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January 2018

Nicholas Poussin (1594–1665) *The Plague at Ashdod,* 1630. Oil on canvas. 58.3 in × 78 in/148 cm × 198 cm. Louvre Museum, Paris, France.

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On the Cover

Nicholas Poussin (1594–1665) *The Plague at Ashdod* (1630) (detail). Oil on canvas. 58.3 in × 78 in/148 cm × 198 cm. Louvre Museum, Paris, France

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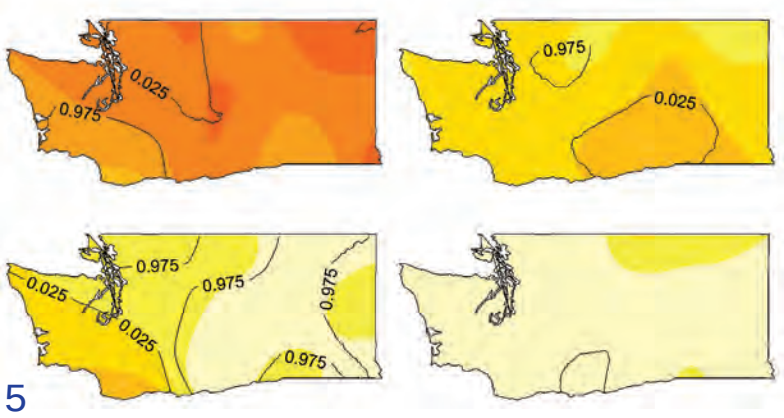

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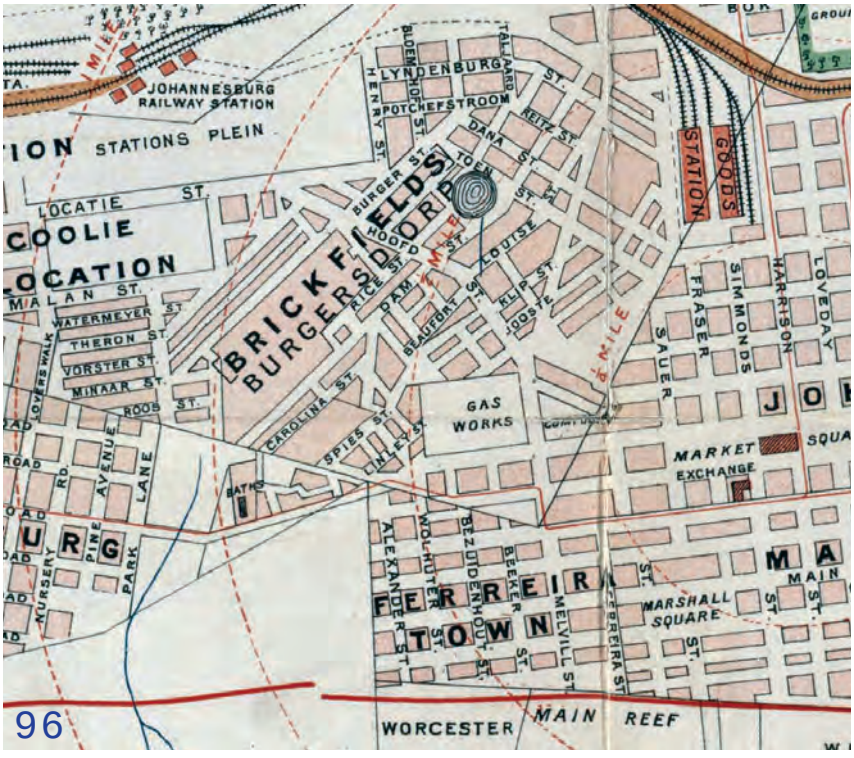

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Corrections

Vol. 21, No.8 **182**
There was an incorrect word in a sentence in *Escherichia coli* O157 Outbreaks in the United States, 2003–2012.

Vol. 23, Supplement **182**
The name of author Melanie E. King was incorrectly listed and several items in text were unclear in Surveillance Training for Ebola Preparedness in Côte d'Ivoire, Guinea-Bissau, Senegal, and Mali.

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Axis labels for Figure 3 were incorrect in Enhancing Workforce Capacity to Improve Vaccination Data Quality, Uganda.

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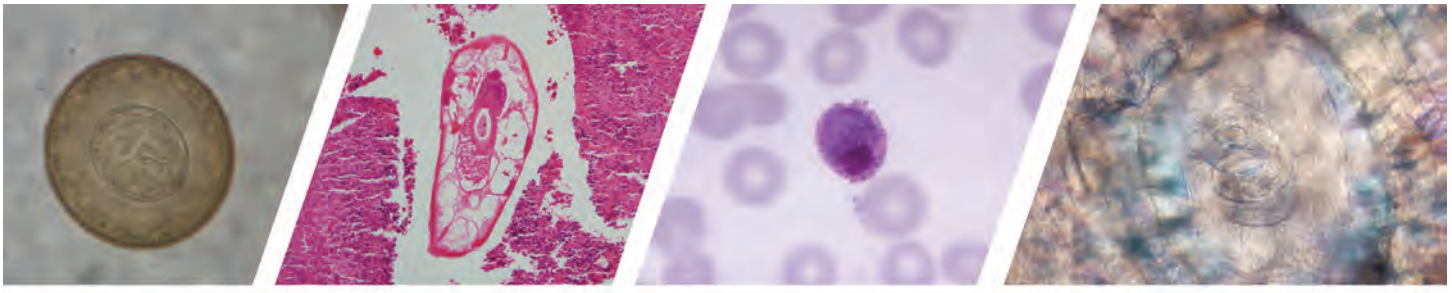

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Zika Virus Testing and Outcomes during Pregnancy, Florida, USA, 2016

Colette Shiu,[1] Rebecca Starker,[1] Jaclyn Kwal, Michelle Bartlett, Anise Crane, Samantha Greissman, Naiomi Gunaratne, Meghan Lardy, Michelle Picon, Patricia Rodriguez, Ivan Gonzalez, Christine L. Curry

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Release date: December 18, 2017; Expiration date: December 18, 2018

Learning Objectives

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

- Assess clinical outcomes of the Zika virus infection during pregnancy, based on a retrospective chart review
- Identify challenges associated with Zika virus screening and testing
- Interpret social factors associated with screening positive for Zika virus infection during pregnancy.

CME Editor

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

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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

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Zika virus infection during pregnancy can lead to congenital Zika syndrome. Implementation of screening programs and interpretation of test results can be particularly challenging during ongoing local mosquitoborne transmission. We conducted a retrospective chart review of 2,327 pregnant women screened for Zika virus in Miami–Dade County,

[1]These authors contributed equally to this article.

Florida, USA, during 2016. Of these, 86 had laboratory evidence of Zika virus infection; we describe 2 infants with probable congenital Zika syndrome. Delays in receipt of laboratory test results (median 42 days) occurred during the first month of local transmission. Odds of screening positive for Zika virus were higher for women without health insurance or who did not speak English. Our findings indicate the increase in screening for Zika virus can overwhelm hospital and public health systems, resulting in delayed receipt of results of screening and confirmatory tests and the potential to miss cases or delay diagnoses.

Zika virus infection during pregnancy can lead to congenital Zika syndrome (*1*), of which microcephaly is one of many possible malformations (*2*). Clinicians can recommend laboratory screening for Zika virus during pregnancy, even in the absence of symptoms of infection, if concern exists about exposure of the pregnant woman or her sex partner(s) because of travel to or residence in an area of ongoing Zika virus transmission (*3*). If a pregnant woman or a partner with whom she has had unprotected sex experiences symptoms, testing is warranted (*4*).

To appropriately evaluate infants born with congenital malformations, pediatricians must be aware of maternal risk for infection during pregnancy. Implementation of screening guidelines and testing for Zika virus is complicated by interpretation of test results and the need for confirmatory testing, which can delay diagnosis during a time-limited situation, such as pregnancy (*5*,*6*). To assess clinical outcomes and challenges associated with Zika virus screening and testing, we analyzed data from 2 tertiary care centers that provided care to women with travel-associated and local Zika virus infection during pregnancy.

Methods

We retrospectively reviewed charts of all 2,327 pregnant women who were tested for Zika virus during January 1, 2016–December 31, 2016, at 2 tertiary care hospitals in Miami–Dade County, Florida, USA: University of Miami Miller School of Medicine and Jackson Memorial Hospital. After institutional review board approval, we manually extracted data from the electronic medical record. Demographic and laboratory data recorded for pregnant women tested for Zika virus consisted of age, patient-reported ethnic group, language preference, insurance status, screening test date, result receipt date, test result, number of tests performed per patient, and timing of test and result relative to delivery date. We also collected delivery outcomes and laboratory and imaging results for infants of these women.

Testing for Zika virus during pregnancy followed current guidelines from the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). The Florida Department of Health (FLDOH) and its contracting laboratory, LabCorp (Burlington, NC, USA), modified the CDC guidelines by using Zika virus real-time reverse transcription PCR (rRT-PCR) and IgM to screen blood (IgM and rRT-PCR) and urine (rRT-PCR) samples simultaneously, regardless of symptoms or time from potential Zika virus exposure. All samples were collected at a regular obstetrics visit and sent in daily batches to the local FLDOH laboratory, which triaged them.

Before local transmission began in July 2016, laboratory testing of pregnant women was based on Zika virus exposure history (i.e., travel or sexual contact). After documented local transmission, we routinely offered laboratory screening for Zika virus to all pregnant women. If a woman had not been tested for Zika virus during her pregnancy, she was offered testing on arrival to labor and delivery. When a woman had any laboratory evidence of Zika virus infection during her pregnancy or when a congenital malformation in her neonate prompted evaluation for congenital Zika syndrome, urine, serum, or other relevant samples were sent from the neonatology inpatient service directly to the FLDOH, which tested samples directly or forwarded them for testing to LabCorp. The ordering clinician did not determine which entity tested the samples or the type of testing done.

If rRT-PCR or IgM testing yielded positive results at LabCorp or FLDOH, specimens were forwarded to CDC for plaque-reduction neutralization testing (PRNT). Women and infants who were eligible for the US Zika Pregnancy Registry were reported by FLDOH (*7*). All results were faxed to the tertiary care hospital where the specimens had been drawn.

We calculated test result delay on the basis of sample collection date and the date the hospital received the results. Women were triaged to consultation with the high-risk obstetrics team and the pediatric infectious disease team if Zika virus RNA was detected by rRT-PCR or if Zika virus IgM was detected by IgM antibody-capture ELISA (MAC-ELISA) in maternal serum. This change in care was done as part of clinical management. Women with rRT-PCR–positive serum or urine were considered to have acute Zika virus infection. Women with positive Zika virus IgM were presumed to have Zika virus infection until PRNT results were returned, after which we followed the CDC guidelines (*6*). Women with negative serum and urine rRT-PCR results and any nonnegative Zika virus IgM and with a PRNT titer for Zika virus <10 were considered to have no evidence of Zika virus. Results for which the rRT-PCR was negative, the IgM was positive, and the PRNT was <10 were considered false-positive (*8*). Results with nonnegative Zika virus IgM, Zika virus PRNT >10, and dengue virus PRNT <10 were considered to be infected with Zika virus, with timing of infection undetermined. Results with Zika virus and

dengue PRNTs >10 were considered to indicate flavivirus infection, specific virus not determined.

Because missing Zika virus infection during pregnancy has consequences for the woman, her infant, and pediatric care, infected patients were managed clinically as having any laboratory evidence of Zika virus infection in pregnancy. Because some patients did not receive PRNT results during the study period, we used CDC guidelines for areas (Puerto Rico) where PRNT was not recommended and relied on the IgM results (*9*).

For women with laboratory evidence of Zika virus infection during pregnancy, we collected data on gravidity, parity, possible Zika virus symptoms, antenatal ultrasonography, length of time patient was positive for Zika virus by rRT-PCR, follow-up status, and pregnancy outcome. We calculated length of time the woman was positive for Zika virus by rRT-PCR on the basis of the date of the first and last positive rRT-PCR result. FLDOH recommended weekly rRT-PCR testing until urine and serum rRT-PCR results were negative, which provided multiple data points. Women or infants were classified as lost to follow-up after 2 missed clinic visits, 3 nonresponses to phone calls, and no response to a certified letter, as was part of our routine clinical protocol.

We also recorded outcomes of infants born to mothers with laboratory evidence of Zika virus infection during pregnancy. We reviewed infant medical records for results of Zika virus testing, neurologic imaging, auditory and ocular testing, head circumference at birth, and follow-up status.

We performed statistical analyses using SAS University Edition (SAS Institute, Inc., Cary, NC, USA). Descriptive statistics were presented as means ± SDs or medians according to the statistical distribution of continuous data and as number of patients and percentages for categorical parameters. To examine the association between insurance status, primary language, race/ethnicity, and clinical result status among the pregnant women, we used χ^2 tests and reported p values for each test. We generated logistic regression models to estimate the effects of insurance status, primary language, and race/ethnicity on women's clinically positive result status. Results were reported as odds ratios (ORs) with 95% CIs. We excluded from the χ^2 and logistic regression analyses women whose test results were still pending. We also excluded women in the Native American and other race/ethnicity categories from the χ^2 test and logistic regression model between race/ethnicity and result status because no women in these groups tested positive for Zika virus. We also excluded from individual statistical analysis patients with missing data.

Results

During 2016, a total of 2,327 pregnant women were tested for Zika virus (Table 1). Based on the ≈32,000 births reported in Miami-Dade County during 2015, the most recent year for which data are available, we estimated that our analysis represents ≈7% of births in the county (*10*). During August 2016, the month when the highest number of women (607) were screened, results were returned within that same month for only 2.6% (Figure 1). The highest number of test results (598) were returned in October, which was also the month when the greatest number of tests returned were positive for Zika virus (Figure 2). Each woman was screened for Zika virus an average of 1.12 times during her pregnancy. For 646 (27.8%) women, Zika virus testing was first performed at delivery (Table 2). Including women tested at delivery, patients with delays in result receipt and women tested in the third trimester, 37% of results were received after delivery (Table 2).

