of cerebral syphilitic gumma is based on the combination of several factors: the prior treatment of asymptomatic syphilis, the clinical presentation of focal seizures, positive serologic status, characteristic MRI findings, and a clinical response to penicillin (3). In some cases, the diagnosis is made by autopsy (4) or biopsy (5,6), but we did not perform a cerebral biopsy because of the degree of invasiveness and because spirochetes are rarely found in cerebral syphilitic gumma (7).

RPR and TPHA can become positive as late as 6 weeks after infection (8). Therefore, based on the results of serum RPR and TPHA 5 months and 3 months before onset, the patient probably contracted syphilis within 5 months before detection of the cerebral syphilitic gumma.

Many reports exist of early cerebral syphilitic gumma. However, in most of those cases, estimating how long patients had syphilis until cerebral syphilitic gumma appeared was based on medical interviews of history of sexual contact or specific symptoms of each stage. Recently, Tsuboi et al. (9) and Koizumi et al. (10) detected gumma in HIV-positive patients, with specific timing. The HIV-negative patient reported here also had early cerebral syphilitic gumma, within 5 months after syphilis infection, diagnosed accurately by confirming seronegativity. This case suggests that cerebral syphilitic gumma should be considered in patients with syphilis who have neurologic signs and symptoms.

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About the Author
Dr. Kodama is a military physician at the National Defense Medical College Hospital in Saitama, Japan. His primary research interests are general respiratory diseases and tropical medicine.

References

Address for correspondence: Tatsuya Kodama, National Defense Medical College, Department of Infectious Diseases and Pulmonary Medicine, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan; email: kodaamn1986@gmail.com
Clusters of Human Infection and Human-to-Human Transmission of Avian Influenza A(H7N9) Virus, 2013–2017

Lei Zhou,1 Enfu Chen,1 Changjun Bao,1 Nijuan Xiang,1 Jiabing Wu, Shengen Wu, Jian Shi, Xianjun Wang, Yaxu Zheng, Yi Zhang, Ruiqi Ren, Carolyn M. Greene, Fiona Havers, A. Danielle Iuliano, Ying Song, Chao Li, Tao Chen, Yali Wang, Dan Li, Daxin Ni, Yanping Zhang, Zijian Feng, Timothy M. Uyeki, Qun Li

Author affiliations: Public Health Emergency Center of the Chinese Center for Disease Control and Prevention, Beijing, China (L. Zhou, N. Xiang, R. Ren, Y. Wang, D. Li, D. Ni, Y. Zhang, Z. Feng, Q. Li); Zhejiang Center for Disease Control and Prevention, Hangzhou, China (E. Chen); Jiangsu Center for Disease Control and Prevention, Nanjing, China (C. Bao); Anhui Center for Disease Control and Prevention, Hefei, China (J. Wu); Fujian Center for Disease Control and Prevention, Fuzhou, China (S. Wu); Hebei Center for Disease Control and Prevention, Shijiazhuang, China (J. Shi); Shandong Center for Disease Control and Prevention, Jinan, China (X. Wang); Shanghai Center for Disease Control and Prevention, Shanghai, China (Y. Zheng); Shaanxi Center for Disease Control and Prevention, Xi’an, China (Y. Zhang); US Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C.M. Greene, F. Havers, A.D. Iuliano, Y. Song, T.M. Uyeki); National Influenza Center of the Chinese Center for Disease Control and Prevention, Beijing (T. Chen); Chinese Center for Disease Control and Prevention, Beijing (Z. Feng)

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To detect changes in human-to-human transmission of influenza A(H7N9) virus, we analyzed characteristics of 40 clusters of case-patients during 5 epidemics in China in 2013–2017. Similarities in number and size of clusters and proportion of clusters with probable human-to-human transmission across all epidemics suggest no change in human-to-human transmission risk.

Since December 2016, the number of human infections with avian influenza A(H7N9) virus in China has increased markedly (1,2), prompting concerns of pandemic influenza. Early signals of greater human-to-human transmissibility might be increased number and size of clusters of epidemiologically linked human infections and clusters of case-patients who are not blood relatives or increased numbers of case-patients with mild illness (3). To elucidate whether the increase in human infections during the fifth epidemic (2016–2017) in China was associated with increased human-to-human transmissibility of A(H7N9) virus, we compared the characteristics of clusters of A(H7N9) case-patients during the fifth epidemic with those of clusters of case-patients identified from the previous 4 epidemics (2013–2016).

The Study

For each laboratory-confirmed A(H7N9) virus infection reported in mainland China, the provincial or local Center for Disease Control and Prevention (CDC) initiated a field investigation to monitor close contacts for illness signs and symptoms for 10 days after the last known exposure to a symptomatic index case-patient (3). Upper respiratory specimens collected from close contacts with respiratory symptoms were tested for A(H7N9) virus as previously described (3). Detailed information (e.g., demographic data, household and family relationships, exposures to index case-patients and poultry, and clinical management and outcomes) was collected from case-patients, close contacts with laboratory-confirmed A(H7N9) virus infection, and their close contacts. Collection and analyses of data from case-patients with influenza A(H7N9) virus infection were part of an ongoing public health investigation of emerging outbreaks and were exempt from institutional review board assessment in China (2).

An epidemic period was defined as September 1 through August 31 of the following year. A cluster was defined as ≥2 epidemiologically linked case-patients with laboratory-confirmed A(H7N9) virus infection and illness onset within 10 days of each other. Within clusters, probable human-to-human transmission was defined as occurrence of secondary infection in a person who had close contact with a symptomatic index case-patient but no known poultry exposure; possible human-to-human transmission was defined as occurrence of secondary infection in a person who had close contact with a symptomatic index case-patient and known poultry exposure.