Of the 2,327 women screened, 1,999 (85.9%) had no laboratory evidence of Zika virus during pregnancy (Table 2). Eight (0.34%) women had evidence of acute Zika virus infection by positive rRT-PCR. For 102 (4.4%) women, IgM results were presumptive for recent Zika virus infection (Table 2). Of the 69 for whom we received PRNT results, 24 (34%) had results <10 for Zika virus, which met CDC criteria for no evidence of Zika virus infection, so the initial tests were considered false-positive (*8*). For 10 (41%) women, PRNT results for dengue were >10. For 33 women with presumptive recent Zika virus infection, PRNT results were not available during the study period, and these women were managed as presumptively positive. The remaining 45 women had PRNT results >10 for Zika virus; for 40 (88%) of these, PRNT results were >10 for dengue virus (Figure 3).

Of women with acute Zika virus infection, rRT-PCR documented prolonged Zika virus in 6 women. Two of these had 1 positive rRT-PCR time point; for the rest, time from first to last positive rRT-PCR result were 13, 55, 48,

Table 1. Demographic characteristics of 2,327 pregnant women tested for Zika virus, Miami–Dade County, Florida, USA, 2016

Characteristic	Result
Age, y, mean ± SD	28.9 ± 6.09
Race/ethnicity,* no. (%)	
Non-Hispanic white	262 (11.3)
Non-Hispanic black	741 (31.8)
Hispanic	996 (42.8)
Haitian	265 (11.4)
Asian/Pacific Islander	42 (1.8)
Native American	2 (0.1)
Other	19 (0.8)
Insurance, no. (%)	
Public	1,535 (66.0)
Private	350 (15.0)
Not insured	442 (19.0)
Primary language, no. (%)	
English	1,472 (63.3)
Spanish	609 (26.2)
Haitian Creole	214 (9.2)
Other	32 (1.4)

*Race/ethnicity listed as "ethnic group" and was patient self-identified.

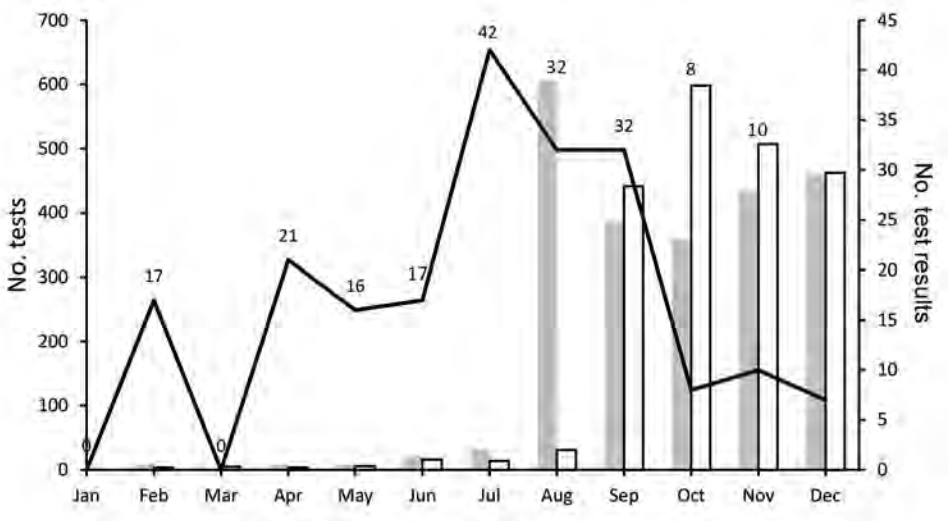

Figure 1. Zika virus screening tests, results, and length of result delay, by month, Miami–Dade County, Florida, USA, 2016. Numbers above line indicate median length of delay (in days) for test conducted in that month.

19, 25, and 13 days. For the remainder of our analysis, pregnant women with evidence acute Zika virus infection, women with evidence of Zika virus (timing of infection undetermined), and women with presumptive Zika virus (IgM positive, PRNT results not available) were analyzed together as having any laboratory evidence of Zika virus infection during pregnancy because their clinical management during pregnancy and the subsequent testing of the infants was the same.

Of the 86 pregnant women with laboratory evidence of Zika virus infection, 59 (68.6%) had ≥2 previous pregnancies (Table 3). Fifty-three (61.6%) of the 86 women were asymptomatic; 14 (16.2%) had documented symptoms suspicious for Zika virus infection. Local acquisition of Zika virus was suspected for 40 (46.5%) women (no documented travel for patient or partner during pregnancy); 26 (30.2%) women were thought to have travel-associated infection. By the end of 2016, 44 (51.1%) pregnant women with laboratory evidence of Zika virus had delivered their infants at term, and 8 (9.3%) women had preterm deliveries (Table 3). Twenty-three (26.7%) women were lost to follow-up for prenatal care.

We assessed outcomes for 52 infants of women with laboratory evidence of Zika virus during pregnancy and 1 infant with laboratory-confirmed Zika virus for whom maternal rRT-PCR and IgM were negative for Zika virus (Table 4). Two infants had probable congenital Zika virus infection (Table 5); because 1 (case-infant 1) was born to a pregnant woman without laboratory evidence of Zika virus infection, we excluded this infant from our calculations, except where indicated. The remaining infants with ocular or imaging abnormalities were negative by rRT-PCR and IgM and therefore were considered negative for congenital Zika virus infection. However, neonatal testing might have been performed after viral RNA and IgM had cleared (*11*).

None of the antenatal ultrasounds for 66 women for whom they were documented showed intracranial calcifications. For 2 infants, intracranial calcifications noted after birth were not detected before delivery (Table 4). For 5 (9.4%) infants, head circumference at birth was reported

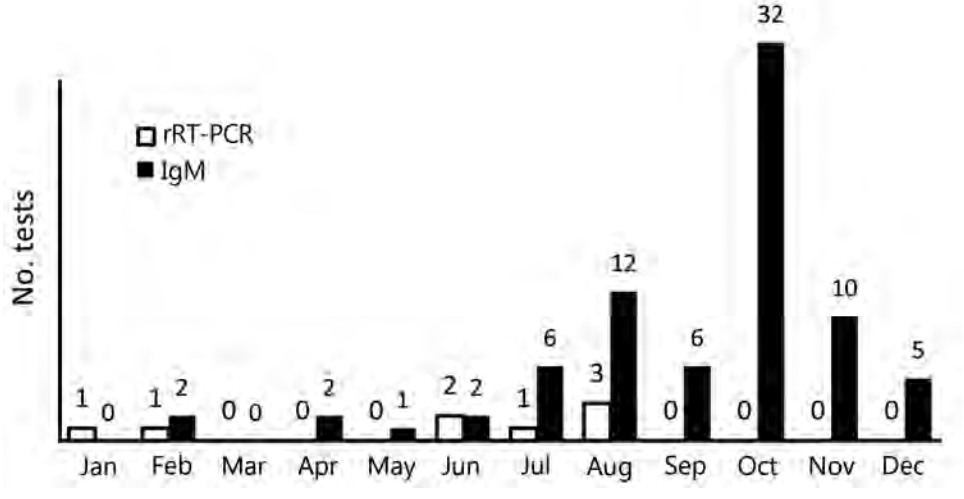

Figure 2. Positive Zika virus test results among pregnant women, by month and testing type, Miami–Dade County, Florida, USA, 2016. rRT-PCR, real-time reverse transcription PCR.

Table 2. Laboratory test results of 2,327 pregnant women for Zika virus, Miami–Dade County, Florida, USA, 2016

Laboratory characteristic	Result
Test results, no. (%)	
IgM positive	102 (4.4)
rRT-PCR–positive	8 (0.3)
Negative	1,999 (85.9)
No. tests per patient, mean ± SD	
Overall	1.12 ± 0.44
Positive	1.76 ± 1.51
Negative	1.10 ± 0.33
Timing of test, no. (%)	
At delivery	646 (27.8)
Before delivery	1,681 (72.2)
Receipt of result, no. (%)	
Before delivery	1,312 (56.4)
After delivery	860 (37.0)
Undetermined	155 (6.7)

as below the third percentile; only 1 (case-infant 1; Table 5) met criteria for microcephaly. Of the 52 infants born to women with evidence of Zika virus infection, Zika virus testing was done on 43. The untested infants were discharged home before receipt of maternal positive Zika virus testing results and did not return for care. Ocular abnormalities were documented for 7 infants (including case-infant 2; Table 5). One infant also had auditory abnormalities (Table 4). Ocular defects were reported as retinal hemorrhage, abnormalities of the optic nerve, severe attenuation of normal retinal vasculature, anomalies of the optic nerve, and abnormal hyaloid artery development. If we consider the 2 infants with probable congenital Zika virus infection, then 2 (3.7%) infants were affected. If we include the additional 6 infants who had ocular/retinal abnormalities but who had negative results, then 8 (15.0%) infants were affected. Twenty-one (39.6%) infants were lost to follow-up.

A total of 65.3% of pregnant women who had laboratory evidence of Zika virus infection had public insurance, 15.8% had private insurance, and the remaining 18.9% were uninsured (Table 6). We found a significant association between insurance status and Zika virus test result ($p<0.0001$ by χ^2 test). Uninsured patients had higher odds of receiving a positive Zika virus test result (OR 3.08, 95% CI 1.95–4.86) than did women with public insurance.

Language preference was significantly associated with Zika virus test result ($p = 0.0001$). Patients speaking Spanish and Haitian Creole had higher odds of receiving a positive Zika virus test result than English speakers (OR 2.62 and 2.91, respectively). Race/ethnicity also was significantly associated with Zika virus test result $p = 0.0013$ by χ^2 test). However, the ORs for each race/ethnicity compared with non-Hispanic white patients were not significant (95% CIs all included 1).

Discussion

We report on the clinical outcomes, challenges in testing, and social factors associated with screening positive for

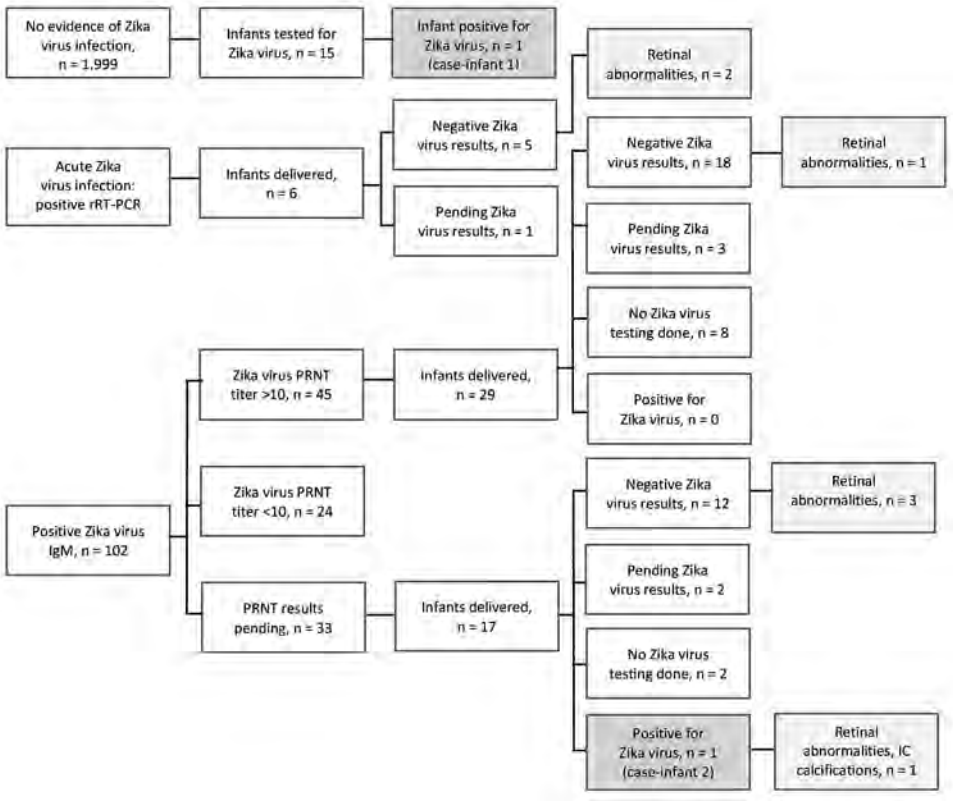

Figure 3. Maternal and infant Zika virus test results and outcomes, Miami–Dade County, Florida, USA, 2016. IC, intracranial; PRNT, plaque-reduction neutralization testing; rRT-PCR, real-time reverse transcription PCR.