We performed descriptive analyses of laboratory-confirmed A(H7N9) case-patient data reported to the China CDC during February 1, 2013–June 30, 2017. We described the number of clusters per epidemic period and compared cluster patient characteristics from the fifth epidemic with those from previous epidemics; specifically, we compared numbers of clusters; case-patients per cluster; and case-patient age, sex, underlying medical conditions, hospitalization, oseltamivir treatment, intensive care unit admission, invasive mechanical ventilation, and deaths. We compared the characteristics of sporadic (noncluster) case-patients with those of index and secondary case-patients; in clusters with

1These authors contributed equally to this article.
probable human-to-human transmission, we also compared index with secondary case-patients. Because of our interest in human-to-human transmission, we focused our analyses on clusters in which secondary case-patients reported no exposure to poultry. We compared categorical variables by using \( \chi^2 \) or Fisher exact tests and median ages by using the rank-sum Wilcoxon test. All tests of statistical significance were 2-sided with a cutoff of \( p = 0.05 \).

As of June 30, 2017, we identified 84 infections comprising 40 clusters among 1,524 A(H7N9) case-patients reported since February 2013 (Table). Most clusters were located in southern and eastern China (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/2/17-1565-Techapp1.pdf). We identified 14 clusters in the fifth epidemic, compared with 4–11 clusters in prior epidemics. The 14 clusters in the fifth epidemic comprised 29 human infections; previous epidemics had clusters of 8–23 human infections per epidemic. In the fifth epidemic, 13 (93%) clusters had 2 infections each and 1 had 3 infections, compared with 23 (88%) clusters with 2 infections each and 3 clusters with 3 infections each among 26 clusters in epidemics 1–4. The proportion of all infections that occurred in clusters differed significantly among all clusters (\( p = 0.023 \)) and was lowest during the fifth epidemic (4%).

Among the 40 clusters for all 5 epidemics, we classified 14 (35%) as probable and 26 (65%) as possible human-to-human transmission. The proportion of clusters with probable human-to-human transmission only did not differ significantly by epidemic (\( p = 0.842 \)) (online Technical Appendix Table). A cluster of 3 infections in the fifth epidemic had possible and probable human-to-human transmission; 2 similar clusters were identified during the fourth epidemic. We identified no cluster with potential spread beyond 2 generations.

Among 14 secondary infections with probable human-to-human transmission during 2013–2017, we linked 4 to household exposures and 10 to exposures in healthcare settings, including 4 during the fifth epidemic. These 10 nosocomial infections included 3 blood relatives exposed to index case-patients (1 each in the second, fourth, and fifth epidemics); 1 unrelated household member exposed to an index case-patient in the second epidemic; and 6 unrelated patients exposed to index case-patients (1 each in the second, third, and fourth epidemics and 3 in the fifth epidemic).

### Table. Features of sporadic and clusters of human infections with avian influenza A(H7N9) virus, mainland China, February 1, 2013–June 30, 2017*  

<table>
<thead>
<tr>
<th>Category</th>
<th>Total</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
<th>Fourth</th>
<th>Fifth</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infections, no.</td>
<td>1,524</td>
<td>134</td>
<td>304</td>
<td>219</td>
<td>118</td>
<td>749</td>
</tr>
<tr>
<td>Deaths, no. (%)</td>
<td>599 (39.3)</td>
<td>44 (32.8)</td>
<td>126 (41.4)</td>
<td>99 (45.2)</td>
<td>47 (39.8)</td>
<td>283 (37.8)</td>
</tr>
<tr>
<td><strong>Sporadic infections</strong>†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infections, no.</td>
<td>1,440</td>
<td>126</td>
<td>281</td>
<td>207</td>
<td>106</td>
<td>720</td>
</tr>
<tr>
<td>Deaths, no.</td>
<td>570 (39.6)</td>
<td>39 (31.0)</td>
<td>120 (42.7)</td>
<td>94 (45.4)</td>
<td>45 (42.5)</td>
<td>272 (37.8)</td>
</tr>
<tr>
<td><strong>Cluster infections</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infections, no. (%)</td>
<td>84 (5.5)</td>
<td>8 (6.0)</td>
<td>23 (7.6)</td>
<td>12 (5.5)</td>
<td>12 (10.2)</td>
<td>29 (3.9)</td>
</tr>
<tr>
<td>Deaths, no. (%)</td>
<td>29 (34.5)</td>
<td>5 (62.5)</td>
<td>6 (26.1)</td>
<td>5 (41.7)</td>
<td>2 (16.7)</td>
<td>11 (37.9)</td>
</tr>
<tr>
<td><strong>All clusters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clusters, no.</td>
<td>40</td>
<td>4</td>
<td>11</td>
<td>6</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Clusters with 2 infections, no.</td>
<td>36</td>
<td>4</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Clusters with 3 infections, no.</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Deaths of index case-patients, no. (%)</td>
<td>19 (46.3)</td>
<td>3 (75.0)</td>
<td>5 (45.5)</td>
<td>2 (33.3)</td>
<td>1 (16.7)</td>
<td>8 (57.1)</td>
</tr>
<tr>
<td>Deaths of secondary case-patients, no. (%)</td>
<td>10 (23.3)</td>
<td>2 (50.0)</td>
<td>1 (8.3)</td>
<td>3 (50.0)</td>
<td>1 (16.7)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>Clusters with probable human-to-human transmission only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clusters, no. (%)</td>
<td>11 (30.0)</td>
<td>2 (50.0)</td>
<td>3 (27.3)</td>
<td>2 (33.3)</td>
<td>1 (20.0)</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>Clusters with 2 infections, no.</td>
<td>11</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Clusters with 3 infections, no.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infections, no.</td>
<td>22</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Index, no.</td>
<td>11</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Secondary, no.</td>
<td>11</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Blood-related family members</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Unrelated persons</td>
<td>10</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Overall deaths, no. (%)</td>
<td>12 (54.5)</td>
<td>3 (75.0)</td>
<td>3 (50.0)</td>
<td>2 (50.0)</td>
<td>1 (50.0)</td>
<td>3 (50.0)</td>
</tr>
<tr>
<td>Of index case-patients, no. (%)</td>
<td>9 (81.8)</td>
<td>2 (100.0)</td>
<td>2 (66.7)</td>
<td>1 (50.0)</td>
<td>1 (100.0)</td>
<td>3 (100.0)</td>
</tr>
<tr>
<td>Of secondary case-patients, no. (%)</td>
<td>3 (27.3)</td>
<td>1 (50.0)</td>
<td>1 (33.3)</td>
<td>1 (50.0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Category variables among the 5 epidemics were compared by using \( \chi^2 \) or Fisher exact tests. Median age was compared by Wilcoxon test. The proportion of infections in clusters was lowest for the fifth epidemic; the proportion of infections in clusters per epidemic differed significantly during 2013–2017 (\( \chi^2 = 11.30; p = 0.023 \)). The remaining items did not differ significantly among the 5 epidemics. The reporting of deaths may be delayed; therefore, these numbers may change as additional deaths are confirmed.

†The first epidemic was defined as February through August 31, 2013; each subsequent epidemic was defined as September 1 through August 31 of the following year.

‡Excludes human infections that were identified in clusters.
The case-fatality proportion for all clusters combined was 35% (29/84), similar to that for all sporadic infections (40%) (Table). The case-fatality proportion for all clusters for the fifth epidemic was 38% and did not differ significantly for epidemics 1–4 (p = 0.23) (Table). The case-fatality proportion for patients with index and secondary infections in probable clusters was not significantly different for the fifth epidemic (p = 0.84) or compared with previous epidemics (p = 0.53). Among all epidemics, the proportion of index case-patients in probable clusters admitted to an intensive care unit was higher than that for case-patients with sporadic infections (87% vs. 56%; p = 0.018), although this difference was not significant when data were limited to the fifth epidemic (online Technical Appendix Table).

For clusters with probable human-to-human transmission, we found no significant differences between index case-patients and patients with secondary infections during the fifth epidemic or during each previous epidemic by median age, sex, underlying medical conditions, proportion hospitalized, proportion who received mechanical ventilation, or oseltamivir treatment (online Technical Appendix Table). However, when we aggregated and analyzed data for probable clusters for all 5 epidemics, index case-patients were significantly more likely than patients with secondary infections to have received mechanical ventilation (60% vs. 14%; p = 0.02) and index case-patients were more likely than patients with secondary infections to be male (93% vs. 57%; p = 0.04) (online Technical Appendix Table).

Conclusions

Despite the surge in human infections with A(H7N9) virus during the fifth epidemic in China, the similarity in number and size of clusters and proportions of clusters with probable human-to-human transmission during 2013–2017 suggest no change in human-to-human A(H7N9) virus transmission risk over time. These findings suggest that the increase in human infections during the fifth epidemic probably reflects an increase in sporadic poultry-to-human A(H7N9) virus transmission over a wide geographic area in China (1).

Although we restricted the assessment of human-to-human A(H7N9) virus transmission in probable clusters to secondary case-patients without identified poultry exposure, we may have overestimated human-to-human transmission in clusters if not all poultry exposures were identified and reported. We could have underestimated human-to-human transmission by excluding infections in possible clusters with exposures to both poultry and symptomatic case-patients. Only symptomatic close contacts of index case-patients were tested, possibly underestimating the size of clusters of patients with asymptomatic infections (4).

Clusters of probable limited human-to-human A(H7N9) virus infections, including in healthcare settings, underscore the value of adhering to recommended infection prevention and control measures to prevent nosocomial A(H7N9) virus transmission (5–8). Ongoing assessment of the epidemiology of human infections with avian influenza A(H7N9) virus to identify any increase in human-to-human transmission will inform pandemic risk assessment, preparedness, and response (9).

Acknowledgments

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About the Author

Dr. Zhou is deputy chief of the Branch for Emerging Infectious Diseases, Public Health Emergency Center, China CDC. Her research interests are prevention and control of emerging infectious diseases and pandemic influenza preparedness and response.

References

Human African Trypanosomiasis in Emigrant Returning to China from Gabon, 2017

Xinyu Wang,1 Qiaoling Ruan,1 Bin Xu,1 Jianfei Gu, Yiyi Qian, Muxin Chen, Qin Liu, Qing Lu, Wenhong Zhang

Author affiliations: Fudan University, Shanghai, China (X. Wang, Q. Ruan, B. Xu, J. Gu, Y. Qian, Q. Lu, W. Zhang); Chinese Center for Disease Control and Prevention, WHO Collaborating Center for Tropical Diseases, Shanghai (M. Chen, Q. Liu)

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Human African trypanosomiasis is endemic to parts of sub-Saharan Africa and should be considered in the differential diagnosis of patients who have visited or lived in Africa. We report a 2017 case of stage 2 Trypanosoma brucei gambiense disease in an emigrant who returned to China from Gabon.

Human African trypanosomiasis (HAT), or sleeping sickness, is a tsetse fly–borne parasitic disease that is endemic to parts of sub-Saharan Africa. In central and west Africa, Trypanosoma brucei gambiense causes the slow-progressing form of the disease, and T. brucei rhodesiense causes the fast-progressing form in east and southern Africa (1). We report a confirmed case of HAT, after a probable tsetse fly bite, in a man who returned to China from Gabon in central Africa.

A previously healthy 60-year-old man from China lived in Gabon for 8 years. He served as a seaman and traveled between Libreville and Kango to transport river sand. In July 2016, when he was working on a rural farm in Libreville, he had a painful, unidentified insect bite on his right lower limb. The bite wound developed into an indurated, erythematous, and painful skin lesion. He received antiviral and antityphoid therapy in Gabon. Although the skin lesion healed, he had intermittent fever (up to 40°C), headache, and fatigue.