Table 3. Characteristics of 86 women with laboratory evidence of Zika virus infection during pregnancy, Miami–Dade County, Florida, USA, 2016

Characteristic	No. (%)
Gravidity	
1	27 (31.0)
≥2	59 (68.6)
Parity	
0	8 (9.3)
1	39 (45.3)
≥2	39 (45.3)
Reason tested	
Asymptomatic	53 (61.6)
Symptomatic	14 (16.2)
Not determined	19 (22.1)
Pregnancy outcome	
Preterm delivery	8 (9.3)
Term delivery	44 (51.1)
Still pregnant	34 (39.5)
Location of virus acquisition	
Local	40 (46.5)
During travel	26 (30.2)
Both	15 (17.4)
Undetermined	5 (5.8)
Follow-up	
Lost to follow-up	23 (26.7)
Continued care	63 (73.3)

Table 4. Outcomes and characteristics of neonates from 86 pregnant women who had laboratory evidence of Zika virus during pregnancy, Miami–Dade County, Florida, USA, 2016

Characteristic	No. (%)
Delivery status	
Delivered	53 (60.9)
In utero	34 (39.1)
Testing status	
Tested	43 (81.1)
Not tested	10 (18.9)
Test result	
Positive	2 (4.7)
Negative	39 (90.7)
Pending	2 (4.7)
Follow-up	
Lost to follow-up	21 (39.6)
Continued care	32 (60.4)
Head circumference at birth	
Abnormal	5 (9.4)
Within normal limits	48 (90.6)
Audiology testing	
Abnormal	1 (1.9)
Normal	43 (81.1)
Not tested	9 (17.0)
Fundoscopic exam results	
Abnormal	8 (13.2)
Normal	9 (16.9)
Pending	13 (24.5)
Not tested	24 (45.3)
Cranial magnetic resonance imaging results	
Abnormal	2 (3.8)
Not tested	51 (96.2)
Cranial ultrasound at birth	
Abnormal	9 (17.0)
Normal	29 (54.7)
Not tested	15 (28.3)

Zika virus infection during pregnancy. Of the 52 infants born to women with evidence of Zika virus infection, 2 (3.7%) had evidence of probable congenital Zika virus infection, both from first trimester Zika virus infections. Difficulty in estimating the true percentage of infants affected by Zika virus is challenging because current testing might not provide laboratory evidence of fetal Zika virus infection after delivery (*12*). Additional challenges to understanding the true incidence of congenital Zika syndrome also might be related to access to Zika virus testing. The mother of case-infant 1 did not have laboratory evidence of Zika virus during pregnancy; astute pediatric care enabled detection. The mother of case-infant 2 had Zika virus IgM without PRNT results and was presumed to have been infected during pregnancy. Broad application of laboratory testing of infants enabled case detection. Current testing modalities make attributing other abnormalities, such as retinal damage, to Zika infection during pregnancy challenging (*13*).

In the cohort we report, delays in receipt of results of Zika virus screening occurred during the first half of 2016. The longest delays occurred in before local mosquitoborne transmission began; delays decreased as the laboratories and public health agencies became accustomed to an increased number of laboratory tests. In the context of pregnancy, delays in result reporting may affect decisions about

Table 5. Clinical and laboratory characteristics of 2 infants and their mothers who had laboratory evidence of Zika virus infection, Miami–Dade County, Florida, USA, 2016*

Characteristic	Case-infant 1	Case-infant 2
Country of exposure	Haiti	Venezuela
Gestational age at time of symptoms	≈10 wk (2015 Nov)	≈12 wk (2015 Dec)
Laboratory results for Zika virus		
Mother	Serum IgM neg (April 2016)	Serum neg rRT-PCR, pos IgM (2016 Apr)
Infant	Serum/CSF neg rRT-PCR, pos IgM, pos PRNT >10 Zika virus (2016 May)	Serum/CSF/CB neg rRT-PCR, pos IgM (2016 Jun)
Antenatal ultrasound	HC <3%, BPD <3% (33.1 WGA)	HC 10%, BPD 34% (36.4 WGA)
HC at birth	30.5 cm (<1%)	34 cm (25%–50%)
Postnatal cranial imaging	Serpiginous calcifications, R; polymicrogyriccortex, BL; simplified gyral pattern (BL, L >R)	Linear calcifications, L; polymicrogyric cortex, R; atrophy cerebral peduncle, R; overall volume loss of entire brain, R >L
Ocular evaluation	Unremarkable	Hypopigmented R superior retinal lesion
Auditory evaluation	Unremarkable	Normal

*BL, bilateral ; BPD, biparietal diameter; CSF, cerebrospinal fluid; HC, head circumference; L, left; neg, negative; pos, positive; PRNT, plague-reduction neutralization test; R, right; rRT-PCR, real-time reverse transcription PCR; WGA, weeks gestational age.

Table 6. Association between race/ethnicity, insurance status, and language among Zika virus–positive pregnant women, Miami–Dade County, Florida, USA, 2016*

Characteristic	No. (%) women	Odds ratio (95% CI)	p value
Race/ethnicity*			0.0013
Non-Hispanic white	231 (11.2)	Reference	
Non-Hispanic black	655 (31.7)	0.60 (0.23–1.54)	
Hispanic	903 (43.7)	1.88 (0.84–4.19)	
Haitian	235 (11.4)	2.34 (0.94–5.79)	
Asian/Pacific Islander	42 (2.0)	1.60 (0.32–7.98)	
Insurance			<0.0001
Public	1,362 (65.3)	Reference	
Private	329 (15.8)	0.76 (0.36–1.64)	
Uninsured	395 (18.9)	3.08 (1.95–4.86)	
Language			0.0001
English	1,316 (63.1)	Reference	
Spanish	554 (26.6)	2.62 (1.63–4.21)	
Haitian Creole	190 (9.1)	2.91 (1.54–5.52)	
Other	26 (1.3)	1.46 (0.19–11.11)	

*The Native American and other categories were excluded because there were no clinically positive results in these racial/ethnic groups (complete prediction).

continuation and termination of pregnancy (*6*,*12*,*14*,*15*). An additional challenge to patient management is the known cross-reactivity of current IgM tests with antibodies from past infections with related flaviviruses (*16*,*17*). Forty-one percent of the women in this study who had false-positive Zika virus IgM test results had PRNT results demonstrating previous infection with dengue virus. During counseling and disclosure of test results, the ability of the patient and provider to tolerate uncertainty cannot be overstated (*18*). Concern about false-positive Zika virus test results should be balanced with concern about missing an infant exposed to Zika virus during pregnancy.

Recent clinical guidelines recommend including rRT-PCR in screening during each trimester of pregnancy to add specificity in detecting Zika virus. Zika virus IgM is detectable for longer than previously anticipated; the median time from seroconversion to IgM negative is 122 days and as long as 210 days (*19*). The longevity of the Zika virus IgM response makes determining trimester of infection, or possible preconception infection, under previous screening guidelines more difficult to interpret (*9*,*19*,*20*). FLDOH has used both IgM and rRT-PCR as part of the screening since recognizing local transmission in July2016. Even with simultaneous IgM and rRT-PCR laboratory testing, only 8 women had positive rRT-PCR results. Therefore, the value of increasing the number of cases detected by adding rRT-PCR to national guidelines is questionable, although specificity is enhanced when the rRT-PCR is positive. Conversely, including Zika virus IgM screening for pregnant women with ongoing exposure carries the risk for false-positive results as the incidence of disease decreases, but such screening should be discussed with patients as a valuable tool because current diagnostic testing options remain limited (*18*,*21*).

As part of the public health response to local transmission of Zika virus in 2016, Florida state authorities made access to Zika virus screening free for all pregnant women through FLDOH. Review of the cohort reported here suggests that removal of financial barriers to screening were important; 18.9% of the women in this study had no insurance and had increased odds of testing positive for Zika virus during pregnancy. We consider removal of financial barriers to screening as an important adjunct to provider counseling. Similarly, pregnant women in this cohort who primarily spoke Spanish or Haitian Creole had increased odds of positive Zika virus screening during pregnancy. These 2 findings are relevant to the design and implementation of public awareness campaigns.

The findings of our study are subject to several limitations. The high rate of loss to follow-up was due in part to screening only at delivery or late during pregnancy, resulting in discharge before receipt of results. Reengagement with this patient population has been difficult. Because our study was a retrospective chart review, we relied on accurate documentation of symptoms potentially attributable to Zika virus, which possibly limited detection of women who might have been symptomatic. Also, as tertiary care centers, we frequently receive patients who were initially managed at outlying clinics, and complete records were often fragmented, particularly in terms of PRNT results. In addition, because not all infants were tested for Zika virus at birth or were fully evaluated, we might not have accurately represented the impact of congenital Zika syndrome in this cohort.

The strengths of this study include the large number of pregnant women screened for Zika virus in a diverse patient population. The wide socioeconomic strata represented by these women enabled identification of factors associated with the odds of screening positive for Zika virus during pregnancy.

Among the multiple patient management and counseling issues our study raises are the caveats in laboratory

result interpretation and the need for initial counseling that provides the most current understanding of Zika virus infection during pregnancy. In addition, our study provides lessons for other regions at risk for local transmission. Specifically, the increase in screening for Zika virus can overwhelm hospital and public health systems, resulting in delayed receipt of results of screening and confirmatory tests. Similarly, delay in penetration of screening guidelines to the medical community may result in lack of screening during pregnancy, which can lead to missed cases or delayed diagnoses. Because the understanding of the effect of Zika virus infection during pregnancy and the guidelines regarding testing interpretation are rapidly evolving, clinicians need to be well-versed on the current national guidelines for Zika virus testing (*9*,*11*).

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Sensitivity and Specificity of Suspected Case Definition Used during West Africa Ebola Epidemic

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Rapid early detection and control of Ebola virus disease (EVD) is contingent on accurate case definitions. Using an epidemic surveillance dataset from Guinea, we analyzed an EVD case definition developed by the World Health Organization (WHO) and used in Guinea. We used the surveillance dataset (March–October 2014; n = 2,847 persons) to identify patients who satisfied or did not satisfy case definition criteria. Laboratory confirmation determined cases from noncases, and we calculated sensitivity, specificity, and predictive values. The sensitivity of the defintion was 68.9%, and the specificity of the definition was 49.6%. The presence of epidemiologic risk factors (i.e., recent contact with a known or suspected EVD case-patient) had the highest sensitivity (74.7%), and unexplained deaths had the highest specificity (92.8%). Results for case definition analyses were statistically significant ($p<0.05$ by χ^2 test). Multiple components of the EVD case definition used in Guinea contributed to improved overall sensitivity and specificity.

The 2014–2016 West Africa Ebola virus disease (EVD) epidemic became the largest filovirus outbreak in history; 28,646 reported cases (suspected, probable, and confirmed) and 11,323 deaths were reported as of March 30, 2016 (*1*). Early identification of suspected EVD cases was needed to prevent additional persons from becoming infected and stop the epidemic. However, a major difficulty in correctly identifying cases is that nonspecific symptoms associated with EVD mimic those of other common febrile diseases, such as malaria and typhoid fever. The early course of EVD might include general signs and symptoms, such as headache, fever, chills, and myalgia, which can progress to vomiting, diarrhea, hemorrhage, shock, and end organ failure (*2*,*3*). Furthermore, variations in clinical presentations between persons can complicate case identification (*4*). Fever, vomiting, diarrhea, and hemorrhage are the most familiar signs associated with EVD. However, fever was either not reported or not observed in 11%–24% of reported cases, and the presence of hemorrhagic symptoms varied widely (1%–51%) (*4*–*10*). Nonetheless, early identification and aggressive supportive care early in an infection might improve the outcome of patients (*4*).

Development and use of appropriate case definitions can help identify suspected EVD cases early. This identification can in turn reduce the number of persons exposed to an infectious patient and ensure quality supportive care early in the illness of a patient. Furthermore, a proper case definition is not only needed from an epidemiologic classification standpoint but also has downstream implications related to identifying cases, controlling an outbreak, and saving lives.

A case definition with a high type 1 error rate (false-positive results) could potentially result in unnecessary exposure of misclassified patients in an Ebola treatment unit (ETU). Likewise, a case definition with a high type 2 error rate (false-negative results) can result in further exposures and infections (e.g., an infected but undetected patient in a community). For these reasons, different case definitions have been developed for EVD, depending on the *Ebolavirus* species and the goals of surveillance.

As an illustration, 1 approach is a highly sensitive (i.e., broad) clinical case definition that enables all possible signs and symptoms of EVD to be detected, with confirmation relying on highly specific diagnostic testing. This approach can be valuable in a setting in which diagnostic testing and healthcare facilities are available to test and safely care for all persons who satisfy the case definition. Another approach is the use of a more stringent clinical case definition for EVD for patients who do not have known risk factors (i.e., contact with EVD cases) and enables a lower threshold for suspecting EVD if a person has had risk for exposure. This strategy could be essential in resource-limited areas where testing facilities are not readily available or where there might be delays in laboratory results. Rapid detection of EVD and institution of appropriate infection control procedures in these areas rely heavily on quick patient identification and presumptive diagnosis before laboratory confirmation.

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Multiple case definitions were used during the West Africa EVD epidemic, as exemplified by EVD surveillance in Sierra Leone, Liberia, and Guinea, which each used variations of the suspected case definition (*9*,*11*–*14*). A commonly used suspected case definition used in Guinea was developed by the World Health Organization (WHO) (*15*). We describe the diagnostic performance of this suspected case definition by using epidemiologic surveillance and diagnostic test data for Guinea.

Methods

Case Definition

The WHO suspected case definition was used in Guinea, and similar versions were used throughout West Africa during the epidemic. This definition was defined as 1) any person, alive or dead, who has (or had) sudden onset of high fever and contact with a suspected, probable, or confirmed EVD case-patient, or a dead or sick animal; or 2) any person with sudden onset of high fever and ≥3 signs/symptoms (headache, generalized or articular pain, intense fatigue, nausea/vomiting, loss of appetite, diarrhea, abdominal pain, difficulty swallowing, difficulty breathing, hiccups, miscarriage); 3) unexplained bleeding; or 4) sudden unexplained death.

Population Dataset

As part of national surveillance for EVD in West Africa, a standard case investigation form was completed for all patients who were suspected of having EVD (*16*), and diagnostic laboratory testing was conducted for patient specimens. Confirmed case-patients were persons who had positive results for Ebola virus (EBOV) RNA by reverse transcription PCR. Non–case-patients were persons tested for EVD and who had negative results by reverse transcription PCR (*17*). These data were compiled nationally by the Guinea Ministry of Health by using the viral hemorrhagic fever application in Epi Info version 7.1.4 (Centers for Disease Control and Prevention, Atlanta, GA, USA), which was put into operation in Guinea in April 2014 (*18*).