He returned to Jiangshu, China, for further treatment in June 2017. Magnetic resonance imaging (MRI) of the brain revealed temporal foci suggestive of white matter demyelination. Brain magnetic resonance angiography and encephalography revealed normal findings. He had daytime somnolence 2 weeks before admission to Huashan Hospital, associated with Fudan University in Shanghai, on August 30. The patient was lethargic during admission and had a temperature of 38.5°C and palpable cervical and inguinal lymph nodes. Hyperpigmentation of the right lower limb was visible. Meningeal irritation and the Babinski sign were absent.

The preliminary diagnosis was suspected HAT. We performed bone marrow puncture, which revealed a few trypanomastigotes (Trypanosoma spp.; Figure 1, panel A). We also found trypanosomes in a peripheral blood smear. We sent a serum sample to the Chinese Center for Disease Control and Prevention (China CDC), which showed a positive result for the T. brucei gambiense antibody test. A cerebrospinal fluid (CSF) sample revealed an open pressure of 15 cm H2O, a leukocyte count of 9 cells/μL, a protein level of 1,412 mg/dL, and a glucose level of 1.6 mmol/L. Direct examination revealed no trypanosomes in the CSF, although next-generation sequencing identified T. brucei gambiense in the CSF and bone marrow (stage 2 disease). Brain MRI revealed hyperintense signal changes in the left basal ganglia, and positron emission tomography–computed tomography suggested reduced glucose metabolism (Figure 1, panels B, C). The World Health Organization (WHO) and China CDC helped obtain nifurtimox and eflornithine, which we administered to the patient within 48 h after the diagnosis. The patient was discharged after 10 days of treatment.

Several HAT cases had previously been imported into China. One case-patient was a 45-year-old man who worked in forests and river valleys in Gabon and was diagnosed with T. brucei gambiense disease by blood smear in 2014, two months after returning to China (2). The second case involved a woman, 41 years of age, who traveled to Tanzania and Kenya, and was diagnosed with T. brucei rhodesiense by blood smear in 2017, one week after returning to China. Both cases were confirmed by molecular
diagnosis. Additional cases may have been imported to China, but we could not find any reports after a review of literature and available national records.

The latest data from WHO indicate that new HAT cases have declined during the past 15 years, reaching a low of 2,184 cases in 2016 (3). Nevertheless, HAT cases exported from Africa have been reported in all continents. In non–HAT-endemic areas, 94 cases of HAT were reported during 2000–2010, including 26 cases of T. brucei gambiense disease (4). T. brucei rhodesiense disease typically involves tourists who have visited national parks and game reserves in eastern and southern Africa, whereas T. brucei gambiense disease is rarer and typically involves migrants and long-term emigrants (1). Persons in Gabon are at risk for T. brucei gambiense infection; 403 cases were reported during 2010–2014 (4). Nine exported HAT cases from Gabon have been reported, including 5 from Libreville; all these cases were T. brucei gambiense disease, and 6 were in immigrants from various countries (2,5–7).

Although HAT is uncommon in nonendemic settings, it should be included in the differential diagnosis of travelers who return from sub-Saharan Africa with fever. T. brucei rhodesiense disease causes acute illness and usually manifests as severe parasitemia; thus, the diagnosis is easier and quicker. In contrast, T. brucei gambiense disease usually has a chronic progressive course, with an estimated untreated infection duration of 2 years (8). Blood film review is essential for the early detection of HAT. Moreover, T. brucei gambiense infection can be reliably diagnosed by testing for specific antibodies, although this test is not available in most areas of China. As in this case, next-generation sequencing technologies are useful for diagnosing unknown tropical febrile illnesses (9). Because rapid diagnosis and treatment of HAT are essential, countries with large populations of travelers to HAT-endemic regions should maintain diagnostic tools and appropriate medication to facilitate rapid clinical management.

Acknowledgments
We thank Gerardo Priotto and Jose Ramon Franco for providing the nifurtimox/eflornithine medication and advice regarding the patient’s management. We also express our thanks to Xiao-nong Zhou, Jia-xu Chen, and Xun-jia Chen.

About the Authors
Dr. Wang is an attending physician in the Department of Infectious Disease, Huashan Hospital, affiliated with Fudan University; primary research interest is travel medicine research. Dr. Ruan is a resident physician in the Department of Infectious Disease, Huashan Hospital, affiliated with Fudan University; primary research interests are infectious diseases, especially tuberculosis. Dr. Xu is an attending physician of Department of Infectious Disease, Huashan Hospital, affiliated with Fudan University; primary research interest is the management of severe infectious diseases.

References
Dengue-Associated Posterior Reversible Encephalopathy Syndrome, Vietnam

Nguyen Thi Hoang Mai, Nguyen Hoan Phu, Ho Dang Trung Nghia, Tran My Phuong, Du Trong Duc, Nguyen Van Vinh Chau, Bridget Willis, Choie Cheio Tchoyoson Lim, Guy Thwaites, Cameron Paul Simmons, Sophie Yacoub

Author affiliations: Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam (N.T.H. Mai, N.H. Phu, H.D.T. Nghia, T.M. Phuong, D.T. Duc, N.V.V. Chau); Oxford University Clinical Research Unit, Wellcome Trust Major Overseas Programme, Ho Chi Minh City (N.T.H. Mai, N.V.V. Chau, B. Willis, G. Thwaites, C.P. Simmons, S. Yacoub); University of Oxford, Oxford, UK (B. Willis, G. Thwaites); National Neuroscience Institute, Singapore (C.C.T. Lim); University of Melbourne, Melbourne, Victoria, Australia (C.P. Simmons); Imperial College London, Hammersmith Campus, London, UK (S. Yacoub)

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Dengue can cause neurologic complications in addition to the more common manifestations of plasma leakage and coagulopathy. Posterior reversible encephalopathy syndrome has rarely been described in dengue, although the pathophysiology of endothelial dysfunction likely underlies both. We describe a case of dengue-associated posterior reversible encephalopathy syndrome and discuss diagnosis and management.