For this analysis, we used deidentified national line list data for all persons with symptom onset or case detection dates during March–October 2014, which was during the early stage of the EVD outbreak. Analyses were limited to this period because all the analyses in this study were conducted in 2015, during the EVD response. From a database of 3,216 persons, we excluded 369 (11.5%) who were missing complete case report forms or laboratory reports. This exclusion resulted in a dataset of 2,847 persons for further investigation.

Initial clinical signs/symptoms and associated epidemiologic risk factors (contact with infected persons or body fluids, handling of bushmeat, attending the funeral of an Ebola case-patient) were presented mostly in closed response formats and had yes, no, and unknown response categories. The clinical data used in the study were generally captured at the date of initial case identification. We conducted 2 types of analyses by using the 2,847 persons who had complete case report forms and laboratory reports: 1) a complete case definition analysis that required satisfying ≥1 of the 4 criteria (epidemiologic risk factor criteria, clinical criteria, unexplained bleeding criteria, and unexplained death criteria); and 2) individual criteria analyses where each of the 4 criteria was assessed separately. For individual criteria analyses, if a person had missing data in the specific criteria of interest, we did not include them in the analysis. Deaths were surmised as unexplained death if the person was declared dead at the time the case report form was completed but no cause of death was reported (n = 157). Data cleaning ensured proper French to English language conversion and that all components were linked to the appropriate patient, including epidemiologic risk factors, laboratory samples, and laboratory test results.

Statistical Analysis

We used SAS software (SAS Institute Inc., Cary, NC, USA) to conduct complete case definition analysis and individual criteria analyses. We calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV). We used a χ^2 test to determine whether the case definition and laboratory confirmation were significantly associated ($p<0.05$).

Results

Of the 2,847 persons included in the analysis, 14.9% were ≤15 years of age and 14.5% were >55 years of age. Within this dataset, 53.5% of persons were reported to have had a fever (tactile or measured); however, only 63 (2.2%) persons had specific temperature readings recorded (Table 1). Fever, fatigue, diarrhea, and nausea/vomiting were the most commonly reported signs/symptoms. Among persons with recorded final outcome data (n = 2,136, 75%), 52.6% died. A total of 1,304 case-patients (45.8%) had a record indicating that laboratory tests confirmed EBOV infection, and 17.3% of participants reported ≥1 epidemiologic risk factor.

Complete Case Definition Performance

Approximately half of the persons in the dataset (1,412 [49.6%]) had complete data fields to satisfy ≥1 of the 4 field case definition criteria (epidemiologic, clinical, unexplained bleeding, or unexplained death) to be included in the analysis of the complete case definition. A total of 801 persons had confirmed cases; 611 persons were classified as having noncases. A total of 552 (64.2%) cases and 308 (35.8%) noncases satisfied the complete definition (Table 2). The sensitivity was 68.9%, the specificity was 49.6%, the PPV was 64.2%, and the NPV was 54.9% ($p<0.0001$) (Table 3).

Epidemiologic Criteria Performance

For the epidemiologic risk factor criteria, 241 (8.5%) of 2,847 persons had complete data fields for the analysis. A total of 162 persons had confirmed cases; 79 persons were classified as having noncases. A total of 128 (82.3%) cases and 26 (17.7%) noncases satisfied the epidemiologic risk factor criteria (Table 2). The sensitivity was 74.7%, the specificity was 67.1%, the PPV was 82.3%, and the NPV was 56.4% (p<0.0001) (Table 3).

Clinical Criteria Performance

For the clinical criteria component, 1,412 (49.6%) of 2,847 persons had complete data fields for the analysis. A total of 801 persons had confirmed cases; 611 persons were classified as having noncases. A total of 458 (66.4%) cases and 232 (33.6%) noncases satisfied the clinical criteria (Table 2). The sensitivity was 57.2%, the specificity was 62.0%, the PPV was 66.4%, and the NPV was 52.5% (p<0.0001) (Table 3).

Unexplained Bleeding Criteria Performance

For the unexplained bleeding criteria, 1,412 (49.6%) of 2,847 persons had complete data fields for the analysis. A total of 801 persons had confirmed cases; 611 persons were classified as having noncases. A total of 79 (49.1%) cases and 82 (50.9%) noncases satisfied the unexplained bleeding criteria (Table 2). The sensitivity was 9.9%, the specificity was 86.6%, the PPV was 49.1%, and the NPV was 42.3% (p = 0.04) (Table 3).

Unexplained Death Criteria Performance

For the unexplained death criteria, 1,404 (49.3%) of 2,847 persons had complete data fields for the analysis. A total of 796 persons had confirmed cases; 608 persons were classified as having noncases. A total of 113 (72%) cases and 44 (28%) noncases satisfied the unexplained death criteria (Table 2). The sensitivity was 14.2%, the specificity was 92.8%, the PPV was 72.0%, and the NPV was 45.2% (p<0.0001) (Table 3).

Discussion

This analysis examined the performance of a case definition used for surveillance during the West Africa EVD epidemic. Developing appropriate case definitions in the setting of an outbreak or epidemic is critical because of the need to balance the strengths of the definition (good sensitivity, specificity, PPV, and NPV) with the utility of the definition in the particular setting. A previous analysis of a simplified 1999 WHO case definition (which consisted of fever and unexplained hemorrhage as a suspected case definition) found that these simple criteria resulted in poor sensitivity and a misclassification of 30% of cases infected with Ebola virus or Marburg virus (*14*). In contrast, our analysis of a complex multipart case definition found a relatively higher sensitivity (68.9%). A more recent study analyzed the diagnostic validity of EVD clinical features of the WHO suspected case definition for patients admitted to an ETU during the second half of the epidemic in Sierra Leone (*19*). That study found that the epidemiologic risk factors (previous contact with an EVD case-patient) were strongly correlated with EVD diagnosis and that the suspected case definition showed low specificity and PPV, in agreement with our analysis.

Table 1. Characteristics of 2,847 persons used for analysis of suspected case definition during West Africa Ebola epidemic, Guinea, March–October 2014

Characteristic	No. (%)
No. persons	2,847 (100.0)
Sex	
M	1,396 (49.0)
F	1,408 (49.5)
Missing	43 (1.5)
Age, y	
0–<5	166 (5.8)
5–15	259 (9.1)
16–25	474 (16.6)
26–35	625 (21.9)
36–45	517 (18.2)
46–55	295 (10.4)
>55	414 (14.5)
Missing	97 (3.4)
Fever (temperature) recorded	
>38.6°C	29 (1.0)
>38°C	34 (1.2)
Missing	2,813 (98.8)
Sign/symptom reported	
Fever, tactile or measured at >38°C	1,524 (53.5)
Fatigue	1,400 (49.2)
Diarrhea	963 (33.8)
Vomiting/nausea	1,019 (35.8)
Anorexia	772 (27.1)
Headache	782 (27.5)
Chest pain	44 (1.5)
Abdominal pain	483 (16.9)
Muscle pain	445 (15.6)
Joint pain	331 (11.6)
Difficulty swallowing	32 (1.1)
Difficulty breathing	35 (1.2)
Hiccups	121 (4.3)
Unexplained bleeding	274 (9.6)
Epidemiologic risk factors in past month	
Contact with any sick person	282 (9.9)
Contact with infectious bodily fluids from known case-patient	36 (1.3)
Physical contact with a case-patient, dead or alive	87 (3.1)
Participated in a funeral touching/carrying a body	83 (2.9)
Contact with bats or nonhuman primates	2 (0.1)
Laboratory confirmation status	
Reverse transcription PCR positive	1,304 (45.8)
Reverse transcription PCR negative	1,233 (43.3)
Unconfirmed/eliminated	310 (10.9)
Final status	
Alive	1,012 (35.5)
Dead	1,124 (39.5)
Status missing	711 (24.9)

Table 2. Analysis of complete Ebola virus disease suspected case definition and individual criteria during West Africa Ebola epidemic, Guinea, March–October 2014*

Criteria choice	Cases, no. (%)	Noncases, no. (%)
Meets complete definition	552 (64.2)	308 (35.8)
Does not meet complete definition	249 (45.1)	303 (54.9)
Meets epidemiologic risk criteria†	121 (82.3)	26 (17.7)
Does not meet epidemiologic risk criteria	41 (43.6)	53 (56.4)
Meets clinical criteria	458 (66.4)	232 (33.6)
Does not meet clinical criteria	343 (47.5)	379 (52.5)
Meets unexplained bleeding criteria	79 (49.1)	82 (50.9)
Does not meet unexplained bleeding criteria	722 (57.7)	529 (42.3)
Meets unexplained death criteria	113 (72.0)	44 (28.0)
Does not meet unexplained death criteria	683 (54.8)	564 (45.2)

*Percentages correlate with rows.
†Contact with infected persons or body fluid, handling of bushmeat, attending the funeral of an Ebola case-patient.

The complete case definition showed poor sensitivity (68.9%) and specificity (49.6%). However, the case definition included subcriteria that showed higher sensitivity and specificity when analyzed individually. Among the 4 criteria, unexplained death (92.8%) and unexplained bleeding (86.6%) had the highest specificity, which indicated that if these patients were identified and tested in the EVD outbreak setting, there was higher likelihood of being EVD laboratory confirmed. The lower sensitivity in the complete case definition could have been affected by the epidemiologic risk criteria for which, among the 2,847 persons evaluated, only 241 (8.5%) had epidemiologic information for assessing whether they satisfied the epidemiologic risk criteria. The incomplete, missing data might have caused a bias. If the dataset had a high proportion of persons who had complete epidemiologic risk criteria, then the complete case definition might have a higher sensitivity and specificity than the subcriteria.

The reasons for a death being classified as unexplained could be caused by incomplete or erroneous data entry of case report forms or poor recall of patient history. It is essential to note that information about unexplained deaths was not reported in the outbreak case report forms. Rather, this criterion was artificially created on the basis of absence of clinical or epidemiologic data, or a cause of death provided, so that we could approximately capture this information for our analysis. The high number of unexplained deaths could reflect the high number of overall deaths reported during the early portion of the EVD epidemic, in which surveillance had potential lags, leading to patients not being detected until they had died.

Although the unexplained bleeding criteria showed a relatively high specificity (86.6%), the sensitivity was the lowest among the analyses (9.9%). These findings could be explained by the observation that although bleeding is certainly a striking presentation for EVD patients, medical documentation in which it was reported that the patient had bleeding varied from 2% to 69%. Lower percentages were reported in nonobservable hemorrhage (e.g., gastrointestinal bleeding) and for survivors (*3*,*4*,*10*). These findings could also explain the low PPV (49.1%) and NPV (42.3%) of unexplained bleeding criteria in this analysis.

The presence of epidemiologic risk factors had the highest sensitivity (74.7%) and high PPV (82.3%), indicating that most laboratory-confirmed case-patients had a reported exposure to EVD case-patients, which is consistent with previous investigations of EBOV transmission (*20*–*22*). However, this subcriteria also had the smallest sample size when compared with other subcriteria. Response bias, as a result of fewer cases with available epidemiologic data, could have caused epidemiologic risk factors to perform better than the other subcriteria. We surmise that if the sample size for epidemiologic risk factors was similar to those for other subcriteria, its performance would not be as high. Along these lines, accurate assessments of exposure of a person to EBOV is not always possible, and it is necessary to include clinical criteria as a component of a case definition. The performance of the clinical criteria was moderate, as might be expected because EVD is known to be difficult to diagnose by only clinical criteria, but performance is especially needed when epidemiologic information might be lacking (*9*). The clinical criteria often guide clinicians to order tests for laboratory confirmation of EVD. This procedure also reduces the risk for missing possible EVD patients and discharging them from treatment where they could shed virus in the community.

Table 3. Analysis of Ebola virus disease suspected case definition during West Africa Ebola epidemic, Guinea, March–October 2014

Component of case definition analyzed	p value	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
Complete definition	<0.0001	68.9	49.6	64.2	54.9
Epidemiologic risk criteria*	<0.0001	74.7	67.1	82.3	56.4
Clinical criteria	<0.0001	57.2	62.0	66.4	52.5
Unexplained bleeding	0.04	9.9	86.6	49.1	42.3
Unexplained death	<0.0001	14.2	92.8	72.0	45.2

*Contact with infected persons or body fluid, handling of bushmeat, attending the funeral of an Ebola case-patient.

The case definition analyzed in this study was implemented in the midst of an unprecedented outbreak. A major advantage of this complete case definition was its broad and complex scope that enabled wider inclusion of suspected cases. The definition enabled persons to be considered as having suspected cases if they did not meet clinical criteria but had an epidemiologic link with another case-patient or if there was an unexplained death. Broad inclusive criteria are needed to identify cases and immediately control further spread of the virus. Once a suspected case is identified, resource-intensive contact tracing can begin even while laboratory confirmation is pending.

The benefits of the Guinea dataset used in our analyses were the large sample size and level of detail. These features enabled an in-depth analysis to determine if specific components of the case definition played roles in their performance. However, the dataset also had limitations. First, it is unknown how representative this database was of all EVD patients. This dataset was dependent on patients identified early in the Ebola outbreak when case identification was still being established. Developing the suspected case definition during the early stages of the outbreak could have been dependent on what commonalities were observed among infected patients, which could also mean some patients who did not exhibit the common presentation could be missed. Therefore, this limitation could cause an overestimation of the performance characteristics in our analyses.

In addition, because the dataset was collected early in the response, the quality of the data collected could be suspect. Many portions of the data were missing, and clinical symptoms and epidemiologic information could have been inaccurate. For example, few patients reported epidemiologic risk factors that could be a source of misclassification bias, which would lead to inaccurate measures of sensitivity and specificity. Also, clinical and epidemiologic data might not have been completely or consistently collected because of various levels of training and a large number of personnel who completed the case report forms. Despite these challenges, future EVD outbreaks would benefit in training public health staff in thorough, consistent data collection and documentation to reduce this problem. Finally, this analysis assessed only 1 case definition used in West Africa. Other case definitions used during the epidemic will perform differently on the basis of the criteria.

Although individual criteria had their strengths in sensitivity and specificity compared with the complete case definition, none should be mutually exclusive. The need for detecting every suspected case in a large EVD epidemic was reliant on a broad case definition that included criteria from all possible scenarios. A complete case definition was especially appreciated when epidemiologic and medical histories for patients were often incomplete or lacking during the height of the epidemic; several cases that were laboratory confirmed did not meet criteria for the complete case definition. An explanation for this dichotomy would be that in the midst of an epidemic, when the threshold to diagnose a possible EVD case was low, clinicians were often prompted to test a patient for EVD even if the case definition was not satisfied. The concern for missing a suspected case might have outweighed strict efforts to follow the case definition. Furthermore, the poor specificity of the case definition reflects its development for public health and epidemiologic purposes and not for clinical screening in the setting of triage (*14*,*23*,*24*). However, because identifying a suspected case could lead the patient to a clinical setting, such as a suspected case-patient arriving at an ETU, its utility should not be limited to only epidemiology. Thus, use of a case definition should not be rigid but should be used as an adaptable tool.

The use of the case definition is a cogent starting point to identify possible cases and, as more information is gathered (i.e., signs, symptoms, clinical status), can eventually aid clinical management decisions. Similarly, case definitions should not be the end-all deciding factor on clinical management of a patient. For example, decisions on admission and treatment for critically ill patients with an illness that might meet the suspected case definition but that is clearly not EVD, such as measles, who arrive at an ETU should be balanced with the clinical judgment of the physician, risk/benefit to patients, and available resources.

Likewise, the sensitivity and specificity of a case definition are not only affected by the criteria included in the definition but by its use during the timing of an outbreak. In the early stages of an outbreak, when case reporting might still be established, it might be more useful to identify true cases. However, in the final stages of the outbreak and as the response is better established, an increase in sensitivity to identify all possible cases might offset a loss of specificity. Therefore, understanding these dynamics is needed in the deployment of appropriate case definitions.

In Guinea, the case definition for suspected cases was last revised in April 2016 to take into consideration the lessons of the last outbreak in Koropara and related new scientific knowledge highlighting the possibility of sexual transmission from the sperm of an Ebola survivor >1 year after his release from an ETU (*25*). Therefore, since that time, the new definition has been broadened to include 2 additional individual epidemiologic conditions: 1) 2 deaths in 1 family in a period of 3 weeks, or 2) 1 death in the family or acquaintances of an Ebola survivor. Also, because fever measurement was missing for 99% of the persons in the dataset, a future consideration is how fever and other clinical criteria (e.g., headache, generalized or articular pain, nausea/vomiting) might be useful factors in the definition of a suspected case of EVD. This exploration should be a fruitful endeavor, especially since the presence of fever is assumed to be an integral part of EVD case definitions.

Modification of surveillance databases to include new variables that take into account evolving case definitions and completeness of data will remain a challenge worth pursuing. Frequent diagnostic performance evaluation and revision of case definitions by using available data have the potential to play a major role in early identification of cases and related improved outcomes. Therefore, understanding of these dynamics is needed during all stages of an outbreak.

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Nipah Virus Contamination of Hospital Surfaces during Outbreaks, Bangladesh, 2013–2014

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Nipah virus (NiV) has been transmitted from patient to caregivers in Bangladesh presumably through oral secretions. We aimed to detect whether NiV-infected patients contaminate hospital surfaces with the virus. During December 2013–April 2014, we collected 1 swab sample from 5 surfaces near NiV-infected patients and tested surface and oral swab samples by real-time reverse transcription PCR for NiV RNA. We identified 16 Nipah patients; 12 cases were laboratory-confirmed and 4 probable. Of the 12 laboratory-confirmed cases, 10 showed NiV RNA in oral swab specimens. We obtained surface swab samples for 6 Nipah patients; 5 had evidence of NiV RNA on ≥1 surface: 4 patients contaminated towels, 3 bed sheets, and 1 the bed rail. Patients with NiV RNA in oral swab samples were significantly more likely than other Nipah patients to die. To reduce the risk for fomite transmission of NiV, infection control should target hospital surfaces.

Nipah virus (NiV) is a batborne paramyxovirus (*1*,*2*) that causes encephalitis in humans. NiV has caused outbreaks almost every year in Bangladesh since 2001; the case-fatality rate is >70% (*3*).The 2 primary pathways of NiV transmission in Bangladesh are drinking raw date palm sap contaminated with excretions from *Pteropus* spp. fruit bats and human-to-human transmission through close contact with infected persons (*4*–*7*). Nearly one third of identified Nipah patients in Bangladesh were infected through person-to-person transmission (*8*); most of these were family caregivers who provided hands-on care to Nipah patients at home and in hospital (*3*,*6*,*9*,*10*).

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Transmission of NiV in hospital settings was first identified in 2001 during an outbreak in Siliguri, India, and in several outbreaks in Bangladesh since 2004 (*6*,*9*,*11*–*13*). In the outbreak in Siliguri, 66 persons were infected, and of the 60 for whom exposure was known, 45 (75%) acquired infection during their hospital stay (11 patients admitted for other illness, 25 hospital staff, and 8 persons who visited an infected patient) (*11*). In Bangladesh, during the 2010–2011 Nipah outbreak, 2 hospital staff (1 physician, 1 hospital cleaner) were infected (*12*,*13*).

NiV RNA has repeatedly been identified in infected patients' oral secretions (*14*,*15*), and epidemiologic evidence suggests that exposure to respiratory secretions is a likely route of NiV transmission from patient to caregiver (*6*). In 2004, during an NiV outbreak with person-to-person transmission in Bangladesh, NiV RNA was found on a hospital wall near where an NiV patient received care (*6*).

Hospital wards in Bangladesh are often overcrowded with patients, family caregivers, and visitors and have a median of 4 persons/10 m^2 of floor space (*16*). The floor is often soiled with bodily secretions, and a median of 5 uncovered coughs or sneezes per 10 m^2 per hour has been observed (*16*). Most wards have intermittent water supply, lack functioning handwashing stations, and have an inadequate number of toilets (*16*,*17*). Hospital staff and family caregivers can acquire infections through direct patient contact or contaminated fomites (*18*,*19*). Healthcare workers (i.e., doctors and nurses) have direct contact with patients; other staff, such as hospital cleaners, and visitors, who are not involved in patient care, might have contact only with hospital surfaces. Possible contamination of nearby hospital surfaces by Nipah patients with infectious bodily secretions, coupled with a lack of infection control measures in low-income hospitals, puts healthcare workers, caregivers, visitors, and other patients in the ward at risk for NiV infection by contaminated hospital surfaces. Propagation of a highly fatal pathogen with the capacity for person-to-person transmission within resource-constrained healthcare settings increases the risk for broader outbreaks (*11*,*20*).

In Bangladesh, resources for infection control in hospitals are severely limited (*16*), and we have limited knowledge about where to focus infection control to optimize use of scarce resources. Identification of fomites for possible NiV transmission would help design interventions prioritizing the area of hospital wards for disinfection to reduce surface contamination and possible risk for fomite transmission. Our objective was to identify whether Nipah patients contaminate nearby hospital surfaces with NiV RNA and, if so, which hospital surfaces are most commonly contaminated and which patients are most likely to contaminate their environment.

Methods

Case Identification and Sample Collection

We conducted this study in 3 Nipah surveillance hospitals at Faridpur, Rajshahi, and Rangpur, Bangladesh, during December 2013–April 2014. Surveillance physicians identified patients admitted with encephalitis, defined as fever or history of fever with axillary temperature >38.5°C (101.3°F) and altered mental status, new onset of seizures, or new neurologic deficit (*21*), and collected blood and oral swab samples. Because of resource constraints, surface sampling for all encephalitis cases was not possible; therefore, a research assistant swabbed hospital surfaces near encephalitis patients with a history of consuming raw date palm sap, contact with another encephalitis patient, or both (*22*). Occasionally, physicians from other nearby hospitals reported suspected Nipah case-patients to public health authorities. These patients also had biological samples collected for laboratory testing but were not included in the surface sampling study.

Blood samples were centrifuged at the local government health facility, and the separated serum was stored and transported to the Institute of Epidemiology Disease Control and Research laboratory in a liquid nitrogen dry shipper (–150°C) and then stored at –20°C until testing. From each patient, 1 oral swab was collected in 1 mL of nucleic acid extraction lysis buffer every consecutive day for 7 days, until hospital discharge or death, whichever occurred first.

A research assistant collected 1 swab sample from up to 5 areas near each patient: the wall beside patient bed, bed rail, bed sheet, clinical record file, and multipurpose towel used by family caregivers for cleaning patient secretions, drying hands, and other caregiving purposes. The research assistant collected surface swab samples at least 12 hours after the patient was admitted to the hospital. With 1 sterile rayon swab stick per surface, the research assistant swabbed the area of the wall in contact with the bed 45 cm high from the level of the bed sheet; all surfaces of the bed rail in the area near the patient's head; half of the bed sheet where the patient's head was, including underneath the patient; front and back covers of the patient file; and both sides of the multipurpose towel. Not all patients had a wall or bed rail near them because some patients were cared for on the floor and some were away from the walls. One swab sample per surface area was collected in separate cryovials with 1 mL of nucleic acid extraction lysis buffer (bioMérieux, Marcy-l'Étoile, France). The vials were kept in a cool box maintaining a temperature of 2°–8°C for up to 30 min after collection and then were placed in a liquid nitrogen dry shipper for storage and transportation.

Testing of Clinical Samples and Surface Swab Samples

Serum samples were tested for NiV IgM using an IgM-capture enzyme immunoassay (*23*). Oral and surface swab samples were tested for NiV RNA by real-time reverse transcription PCR (rRT-PCR). Viral RNA was extracted using a Kingfisher Flex 96 (Thermo Scientific, Waltham, MA, USA) automatic extractor using InviMagVirus DNA/RNA Mini Kit/KF 96 (STRATEC Molecular, Birkenfeld, Germany). The rRT-PCR was performed on the CFX96 system (Bio-Rad, Inc., Hercules, CA, USA) and ABI7500 platform (Applied Biosystems, Foster City, CA, USA) using AgPath-ID One-Step RT-PCR Kit (Applied Biosystems). The following primers were used for detecting the NiV N gene: forward primer NVBNF2B 5′-CTG-GTCTCTGCAGTTATCACCATC GA-3′, reverse primer NVBN593R 5′–ACGTACTTAGCC CAT CTT CTA GTTTCA-3′, and probe NVBN54P2 5′–Fam-CAG CTC CCGACACTGCCGAGG AT-BHQ–3′ (*24*). To provide evidence that similar viruses were present in human specimens and the environmental swab samples, we performed PCR-based direct sequencing using nucleic acids obtained from patients' oral swab samples and their corresponding surface swab samples by using NiV-specific primers (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/1/16-1758-Techapp1.pdf). The sequencing was performed using the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit in an automated ABI 3500 XL genetic analyzer (both from Applied Biosystems). Nucleotide sequence similarity searches were performed using BLAST (https://www.ncbi.nlm.nih.gov/BLAST/).

Community Investigation

An investigation team visited the communities of encephalitis patients identified at surveillance hospitals who had NiV IgM in serum to identify any other associated encephalitis cases. The team interviewed identified encephalitis patients and their caregivers using a structured questionnaire. Identified patients were asked about the nature of their contact with hospitalized patients (i.e., touching, being in the same room, feeding, sharing a bed, or cleaning body

secretions) to find evidence of person-to-person transmission. The team also collected blood from the encephalitis patients identified in the community investigation.

Classification of Cases

We classified an encephalitis case as laboratory-confirmed Nipah in a patient with NiV IgM in serum and a probable Nipah case as a case with an epidemiologic link with a laboratory-confirmed Nipah case in a person who died before blood could be collected for testing. We defined a Nipah spreader as a person with a probable or confirmed case who had close contact with at least 1 person in whom Nipah illness developed 5–15 days after contact (*5*).

Statistical Analysis

We summarized the data using frequency and percentages. We assessed the difference in proportions using χ^2 test or Fisher exact test when appropriate. We considered $p<0.05$ as statistically significant.

Ethical Consideration

Study participants or their legal guardian provided informed written consent. The Ethical Review Committee of icddr,b (Dhaka, Bangladesh) reviewed and approved the study protocol. The Institutional Review Board at the Centers for Disease Control and Prevention (Atlanta, GA, USA) deferred to icddr,b's approval.

Results

Surveillance physicians identified 332 encephalitis cases in the 3 surveillance hospitals. One encephalitis case was reported from a nearby hospital, and we identified an additional 2 encephalitis cases from the community investigations. Of the 332 encephalitis cases identified in surveillance hospitals, we tested blood samples and oral swab samples from 312 (94%) case-patients and collected hospital surface swab samples from 49 case-patients who had a history of consuming raw date palm sap or contact with other encephalitis patients as reported by their caregiver on admission at the hospital. Of the 312 patients tested from surveillance hospitals, 9 (3%) had NiV IgM. All 3 case-patients identified during community investigations were hospitalized at nonsurveillance hospitals, and all had detectable NiV IgM (Figure 1). Through the community investigation, we identified an additional 4 probable Nipah case-patients who died before specimens could be collected. Thus, we identified a total of 16 Nipah cases from hospital and community investigations. Four cases occurred in isolation, but 12 clustered in 4 outbreaks. The 4 clusters comprised 8 laboratory-confirmed and 4 probable cases. Two of the 4 clusters involved person-to-person transmission (online Technical Appendix).