On December 20, 2015, a 55-year-old woman in Vietnam sought medical care at her local hospital near Ho Chi Minh City with 1 day of fever, muscle aches, and anorexia. She had no remarkable medical history; she did not take any medications and had not received any recent vaccinations. Initial hematology and biochemistry tests were normal, but a rapid test for dengue nonstructural protein 1 antigen was positive. She remained hemodynamically stable and did not experience any bleeding or have evidence of plasma leakage. On December 24, she had a generalized convulsion and was transferred to the intensive care unit at the Hospital for Tropical Diseases.

At admission, her Glasgow Coma Scale score was 10/15 (eyes 3, motor 4, voice 3); she was confused, and speech was slurred. Her temperature was 37.5°C, heart rate was 100 beats/min, blood pressure was 140/90 mmHg, and respiratory rate was 18 breaths/min. Cardiovascular and abdominal examinations were normal. Neurologic examination demonstrated increased tone in upper and lower limbs and bilateral upgoing plantar reflexes; assessment of power was difficult because of generalized rigidity. Examination of her eye movements demonstrated vertical gaze palsy, but other cranial nerves were intact.

Blood tests showed hemoglobin level of 14 g/dL, hematocrit level of 42.2%, a white cell count of 11 × 10⁹ cells/L, and a platelet count of 100 × 10⁹/L. A test for dengue nonstructural protein 1 antigen remained positive, but PCR was negative. Urea and electrolyte levels were normal, but liver transaminases showed elevated aspartate aminotransferase of 386 U/L and alanine aminotransferase of 242 U/L. Chest radiograph was unremarkable, but a brain computed tomography scan demonstrated bilateral cerebral and cerebellar white matter hypodensities. Lumbar puncture demonstrated an opening pressure of 12 cm H₂O, a cerebrospinal fluid (CSF) cell count of 7 cells/µL, a red cell count of 4 cells/µL, protein level of 4.4 g/L, glucose level of 10.4 mmol/L (10.4 mmol/L in blood), and lactate level of 3.25 mmol/L. Dengue virus IgM was detected in CSF; Japanese encephalitis virus IgM was negative. CSF dengue and herpes simplex virus PCR were negative. Microbiologic cultures performed on blood and CSF were sterile.

Magnetic resonance imaging (MRI) of the brain, performed the following day, demonstrated bilateral symmetric high signal on T2-weighted and fluid attenuation inversion recovery images, involving periventricular and deep cerebral white matter (Figure, panel A). The differential diagnoses included encephalitis or acute demyelinating encephalomyelitis (ADEM), and a course of intravenous methylprednisolone was started (1 g/d). After 5 days, this regimen was converted to oral prednisolone (60 mg/d), tapering over 5 days. Phenobarbital was administered for 1
week for seizure control. Repeated neurologic assessment on day 5 revealed normal eye movements and improved rigidity but total left-sided hemiplegia. The patient gradually improved over the next 4 weeks. A repeated MRI 7 weeks later (February 17) demonstrated almost complete resolution, with minimal residual white matter abnormalities (Figure, panel B). The patient was discharged for rehabilitation on February 21.

The diagnosis did not fit with dengue encephalitis because of a lack of CSF pleocytosis and the high protein levels; the presence of dengue IgM in the CSF was likely secondary to vascular disruption rather than intrathecal production. Review of the MRIs, which demonstrated early reversible white matter changes rather than delayed multifocal discrete lesions associated with ADEM, were diagnostic of dengue-associated posterior encephalopathy syndrome (PRES) (1).

PRES is an acute neurologic syndrome, typically in patients with blood pressure fluctuations or metabolic derangement (1). However, PRES has been recognized to complicate various infections accompanied by normal blood pressure (2,3). Characteristic radiographic findings include bilateral white matter changes in areas supplied by the posterior circulation but can be diffuse, as described in this case, and resolve over weeks. High CSF protein levels correlate with cerebral edema and disease severity (4). The pathophysiology of PRES is thought to involve disruption to cerebral blood flow autoregulation, endothelial dysfunction, and vasogenic edema (1).

Most dengue infections cause a self-limiting febrile illness; however, life-threatening complications can occur, including increased capillary permeability, causing plasma leakage and shock. Like PRES, endothelial dysfunction is thought to underlie the capillary leak (5). Severe dengue can also occur with specific organ involvement (including neurologic) and without other severe features, as defined by the 2009 World Health Organization classification (6). Various neurologic manifestations have been described in dengue; however, PRES has been suspected in only 2 other reported cases (7,8), possibly because of underreporting or misdiagnosis, especially given the limited access to neuroimaging services in dengue-endemic areas and the common assumption that PRES diagnosis requires hypertension or metabolic derangement to be present. Unlike ADEM, PRES usually only requires supportive treatment.

This case highlights the need to consider PRES in dengue patients with neurologic symptoms and that PRES should be distinguished from encephalitis or ADEM. The high CSF protein levels and characteristic MRI findings we have described could assist clinicians in dengue-endemic areas.

Figure. Fluid-attenuated inversion recovery magnetic resonance images of the brain of a 55-year-old woman with dengue-associated posterior reversible encephalopathy syndrome, Ho Chi Minh City, Vietnam. A) Bilateral abnormal nonenhancing, confluent high signal in the periventricular and deep cerebral white matter of the high frontal parietal area and cerebellar hemispheres, thalamus, andpons. B) Almost complete resolution of abnormal findings 7 weeks later, after treatment.
About the Author

Dr. Mai is a neurologist specializing in infectious diseases of the central nervous system and serves as a clinical researcher at the Oxford University Clinical Research Unit in Vietnam and a clinician at the Hospital For Tropical Diseases in Ho Chi Minh City. Her research interests are clinical therapeutic trials of encephalitis, tuberculosis, and bacterial meningitis.

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LETTERS

Relative Risk for Ehrlichiosis and Lyme Disease Where Vectors for Both Are Sympatric, Southeastern United States

Marcia E. Herman-Giddens

Author affiliation: University of North Carolina, Chapel Hill, North Carolina, USA

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To the Editor: The timely study on the relative risk for ehrlichiosis and Lyme disease in which the tick vectors, *Amblyomma americanum* and *Ixodes scapularis*, are sympatric notes that knowledge of tickborne diseases is “startlingly low” (1). The call for more research in diseases other than Lyme disease (LD) is long overdue. In the southern United States, 5 species of ticks bite humans (2). At least 11 associated human pathogens have been identified; all may cause tick paralysis (2,3).

This study also prompts comment on drawbacks. First, even where *A. americanum* ticks outnumber *I. scapularis* in high-incidence LD areas (1), there is no mentioned concern about inflated LD case numbers resulting from reporting patients with erythema migrans (EM) from *A. americanum* tick bites (4). Second, there is no evidence for or against a 1:1 transmissibility factor. Bites from infected ticks may not result in illness because of various factors. Subclinical cases may occur. Finally, LD may be reported more frequently because of EM occurrence compared with ehrlichiosis, which depends on laboratory criteria (5).

In addition, this study prompts pertinent observations. *A. americanum* ticks are known vectors of numerous pathogens and conditions, including several not yet reportable—for example, α gal allergy, Southern tick-associated rash illness, and Heartland virus—and no prevalence studies have been conducted, so their impact is unknown. It is notable that Monmouth County, New Jersey, USA, tests *I. scapularis* but not *A. americanum* ticks, which are more numerous, carry a greater number of pathogens, and are aggressive biters of humans.

Even though the Southeast United States has more tick species and tickborne pathogens, tick education campaigns, such as those conducted in the Northeast, are absent. The Southeast is experiencing human misery and economic impact from the increase in tick species and diseases. Attention to diseases other than LD is needed and is gratifying to see.

References

Invasive Serotype 35B Pneumococci Including an Expanding Serotype Switch Lineage


Author affiliations: Baylor College of Medicine, Houston, Texas, USA (L. Olarte, S.L. Kaplan, E.O. Mason, K.G. Hultén); Ohio State University College of Medicine, Columbus, Ohio, USA (W.J. Barson); University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA (J.R. Romero); Children’s Hospital of Pittsburgh of the University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA (P.L. Lin); Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA (T.Q. Tan); University of Southern California School of Medicine, Los Angeles, California, USA (J.A. Hoffman); Rady Children’s Hospital San Diego, San Diego, California, USA (J.S. Bradley); Wake Forest School of Medicine, Winston-Salem, North Carolina, USA (L.B. Givner)

DOI: https://doi.org/10.3201/eid2402.170982

1Current affiliation: Children’s Mercy Hospital, Kansas City, Missouri, USA.

To the Editor: We read with interest the article by Chochua et al. from the Centers for Disease Control and Prevention (1). We appreciate that the authors cited our recent publication on the same topic from 8 children’s hospitals across the United States (2). Our study encompassed pneumococcal serotype 35B invasive and non-invasive infections, spanning more than 2 decades. We believe, however, that their reference to our study needs some clarification. We did not report that clonal complex (CC) 156 was the major contributor to antimicrobial resistance among serotype 35B isolates, as stated by Chochua et al. We reported a predominance (69.2%) of 35B sequence type (ST) 558 among invasive isolates across the entire study period (before and after introduction of the 13-valent pneumococcal conjugate vaccine [PCV13]); 95% of the ST558 isolates were penicillin nonsusceptible. We noted that clonal expansion of ST558 was the major contributor to the increase in prevalence of serotype 35B, as did Chochua et al. Furthermore, we observed the emergence of 35B-CC156 after introduction of PCV13 and noted that 35B-CC156 isolates were multidrug-resistant (penicillin nonsusceptibility plus resistance to ≥2 non-β-lactam antimicrobial drugs), similar to previous observations of CC156 associated with other pneumococcal polysaccharide capsules (e.g., serotypes 9V and 14). Thus, the increase in multidrug resistance among serotype 35B isolates in the post-PCV13 era was strongly associated with the emergence of CC156.

Our conclusion that both clonal expansion and diversification had occurred in the post-PCV13 era is validated by the results of Chochua et al. During 2015–2016, we observed no further increase in serotype 35B. We agree with Chochua et al. that the emergence of serotype 35B is of concern and the development of a new generation pneumococcal vaccine is necessary. We will continue to monitor and report data regarding ongoing changes in pneumococci; although our study is not population based, we believe it provides reliable data that are useful for clinical, epidemiologic, and vaccine-related considerations.

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Address for correspondence: Kristina G. Hultén, Texas Children’s Hospital, Feigin Center, 1102 Bates Ave, Ste 1150, Houston, TX 77030, USA; email: khulten@bcm.edu
In 2006, Elio Schaechter began a blog, titled *Small Things Considered*, about the microbial world for the American Society for Microbiology (ASM). The book, *In the Company of Microbes: Ten Years of Small Things Considered*, is a selected compilation of 70 of the more than 1,000 blog entries that were posted through 2015. Contributions from 33 writers in addition to Dr. Schaechter are included. These writers are past presidents of ASM and microbiologists from academic institutions around the world. The selections were chosen mostly because they were the authors’ personal or historical reflections on interactions with microbes. As such, these are aimed to be enjoyable reads, without bogging the reader down in obscurely technical scientific, often ephemeral, data.