Of the 12 case-patients with laboratory-confirmed Nipah, 10 (83%) had NiV RNA in ≥1 oral swab sample (Figure 2). We collected 19 oral swab samples from these

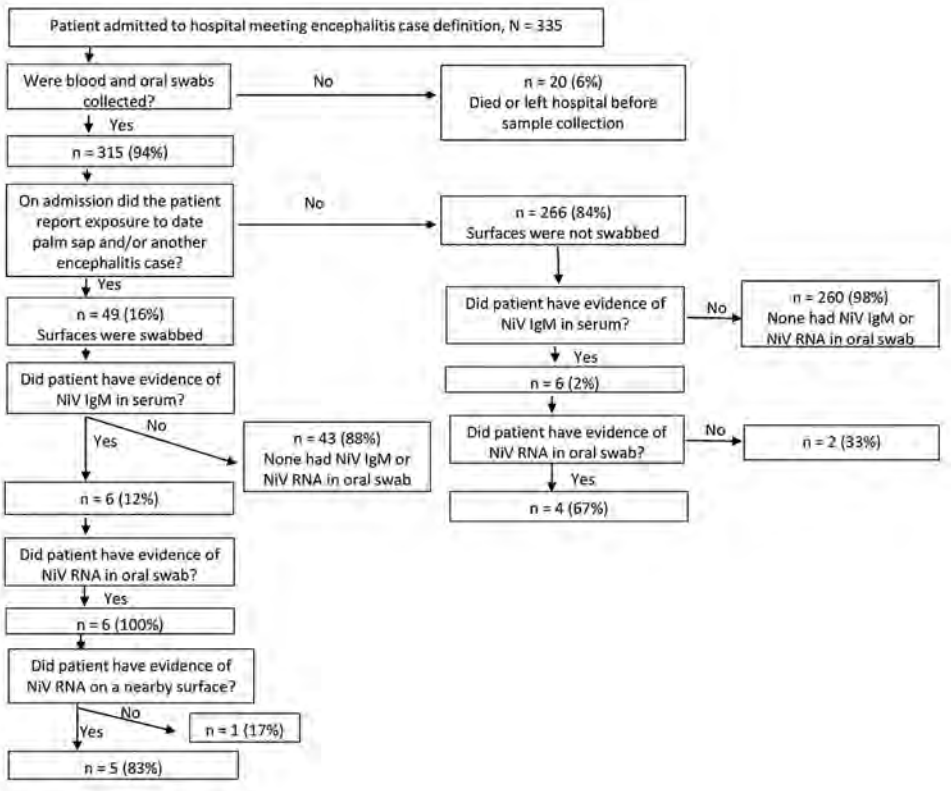

Figure 1. Number of blood samples, oral swab samples, and surface swab samples collected and tested from encephalitis patients identified in hospitals, Bangladesh, December 2013–April 2014.

Table 1. Laboratory results of swab samples of 6 patients with detectable Nipah virus RNA from 3 surveillance hospitals, Bangladesh, December 2013–April 2014*

	Days after hospitalization collected and result												
				Surface swab sample									
				1					2				
	Oral swab sample						Clinical					Clinical	
Patient	1	2	3	Towel	Bed sheet	Bed rail	file	Walls	Towel	Bed sheet	Bed rail	file	Walls
1	Pos	Pos							Pos	Neg	Pos	Neg	
2	Pos	Pos							Pos	Pos		Neg	Neg
3	Pos	Pos	Pos	Pos	Pos		Neg	Neg					
4	Pos				Pos	Neg	Neg						
5	Pos	Pos							Pos	Neg	Neg	Neg	Neg
6	Pos	Pos		Neg	Neg	Neg	Neg	Neg					

*Pos, positive; neg, negative. Blank cells indicate no sample collected.

10 case-patients; all 19 samples had evidence of NiV RNA. None of the 303 patients identified at surveillance hospitals without NiV IgM in serum had detectable NiV RNA on an oral swab sample. Of the 49 patients identified in surveillance hospitals for whom surface swab samples were collected, 6 had laboratory-confirmed Nipah (Table 1). We did not collect nearby surface swab samples from the other laboratory-confirmed Nipah patients with detectable NiV RNA in oral swab samples because during hospital admission they reported no history of consuming raw date palm sap or contact with other encephalitis patients. All of the 6 laboratory-confirmed Nipah patients from whom we collected nearby surface swab samples had detectable NiV RNA in their oral swab samples, and 5 of these had evidence of NiV RNA on ≥1 nearby surface. Of the 5 patients who contaminated nearby hospital surfaces, 4 contaminated their towels, 3 contaminated their bed sheets, and 1 contaminated the bed rail. We detected no evidence for NiV RNA–contaminated walls or clinical files (Table 2).

We retrieved data on the partial N gene sequence (361 bp) from 4 patients' oral swab samples and 3 surface swab samples surrounding 2 of these 4 patients: from the towel surface for 1 patient and the towel and bed rail for 1 patient (GenBank accession nos. KY887670–1, MF133373–6, and MF13337). The sequence recovery was 40% (4/10) for oral swab samples and 38% (3/8) for surface swab samples. The sequences from patients' oral swab samples and corresponding surface swab samples were indistinguishable over the length of the sequenced fragments, and BLAST analysis indicated they were >99% similar to that of the NiV sequences (GenBank accession nos. JN808857, JN808859, JN808860, JN808864, JN808862) reported from Bangladesh.

Our investigation identified 3 Nipah patients who were infected through person-to-person transmission. Two of these patients were infected by 1 probable case-patient who died before specimens were collected. The third case-patient had close contact with 2 laboratory-confirmed case-patients over the same time period, but we were unable to determine the source of infection. Both possible sources had evidence of NiV RNA in oral swabs; however, only 1 of the possible infectors contaminated the hospital surfaces and therefore might be more likely to be the infector (online Technical Appendix Table 2, Figure 1). Laboratory-confirmed Nipah patients with detectable NiV RNA in oral swab samples were more likely to die than were patients with undetectable NiV RNA (90% [9/10] vs. 0% [0/2]; p = 0.04).

Discussion

Nipah patients frequently contaminated hospital surfaces near them with detectable NiV RNA, posing a risk for fomiteborne Nipah transmission. The most commonly contaminated surfaces were the bed sheets and the towels used by caregivers for patient care. In Bangladesh, family caregivers, rather than trained healthcare workers, provide 24-hour hands-on care to hospitalized patients (*17*,*25*). The more severe the patient's illness, the more hands-on care he or she receives (*17*). Most Nipah patients in Bangladesh are unconscious when they are brought in for care and have cough and difficulty breathing (*21*), requiring close attention and care. Nipah patients often dribble frothy oral secretions, soiling themselves and contaminating their bed sheets. Caregivers frequently use a towel brought from home to clean patient oral secretions (*17*) and often use the same towel throughout the hospital stay. They also frequently use the same towel for cleaning their own hands and face. The lack of running water in healthcare settings in Bangladesh makes it difficult for caregivers to wash their hands or wash the items used for patient care (*16*). One Nipah patient we identified was infected after caring for 2 other patients, 1 of whom had a towel contaminated with detectable NiV RNA, highlighting the possibility of this fomite as a vehicle of NiV transmission from patient to caregiver. The caregiver also contaminated nearby surfaces during his illness, including the towel, although no further transmission was evident.

We did not detect NiV RNA on the patient clinical file and nearby wall surfaces, most likely because of the distance and infrequency of contact of these surfaces with a patient's oral secretions. Although in Bangladesh hospitals patient clinical files are commonly kept on the bed or under

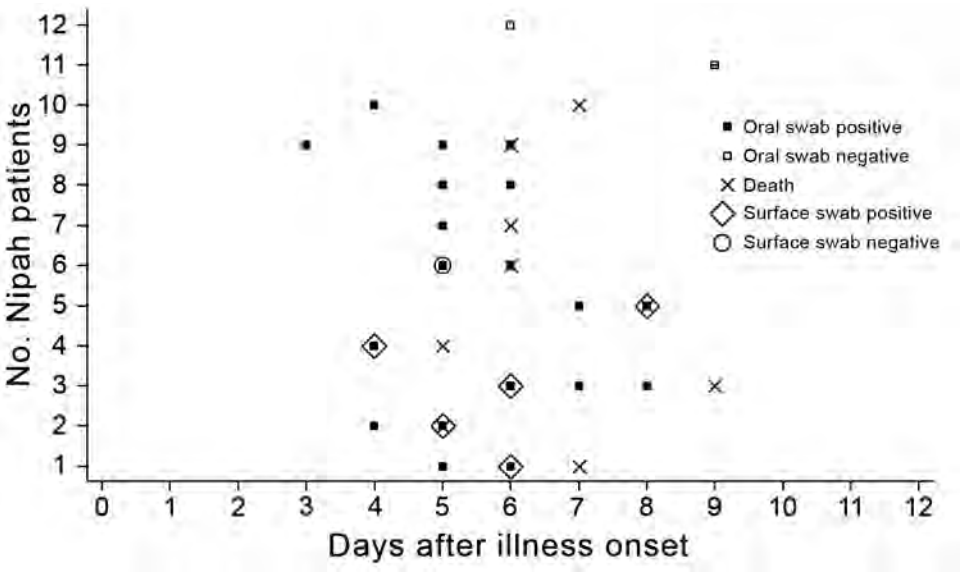

Figure 2. Timing of Nipah virus detection in oral swab and surface swab samples in relation to illness onset for 12 patients with laboratory-confirmed Nipah identified in hospitals, Bangladesh, December 2013–April 2014. Nearby surface swabs were not collected for 6 patients (nos. 7–12).

the bed sheet or pillow, they are also sometimes kept at the nurses' station, reducing the frequency of the file coming into contact with patient oral secretions. We also found that the walls were the surfaces farthest from the patient and for this reason might have been less frequently contaminated with oral secretions.

Transmission of NiV through fomites is plausible. Many paramyxoviruses, including respiratory syncytial virus, parainfluenza viruses 1–4, and human metapneumoviruses, have been identified on hospital surfaces, and fomiteborne transmission of these pathogens has been reported (*26–30*). Past studies have indicated that other paramyxoviruses can survive on surfaces for up to 10 hours and be a source of infection for patients, healthcare workers, and hospital visitors (*26*,*31–33*). Animal experiments with NiV in a hamster model also showed that NiV can be transmitted through fomites (*34*). Although it is not known how long NiV remains infectious in the environment, we hypothesize that surfaces might play an important role in NiV transmission for several reasons: hospital surfaces in Bangladesh are not routinely cleaned (*16*); new patients frequently use the same bed sheets used by previous occupants (*16*); caregivers and healthcare workers frequently come into contact with contaminated surfaces (*16*); and handwashing by caregivers and healthcare workers occurs infrequently because of several barriers, including a lack of running water in hospitals (*16*,*17*).

Investigations of earlier outbreaks showed that only 7% of all Nipah patients were Nipah spreaders (*5*,*35*). During our 5-month study, we identified 16 Nipah patients and 2 likely spreaders. The 2 spreaders we identified both infected their primary caregivers (online Technical Appendix Table 2, Figure 1). This finding provides additional evidence that exposure to contaminated oral secretions drives person-to-person transmission of NiV. Caregivers can be exposed to oral secretions through direct patient contact, contaminated surfaces, or both. Family care providers maintained close physical contact with Nipah patients, including sharing eating utensils and drinking glasses, sleeping in the same bed, and hugging and kissing near the time of death, which highlights that contact transmission might play a major role in NiV transmission (*36*). Our investigation showed similar patterns of caregiving practices in this outbreak (online Technical Appendix Table 2). Reports from earlier outbreaks also demonstrated that Nipah patients who had respiratory involvement (difficulty breathing and cough) were more likely to become Nipah spreaders (*5*,*6*,*9*,*12*). However, our current understanding is limited about why some Nipah patients shed NiV in their oral secretions (and for how long they shed) but others do not. Virus replication in the respiratory epithelium of hamsters infected with a high dose of NiV was 2 logs higher than in those infected with a low dose, suggesting dose of exposure might affect viral shedding in respiratory secretions (*36*). All NiV case-patients who had evidence of NiV RNA in their oral secretions died, and those without NiV RNA survived, suggesting that virulence also might be associated with tissue tropism or viral

Table 2. Proportion of surfaces contaminated with Nipah virus RNA associated with 6 laboratory-confirmed Nipah cases in 3 surveillance hospitals, Bangladesh, December 2013–April 2014*

Surface type	No. surface samples collected	No. (%) positive
Walls beside patient bed	4	0
Bed rails	4	1 (25)
Bed sheet	6	3 (50)
Clinical record file	6	0
Multipurpose towel	5	4 (80)

*Two patients were on the floor and had no bed rail surface; 2 patients did not have an adjacent wall; 1 patient did not have a towel sample.

load. A better understanding of the factors that determine variations of viral shedding between Nipah patients might explain the drivers of person-to-person transmission of NiV. Given limited resources for infection control in low-income settings, early identification of patients who shed NiV could help focus resources to reduce subsequent transmission of NiV from person to person. NiV surveillance in Bangladesh relies on a central laboratory located in the capital city; thus, confirming a diagnosis can take several days or weeks and limits the ability for an early intervention. A rapid diagnostic test that could quickly identify NiV patients at the bedside could be a powerful tool in the early identification of potential NiV spreaders, formulating early intervention and thereby preventing NiV transmission in hospitals.