The pieces are divided into 7 sections, including personal musings of microbiologists, historical reviews, specifics of the microbial world, thoughts on being a microbiologist, teaching, and technical essays. The blog entries cover a breadth of information but have been selected because they emphasize the unusual or unexpected, often prompting a reaction of surprise, amusement, or curiosity to the reader. Interspersed throughout are Dr. Schaechter’s finely detailed questions, designed to provoke further thought and discussion. For example: “Given so many bacteria are intimately associated with animals and plants, why are so relatively few pathogenic?” “How does *Clostridium botulinum* benefit from making botulinum toxin?” “Is global warming likely to result in a significant net increase, decrease, or no change in the microbial biomass?” “Why are there so many species of microbes on earth?”

Because these are blogs, by design, they are very readable short selections; the book can be picked up and read and put down again and again without missing a beat. It certainly makes for good reading while traveling, sipping coffee, or during other quiet times.

Microbiologists shouldn’t be the only readers of this book. Anyone interested in the microbial world, including those in public health and clinical medicine, will appreciate many of the essays. There are some selections that those with less knowledge of molecular biology or microbial ecology will not understand and thus not benefit from reading. However, because this collection of blog essays, a reader can simply move on to the next selection. Only a few readings relate closely to infectious diseases, including some on medical microbiology, typhus, retroviruses, fecal transplants, and HIV vaccines.

Modern microbiology is a wide field that has rapidly progressed during the past 50 years, encompassing the gamut from basic biology, biochemical engineering, applied mathematics, and theoretical physics. Centered on the ubiquity of the microbial world, these discussions touch everyone in the field and others interested in the disease effects of microbes on humans. This collection of well-written short essays is a notable accomplishment.

Richard Danila
Author affiliation: Minnesota Department of Health, Infectious Disease Epidemiology, Prevention and Control, St. Paul, Minnesota, USA
DOI: https://doi.org/10.3201/eid2402.171664
Address for correspondence: Richard Danila, Minnesota Department of Health, Infectious Disease Epidemiology, Prevention and Control, 625 Robert St, PO Box 64975, St. Paul, MN 55164-0975, USA; email: richard.danila@state.mn.us

Correction: Vol. 23, No. 10

The author list has been corrected for Enterovirus D68–Associated Acute Flaccid Myelitis in Immunocompromised Woman, Italy (E. Giombini et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/10/17-0792_article).
“What is more dangerous than to become a poet? which is, as some say, an incurable and infectious disease.”

—Miguel de Cervantes Saavedra, Don Quixote

For the first half of the 20th century, Spanish artist Pablo Picasso dominated the art world in a great variety of creative styles and forms, creating more than 20,000 paintings, prints, drawings, sculptures, and other works. His widely recognized and now iconic black and white sketch of Don Quixote proved a departure from his intricate surreal and cubist paintings. This briskly rendered drawing...
in the mid–fourth millennium BCE. Though horses may have initially been a source of food, their domestication profoundly advanced human transportation, agriculture, communication, and warfare. In much of the world today, leisure and competitive riding, often at large-scale events, has supplanted earlier uses, and contemporary human interactions with horses frequently involve riders, caretakers, veterinarians, and therapists.

A wide range of infectious diseases affect horses, caused by organisms varying from common equine herpesviruses to a newly identified parvovirus associated with serum hepatitis reported in this issue. Some of those diseases can cause significant economic losses to local economies as well as the global horse industry. Another foreseeable consequence from those many close connections between horses and humans is the potential for myriad zoonotic conditions, including anthrax, cryptosporidiosis, Eastern equine encephalitis, Western equine encephalitis, and Venezuelan equine encephalitis, glanders, Hendra virus disease, leptospirosis, and rabies.

Misadventurers Don Quixote and Sancho Panza were blissfully unconcerned about matters such as zoonotic infections when they trotted off on their humble mounts toward the next windmill on the horizon. Their solitary saga, frequently cited for its futility, contrasts with collaborative approaches such as the One Health Initiative, which emphasizes interdisciplinary collaboration for protecting and preserving human and animal health.

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Address for correspondence: Byron Breedlove, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C19, Atlanta, GA 30329-4027, USA; email: wbb1@cdc.gov
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Upcoming Issue

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- Emergence of Streptococcus pneumoniae Serotype 12F Following the Sequential Introduction of PCV7/PCV13 in Israel
- Use of Verbal Autopsy to Determine Underlying Cause of Death during Treatment for Multidrug-Resistant Tuberculosis, Karnataka, India
- Major Threat to Malaria Control Programs by Plasmodium falciparum Lacking Histidine-Rich Protein 2, Eritrea
- Uses of the Influenza Risk Assessment Tool (IRAT) in Pre-Pandemic Influenza Preparedness
- Insights from a Genetic Spatiotemporal Anatomy of Episodes of Introduced Plasmodium vivax Malaria, Greece 2009–2013
- Increasing Prevalence of Nontuberculous Mycobacteria in Respiratory Specimens from United States–Affiliated Pacific Island Jurisdictions
- The Role of Genome Sequencing in Defining Institutional Influenza Outbreaks
- Associations of Influenza Vaccination with Incident Tuberculosis among Elderly Taiwanese Patients
- Epidemiology and Molecular Identification and Characterization of Mycoplasma pneumoniae, South Africa, 2012–2015
- Prospective Observational Study of Incidence and Preventable Burden of Childhood Tuberculosis, Kenya
- Characteristics Associated with Negative IGRA Results in Cultured-Confirmed Tuberculosis Patients, Texas, 2013–2015
- Genome-Wide Analysis of Mycobacterium tuberculosis Strains Isolated from Russian Patients with Tuberculous Spondylitis
- Two New Cases of Invasive Infections Caused by Nannizzioopsis Molds in Immunocompromised Patients Returning from Africa
- Artificial Differences in Clostridium difficile Infection Rates Associated with Disparity in Testing