Our study had limited power to detect a significant difference in characteristics of patients with and without detectable NiV RNA in oral swabs because of the small number of laboratory-confirmed Nipah patients we identified. However, despite low power and small number of observations, we found a significant association between having detectable NiV RNA in an oral swab sample and dying from illness. In addition, although we identified NiV RNA on various surfaces, the presence of nucleic acid does not confirm contamination with a viable virus nor does it indicate that fomites are important for NiV transmission. However, laboratory evidence suggests that paramyxoviruses can survive on surfaces and have been a source of transmission in healthcare settings (*26*,*27*). Studies on NiV survival in environmental condition have shown that NiV survival varies from a few hours to ≈2 days and is highly dependent on pH, temperature, and desiccation (*37*,*38*). Previous studies suggest that persons at highest risk for infection from patients with NiV are family caregivers who provide continuous care, even during hospitalizations (*9*,*10*,*20*). Therefore, even if the virus remains viable for only a short time, it still could pose a major risk for these caregivers.

Efforts to reduce the risk for person-to-person NiV transmission in healthcare settings should target patient caregiving practices related to the use of towels. Resources are limited for hospitals and for patients' families; however, affordable options exist that deserve additional investigation to determine their acceptability and feasibility. For example, families could be counseled to purchase a separate towel for patients, which costs ≈US $0.50. Also, hospitals could provide low-cost disinfectants, such as 0.5% sodium hypochlorite, for ≈US $1/liter to use to disinfect towels and caregiver hands. We also advocate for the development of a rapid test to identify NiV patients, who represent only ≈3% of all encephalitis patients, to most efficiently focus infection control efforts for NiV prevention (*6*,*39*,*40*).

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Detection and Circulation of a Novel Rabbit Hemorrhagic Disease Virus in Australia

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The highly virulent rabbit hemorrhagic disease virus (RHDV) has been widely used in Australia and New Zealand since the mid-1990s to control wild rabbits, an invasive vertebrate pest in these countries. In January 2014, an exotic RHDV was detected in Australia, and 8 additional outbreaks were reported in both domestic and wild rabbits in the 15 months following its detection. Full-length genomic analysis revealed that this virus is a recombinant containing an RHDVa capsid gene and nonstructural genes most closely related to nonpathogenic rabbit caliciviruses. Nationwide monitoring efforts need to be expanded to assess if the increasing number of different RHDV variants circulating in the Australian environment will affect biological control of rabbits. At the same time, updated vaccines and vaccination protocols are urgently needed to protect pet and farmed rabbits from these novel rabbit caliciviruses.

Rabbit hemorrhagic disease virus (RHDV) is a calicivirus in the genus *Lagovirus* causing acute hepatic necrosis in European rabbits (*Oryctolagus cuniculus*), leading to disseminated intravascular coagulation and death in ≥90% of susceptible animals (*1*). RHDV was first reported in China in 1984 and subsequently spread to South Korea by 1985, various European countries by 1986, and Mexico in 1988 (*1*). RHDV is now enzootic in domestic and wild rabbits in parts of Asia and Europe, and sporadic outbreaks continue to occur in the Americas, the Middle East, and Africa (*1*). RHDV also has had substantial ecological impacts in certain regions such as the Iberian Peninsula, where the rabbit is a keystone species, serving as a major prey species and actively altering the environment through grazing and burrowing behaviors (*2*).

Conversely, in Australia and New Zealand, the European rabbit is a major vertebrate pest, threatening the survival of native plants and animals, facilitating erosion through burrowing and grazing, and causing massive economic losses to agricultural sectors. In 1991, a strain of RHDV from Czechoslovakia (CAPM V-351) was imported into Australia for assessment of its suitability as a rabbit biocontrol agent; it has been used for this purpose since 1995, resulting in substantial benefits for both the economy and the environment (*3*–*5*). The same CAPM V-351 strain appeared in New Zealand in 1997, after it was presumably deliberately released by farmers (*6*). Circulating field strains (descendants of CAPM V-351) now cause regular natural outbreaks in both countries, and the original CAPM V-351 strain is continually rereleased through rabbit control programs (*7*).

RHDV has a 7.4-kb single-stranded positive-sense RNA genome (gRNA) comprising 2 open reading frames (ORFs) (*1*). ORF1 encodes a single polyprotein that is cleaved by the viral protease into 7 nonstructural proteins and the major capsid protein, viral protein (VP) 60, whereas ORF2 encodes a minor structural protein, VP10 (Figure 1) (*1*). Ninety dimers of VP60 self-assemble to form the RHDV capsid, whereas VP10 is present in much smaller amounts, and its function is not well established (*1,8*). In addition to the gRNA, a 2.1-kb subgenomic RNA (sgRNA) can also be detected in virus preparations and encodes both structural proteins VP60 and VP10 (Figure 1) (*1*). The first 16 nt at the 5′ ends of the RHDV gRNA and sgRNA are identical, with the exception of 2 sites; this structure may facilitate template switching at the junction of RNA-dependent RNA polymerase (RdRp) and VP60 (*9*).

RHDV strains are currently all classified as a single serotype, although phylogenetic analyses have led to the viruses being subclassified into 6 genogroups (*10,11*). The CAPM V-351 strain used for biocontrol in Australia and its

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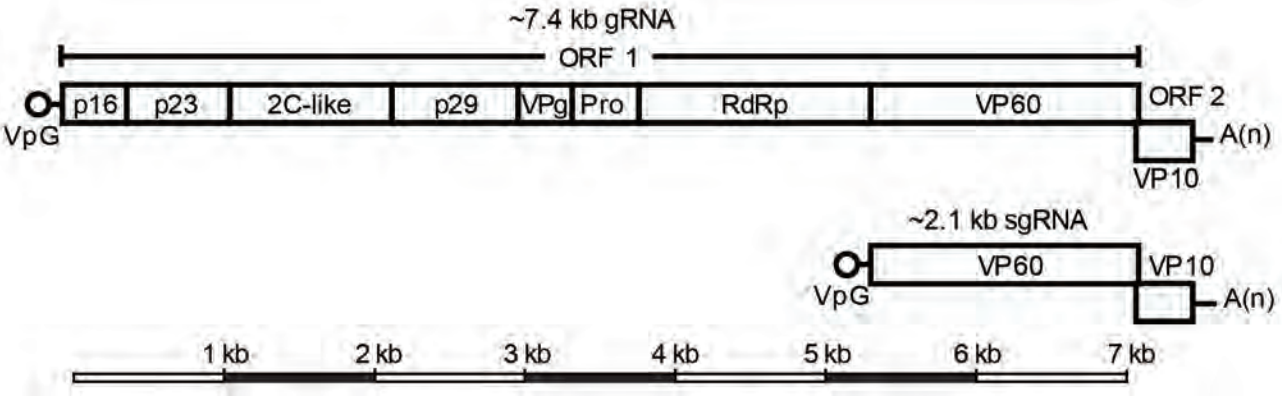

Figure 1. Genomic organization of RHDV. Top panel: RHDV has a polyadenylated single-stranded positive-sense gRNA of ≈7.4 kb consisting of 2 ORFs (open boxes), with the VPg (open circle) covalently attached to the 5′ end. ORF1 encodes a polyprotein that is proteolytically cleaved to produce the major capsid protein, VP60, and the nonstructural proteins p16, p23, 2C-like protein (2C-like), p29, VPg, the viral protease, and the RdRp. ORF2 encodes the minor structural protein, VP10. Bottom panel: A 2.1-kb, VPg-linked, polyadenylated sgRNA is collinear with the 3′ end of the gRNA and encodes VP60 and VP10. High homology between the 5′ end of the sgRNA and the corresponding region of the gRNA (the RdRp and capsid junction) may facilitate template switching during replication. gRNA, RNA genome; ORF, open reading frame; Pro, protease; RHDV, rabbit hemorrhagic disease virus; RdRp, RNA-dependent RNA polymerase; sgRNA, subgenomic RNA; VP, viral protein; VPg, viral genome-linked protein.

descendants are genogroup G2 viruses. In the late 1990s, new genetically and antigenically distinct RHDV variants were reported and were designated as subtype RHDVa or genogroup G6 on the basis of differences in reactivity profiles with monoclonal antibodies and on sequence divergence (*10*,*12*,*13*). Despite this difference, vaccines developed for classic RHDV strains were still found to be protective against RHDVa when administered at the correct dose and within recommended intervals (*10*,*12*,*13*). RHDVa strains were first detected in Italy and were subsequently detected in multiple countries, including Portugal, the United States, China, Cuba, and South Korea (*12;* reviewed in *1*). In some geographic regions, it was suggested that RHDVa had replaced previous enzootic viruses (*14*–*17*).

Subsequently, a novel lagovirus, designated RHDV2, was reported from France in 2010 (*18*). This virus showed greater genetic and antigenic diversity than RHDVa compared with classic RHDV, with ≈82% nucleotide sequence similarity in VP60 between wild-type RHDV2 and RHDV (*11*,*18*,*19*). Early RHDV2 pathogenesis studies demonstrated that this virus had a longer disease duration, lower virulence, and lower mortality rates than RHDV and that it could cause disease in vaccinated rabbits (*18*). RHDV2 rapidly spread to Italy, Spain, Portugal, Great Britain, Scotland, the Azores, and Australia, and ongoing surveillance suggests that in many areas RHDV2 replaced existing circulating viruses, including RHDVa (*18*–*26*). RHDV2 has also been shown to cause disease in other lagomorph species, in contrast to RHDV and RHDVa, which are considered species-specific for the European rabbit (*27*–*30*).

In addition to the virulent RHDV, RHDVa, and RHDV2, several nonpathogenic lagoviruses have been described that are genetically distinct and have historically been called rabbit caliciviruses (RCVs). RCVs have been reported from various geographic locations; in contrast to the pathogenic hepatotropic lagoviruses, most RCVs mainly cause a nonclinical infection of the small intestine (*1*). Until 2014, only the benign rabbit calicivirus Australia-1 (RCV-A1) and the RHDV biocontrol strain CAPM V-351 (and its descendants) were circulating in Australia (*7*,*31*). In May 2015, RHDV2 was detected in Australia for the first time (*23*). Before the detection of RHDV2, another exotic RHDV incursion was reported to the World Organisation for Animal Health (OIE) in January 2014 (*32*). We describe the detection, spread, and evolution of this first exotic virus, RHDVa, in Australia during December 2013–May 2015, before the arrival of RHDV2.

Materials and Methods

Samples

Liver samples collected from rabbits suspected to have died from RHDV were submitted to the Elizabeth Macarthur Agricultural Institute (EMAI) (Menangle, New South Wales [NSW], Australia) or to CSIRO (Black Mountain, Australian Capital Territory [ACT], Australia) by veterinarians or NSW Local Land Service rangers. Samples were collected during December 1, 2013–April 30, 2015. No animal ethics permit is required in Australia for sampling of rabbits that are found dead.

For serologic analysis of healthy wild rabbits, we collected 20 wild rabbits from the ACT; these animals were shot from a vehicle using a 0.22-caliber rifle targeting the head or chest. We collected samples (blood, liver,

duodenum, and bile) postmortem; all sampling was conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes as approved by the CSIRO Ecosystem Sciences animal ethics committee (approval #12–15).

RHDV Antigen Capture ELISA

Liver samples submitted to EMAI were prepared by homogenizing ≈1 g of tissue in 5 mL of phosphate-buffered saline and centrifuging at 3,000 rpm for 10 minutes. Supernatant was tested in the RHDV antigen-capture ELISA as previously reported (*33*).

Real-time Reverse Transcription PCR Assay

We screened samples for RHDV and RHDVa using real-time reverse transcription PCR (rRT-PCR). We applied sterile cotton swabs to the freshly cut surface of liver samples and transferred them to 5-mL vials containing 3 mL of sterile phosphate-buffered gelatin saline, then extracted viral RNA from 50-μL samples from these vials using the MagMAX-96 Viral Isolation System (Ambion, Austin, TX, USA) on a Kingfisher 96 magnetic particle handling system (ThermoFisher Scientific, Waltham, MA, USA).

We tested purified viral RNA (5 μL) in an RHDV-specific Taqman rRT-PCR assay using the AgPath-ID One-Step RT-PCR kit (ThermoFisher Scientific), based on detection of a conserved sequence of the VP60 gene with modifications (Table 1) (*34*). We developed an in-house Taqman rRT-PCR to specifically detect the exotic RHDVa virus (Table 1). Cycling conditions are available from the authors upon request.

Sequencing

We extracted RNA from 20–30 mg of liver tissue or bile using the RNeasy Mini Kit (QIAGEN, Hilden, Germany); the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI, USA); or the Invitrogen Purelink viral RNA/DNA Mini Kit (ThermoFisher Scientific) following the manufacturers' instructions. We performed first-strand cDNA synthesis from 5 μL of RNA with 500 ng of OligodT (18mer) (Geneworks, Thebarton, South Australia, Australia) using Invitrogen Superscript III (Life Technologies, Carlsbad, CA, USA) following the manufacturer's directions. We conducted initial detection and typing of RHDV samples submitted to CSIRO (≥1 isolate from each outbreak) using the universal lagovirus RT-PCR, as described previously (*35*), followed by Sanger sequencing of amplified products. RHDV G2 genomes were amplified as described previously (*7*). We amplified overlapping fragments of the RHDVa genome using Platinum Taq DNA polymerase high fidelity (Life Technologies) using primer sets detailed in Table 2 (cycling conditions available from the authors upon request). We purified PCR products using the QIAquick PCR purification kit (QIAGEN), quantified with the Qubit dsDNA BR assay (Life Technologies) and pooled in equimolar ratios.

Library Preparation and Illumina Sequencing

We prepared DNA libraries of PCR amplicons using the Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina MiSeq using the 300-cycle paired-end MiSeq Reagent Kit v2 (Illumina), as described previously (*7*).