Complete list of articles in the March issue at http://www.cdc.gov/eid/upcoming.htm

NEWS AND NOTES

Upcoming Infectious Disease Activities

March 1–4, 2018
18th International Congress on Infectious Diseases (ICID)
Buenos Aires, Argentina
http://www.isid.org/icid/

March 4–7, 2018
CROI
Conference on Retroviruses and Opportunistic Infections
Boston, MA, USA
http://www.croiconference.org/

March 7–9, 2018
ISIRV
2nd International Meeting on Respiratory Pathogens
Singapore
https://www.isirv.org/site/

March 12–14, 2018
International Conference on (Re-) Emerging Infectious Diseases
Addis Ababa, Ethiopia
http://www.icreid.com/

April 18–20 2018
ISIRV
International Society for Influenza and Other Respiratory Virus Diseases
Neglected Influenza Viruses Group
https://science.vla.gov.uk/flu-lab-net/index.html
Brighton, UK

May 6–9, 2018
ASM Clinical Virology Symposium
West Palm Beach, FL

June 7–11, 2018
ASM Microbe
Atlanta, GA, USA
https://www.asm.org/index.php/asm-microbe-2018

August 26–29, 2018
ICEID
International Conference on Emerging Infectious Diseases*
Atlanta, GA, USA
https://www.cdc.gov/iceid/index.html

* The World Academy of Science, Engineering and Technology (WASET) is sponsoring a similarly named event in London in February 2018. Please note that CDC is not affiliated with this event.

Announcements

Email announcements to EIDEditor (eideditor@cdc.gov). Include the event’s date, location, sponsoring organization, and a website. Some events may appear only on EID’s website, depending on their dates.
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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 75% passing score) and earn continuing medical education (CME) credit, please go to http://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 http://www.medscape.org. If you are not registered on http://www.medscape.org, please click on the “Register” link on the right hand side of the website.

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 this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.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 go to https://www.ama-assn.org. 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 AMA PRA CME credit certificate, and present it to your national medical association for review.

Article Title

Hypervirulent Klebsiella pneumoniae in Cryptogenic Liver Abscesses, Paris, France

CME Questions

1. You are called to evaluate a 63-year-old man with a 5-day history of right abdominal pain and fever. Results on ultrasound examination of his abdomen suggest a liver abscess. What should you consider regarding the pathogenesis of pyogenic liver abscesses (PLAs)?
   A. Most cases of PLAs are related to recent abdominal surgery
   B. Most cases of PLAs originate in the biliary tract
   C. Most cases of PLAs are cryptogenic in origin
   D. Klebsiella pneumoniae is the organism most commonly involved in PLAs

2. The patient undergoes drainage of his abscess, and a culture of the abscess grows K. pneumoniae. According to the results of the current study, what might you expect regarding clinical features of his infection?
   A. The majority of patients with positive culture results for K. pneumoniae had a history of liver transplantation
   B. K. pneumoniae was more common in cryptogenic vs. noncryptogenic infections
   C. Most patients with cryptogenic PLAs associated with K. pneumoniae infection experienced severe septic metastases
   D. Relapses were common among patients with cryptogenic PLAs associated with K. pneumoniae infection

3. What should you consider regarding microbiologic outcomes in the current study as you consider treatment options for this patient?
   A. Cryptogenic cases featuring K. pneumoniae were more likely than noncryptogenic cases to be polymicrobial
   B. K. pneumoniae isolates from cryptogenic cases of PLAs were much more likely to demonstrate antimicrobial resistance vs noncryptogenic cases
   C. Hypervirulent K. pneumoniae was found in only a small percentage of cases of cryptogenic PLAs
   D. All K. pneumoniae isolates from cryptogenic PLAs featured the prmpA gene
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**Article Title**

Clinical and Molecular Epidemiology of Staphylococcal Toxic Shock Syndrome in the United Kingdom

**CME Questions**

1. Your patient is a 16-year-old girl with toxic shock syndrome (TSS). According to the study using UK national surveillance data by Sharma and colleagues, which of the following statements about the clinical features and epidemiology of TSS in England, Wales, and Northern Ireland is correct?
   A. Menstrual TSS (mTSS) is more prevalent than nonmenstrual TSS (nmTSS)
   B. Patients with nmTSS were older than those with mTSS and had higher mortality rates
   C. An estimated 39% of TSS cases occurred in children age 16 years or younger, mostly caused by burns and skin and soft tissue infections
   D. This study most likely overestimated the incidence of TSS

2. In the study using UK national surveillance data by Sharma and colleagues, which of the following statements about the molecular epidemiology of TSS in England, Wales, and Northern Ireland is correct?
   A. Toxic shock syndrome toxin 1 (TSST-1)–producing (tst+) CC30 lineage methicillin-sensitive Staphylococcus aureus (MSSA) strains were the leading cause of mTSS but not nmTSS
   B. Improved understanding of the tst+ CC30 MSSA lineage could help focus future preventive interventions in children (eg, vaccination with recombinant TSST-1 or S aureus screening and decolonization)
   C. The MSSA-ST30 clone is uncommon in the United Kingdom
   D. CC30 MSSA was not isolated from pediatric cases of TSS

3. According to the study using UK national surveillance data by Sharma and colleagues, which of the following statements about superantigen production by dominant TSS strain types and antimicrobial sensitivity of isolates is correct?
   A. tst+ CC30 lineage MSSA produced more TSST-1 (88.5 ±48.3 vs. 31.4 ±18.1 ng/mL; p<0.0001) and superantigen bioactivity than tst+ CC30 MRSA strains
   B. Most isolates were resistant to methicillin
   C. The findings call for a change in current antimicrobial recommendations for TSS
   D. tst was the only superantigen gene among TSS isolates
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Types of Articles

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Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article’s publication.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

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

Etiology. Etiology (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 eidditor@cdc.gov.
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