NGS Genome Assembly and Data Analyses

We performed read quality assessment and trimming and merged overlapping paired-end reads, as described previously (*7*). We then mapped individual cleaned reads to the RHDV reference genome (Genbank accession no. M67473.1) using the Geneious in-built mapper tool as available in Geneious version 8.1.6 (*39*) and generated a majority consensus sequence. Primer sequences were trimmed from the 5′ and 3′ genomic termini. We named sequences using the syntax country/state/isolate identifier/date, with date in the format yyyy/mm (e.g., AUS/NSW/BER-1/2013/12). Consensus sequences were aligned with representative RHDV, RHDV2, and RCV-A1 sequences for recombination and phylogenetic analyses. We submitted all sequences generated in this study to GenBank (accession nos. KY628306–21.

We inferred maximum likelihood phylogenies for both the nonstructural genes (44 sequences, 5,238 nt) and the VP60 gene (47 sequences, 1,743 nt) using PhyML (*40*). We estimated trees using the general time reversible model with rate heterogeneity among sites (4 discrete rate categories) and a proportion of invariant sites (determined as most appropriate using jModel test [*41*]), with a combination of nearest neighbor interchange and subtree pruning

Table 1. Primers and probes used for RHDV and RHDVa detection by real-time reverse transcription PCR in isolates from rabbits, Australia*

Name	Sense	Sequence, 5′ → 3′	Strain	Reference
vp60–7_FOR	+	ACY TGA CTG AAC TYA TTG ACG	RHDV	(*34*)
vp60–8_REV	–	TCA GAC ATA AGA AAA GCC ATT GG		(*34*)
vp60–9_FAM	Probe	CCA ARA GCA CRC TCG TGT TCA ACC T–FAM-BHQ1		Modified from (*34*)
RHDVXa2010-F1	+	GCACCCGGCAGTATTCTC	RHDVa	This study
RHDVXa2010-R1	–	CCCAGCCAGCGTACATCTG		This study
RHDVXa2010-P1	Probe	ACTGTCCAACACTCTCCACAGAACA–FAM-BHQ1		This study

*RHDV, rabbit hemorrhagic disease virus; vp, viral protein; +, positive; –, negative.

Table 2. Primer sequences used for the amplification of RHDVa genomes for full-genome sequencing of isolates from rabbits, Australia*

Fragment	Primers	Sense	Sequence, 5′ → 3′	Reference
1	MRCV-F1	+	AAC TGC TAT TCT CCC AGA AAA GAA ACC CTT	This study
	RCVr1.2	–-	TGA GCT TSC CAG CDC CYT TCA TG	(*31*)
2	RCVf0.8	+	AAT GCT GTT GCT GTG GAY ACA AC	(*31*)
	RCVr3.3	–	GGR AGY CCY TCA TAG TCA TTG TCA T	(*31*)
3	RCVf3.0	+	GGY AAT GAY GAG TAT GAY GAG TGG CA	(*31*)
	RCVr4.7	–	ATR CCA CTT GGR AGY CCT CTT TTR G	(*31*)
4	Lago9	+	TGG NCC NAT YGC AGT YGG VRT TGA CAT GAC	(*36*)
	RCVr6.1	–	ACT ATC TGR CCR TTC CAY CTG TTG TC	(*31*)
5	Rab1b	+	CAG CDS GCA CTG CYA CCA CAG CAT C	(*35*)
	RHDV-12rev	–	ARC CTA ACT CAT ARG CCT GCA CAG TCG	(*37*)
6	RHDVf2	+	GTT TTG GTA CGC TAA TGC TGG ATC TGC	(*37*)
	RHDV_end	–	TTT TTT TTT TTT TTT TTT TTT TTT TTT TTA TAG CTT ACT TTA AAC TAT AAA CCC AAT TAA ACC	(*38*)

*MRCV, Michigan rabbit calicivirus; RCV, rabbit calicivirus; RHDV, rabbit hemorrhagic disease virus; +, positive; –, negative.

and regrafting branch swapping methods employed to search for the optimal topology. We rooted trees using a European brown hare syndrome virus sequence (EBHSV; GenBank accession no. Z69620) and estimated branch support using 1,000 bootstrap replicates.

We screened a full genome alignment containing the 15 newly acquired sequences and 12 reference genomes (27 sequences, 7,303 nt) for recombination using the RDP, GENECONV, MaxChi, and BootScan methods available in the RDP4 package (*42*), with $p<0.05$ representing significant evidence of recombination. We confirmed recombination events by phylogenetic analyses on either side of the proposed breakpoint.

Serologic Analysis

We tested serum specimens from the shot wild rabbits for the presence of RCV-A1 antibodies using a specific blocking ELISA (*43*). We also tested for antibodies against RHDV using a competition ELISA and used ELISAs for RHDV IgA and IgM subclass antibodies as described previously (*44*).

Results

In late December 2013 and January 2014, a property in northern Sydney, NSW, Australia, experienced the sudden deaths of 30 domestic rabbits from a population of 80. The treating veterinarian submitted liver samples to EMAI for routine RHDV testing in January 2014. The samples showed little or no reactivity in the RHDV antigen capture ELISA but tested strongly positive in the RHDV-specific rRT-PCR. This result was in contrast to the typical reactivity of field strains of RHDV from Australia, which react strongly in both the RHDV antigen capture ELISA and the RHDV-specific rRT-PCR (Table 3). To further characterize this calicivirus, a 326-bp fragment of the VP60 capsid gene was amplified using a universal lagovirus PCR (*35*). Sanger sequencing of this fragment identified the virus as a member of the RHDVa group (RHDV G6), a virus not previously reported in Australia.

From January 2014 through March 2015, the exotic RHDVa strain was detected by rRT-PCR in 7 reported outbreaks in domestic rabbit breeding facilities (outbreaks 1, 2, 3, 5, 8, 9, and 10; Table 4; Figure 2), as well as in wild rabbits from 2 locations (outbreaks 7 and 11; Table 4; Figure 2). Overall, >70 rabbit deaths were reported in domestic rabbits from properties where RHDVa was confirmed.

Outbreak 11, from Mulligan's Flat Nature Reserve near Canberra, ACT, Australia, was suspected when rangers detected a sudden reduction in rabbit numbers during their rabbit eradication program, although no rabbit carcasses were recovered. Subsequent serologic analysis of 20 healthy shot rabbits revealed a high proportion of rabbits positive for RHDV-specific IgM (7/20) and IgA (15/20), indicating a recent virus outbreak (*45*). However, despite moderate to high IgA and IgM titers, the serum specimens tested low or negative in the RHDV competition ELISA and RCV-A1 blocking ELISA, which are known to be more strain-specific than the IgM and IgA ELISAs (data not shown). This finding indicated possible exposure of the Mulligan's Flat rabbit population to a lagovirus that was antigenically distinct from both RHDV and RCV-A1. Because RHDV RNA can be detected in the bile of recovering animals for as long as 15 weeks postinfection (*34*), we extracted RNA from the bile of IgM-positive individuals and

Table 3. Cross-reactivity of different diagnostic tests used to identify novel RHDVa variant in rabbits, Australia*

Test	RHDV	RHDVa	RCV-A1
RHDV antigen capture ELISA	+++	–/+	–
RCV-A1 blocking ELISA	–	–	+++
RHDV competition ELISA	+++	++	–/+
RHDV IgA ELISA	+++	+++	++
RHDV IgM ELISA	+++	+++	++
VP60 rRT-PCR	+++	+++	+++
RHDVXa-2010 rRT-PCR	–	+++	–
Lagovirus RT-PCR	+++	+++	+++

*All tests used the VP60 capsid protein coding region as target region. RCV, rabbit calicivirus; RHDV, rabbit hemorrhagic disease virus; rRT-PCR, real-time reverse transcription PCR; VP, viral protein; –, no cross-reactivity; –/+, minimal cross-reactivity; ++, moderate cross-reactivity; +++, marked cross-reactivity.

Table 4. Outbreaks of RHDV in rabbits, Australia, January 2014–March 2015*

Outbreak no.*	Isolate name	Collection date	Location, state	Variant	Rabbit origin	GenBank accession no.
1	AUS/NSW/BER-1/2013/12†	Dec 2013‡	Berowra, NSW	RHDV G2	Domestic	KY628307
1	AUS/NSW/BER-2/2013/12†	Dec 2013‡	Berowra, NSW	RHDVa	Domestic	KY628309
2	AUS/NSW/BER-3/2014/01†	Jan 2014	Berowra, NSW	RHDVa	Domestic	KY628310
2	AUS/NSW/BER-4/2014/01†	Jan 2014	Berowra, NSW	RHDVa	Domestic	KY628311
2	AUS/NSW/BER-5/2014/01	Jan 2014	Berowra, NSW	RHDVa	Domestic	NA
2	AUS/NSW/BER-6/2014/01	Jan 2014	Berowra, NSW	RHDVa	Domestic	NA
2	AUS/NSW/BER-7/2014/01	Jan 2014	Berowra, NSW	RHDVa	Domestic	NA
2	AUS/NSW/BER-8/2014/01†	Jan 2014	Berowra, NSW	RHDVa	Domestic	KY628312
2	AUS/NSW/BER-9/2014/01	Jan 2014	Berowra, NSW	RHDVa	Domestic	NA
2	AUS/NSW/BER-10/2014/01†	Jan 2014	Berowra, NSW	RHDVa	Domestic	KY628308
2	AUS/NSW/BER-11/2014/01	Jan 2014	Berowra, NSW	RHDVa	Domestic	NA
3	AUS/NSW/KYO-1/2014/01†	Jan 2014	Kyogle, NSW	RHDVa	Domestic	KY628316
3	AUS/NSW/KYO-2/2014/01	Jan 2014	Kyogle, NSW	RHDVa	Domestic	NA
4	AUS/NSW/BlueGums1/2014/03	Mar 2014	Murrumbateman, NSW	RHDV G2	Wild	KT006732.1
5	AUS/NSW/ANN-1/2014/04†	Apr 2014	Annangrove, NSW	RHDVa	Domestic	KY628306
6	AUS/NSW/OUR-1/2014/06†	June 2014	Ourimbah, NSW	RHDV G2	Wild	KY628318
6	AUS/NSW/OUR-2/2014/06†	June 2014	Ourimbah, NSW	RHDV G2	Wild	KY628319
7	AUS/NSW/GIR-1/2014/07†	July 2014	Girvan, NSW	RHDVa	Wild	KY628314
7	AUS/NSW/GIR-2/2014/07†	July 2014	Girvan, NSW	RHDVa	Wild	KY628315
8	AUS/NSW/BLA-1/2014/07†	July 2014	Blacktown, NSW	RHDVa	Domestic	KY628313
9	AUS/NSW/WAL-1/2015/01†	Jan 2015	Walcha, NSW	RHDVa	Domestic	KY628320
10	AUS/NSW/OAK-1/2015/02†	Feb 2015	The Oaks, NSW	RHDVa	Domestic	KY628317
11	AUS/ACT/MF-109/2015/03	Mar 2015	Mulligan's Flat, ACT	RHDVa	Wild§	KY628321

*Samples are numbered according to the date on which they were collected. These numbers correspond to those used on the map in **Figure 2**. ACT, Australian Capital Territory; NSW, New South Wales; RHDV, rabbit hemorrhagic disease virus.
†Samples for which the full genome was sequenced in this study.
‡Detection of RHDVa confirmed in January 2014.
§Virus sequence obtained from a healthy shot rabbit.

analyzed it using the universal lagovirus PCR (*35*). We amplified a lagovirus-specific fragment from 1 animal (AUS/ACT/MF-109/2015/03; Table 4), and Sanger sequencing confirmed the presence of the exotic RHDVa in this rabbit.

We also detected Australian field strains (RHDV G2) during the same sampling period on 3 occasions, 2 from wild rabbits (outbreaks 4 and 6; Table 4), and 1 from a domestic rabbit facility (outbreak 1; Table 4). The strain from the domestic rabbit facility was identified in samples from the first reported outbreak of the exotic RHDVa, indicating that both viruses were active simultaneously on this property.

We conducted full-genome sequencing for 15 RHDV isolates we sampled during December 2013–March 2015. We could not recover a full-length genome from AUS/ACT/MF-109/2015/03. Recombination and phylogenetic analyses of the nonstructural genes indicated that all RHDVa viruses were recombinants between RHDVa and an RCV-A1–like virus, with strong bootstrap support (Figures 3, 4). Specifically, we found strong evidence for recombination with a putative breakpoint located between the RdRp and VP60 genes (genome position 5,304 in Genbank accession no. M67473.1 sequence numbering), detected by all recombination detection methods in RDP4 (Figure 3). Phylogenetic analysis of the complete VP60 capsid gene revealed that the RHDV G2 viruses clustered with other recent RHDV G2 samples from Australia and that the RHDVa samples clustered (99% nucleotide sequence identity) with exotic RHDVa variants, particularly a variant from China, XA/China/2010 (Genbank accession no. JN165234) (Figure 4, panel A) (*14*). In contrast, the nonstructural genes of the RHDVa viruses found in Australia clustered with the RCV-A1–like clade that contains several other lagoviruses, including RCV-A1/RHDV2 recombinant viruses (*46*) and Michigan RCV (*47*), neither of which have been detected in Australia (Figure 4, panel B). Although the nonstructural genes of these viruses cluster most closely with RCV-A1, they are quite distinct, sharing only 83.3%–84.4% nucleotide identity with RCV-A1 sequences in this region, and could therefore represent a novel lagovirus genotype.

Discussion

We describe the detection and characterization of RHDVa in Australia, the first of 2 recent incursions of exotic lagoviruses (*23*). The route of entry of these viruses into Australia is unclear. The spread of RHDVa from Europe to distant locations such as Asia, the Americas, Reunion Island, and Australia (*1*) highlights the ease with which this virus can be disseminated, despite strict quarantine regulations. Caliciviruses are known to be highly environmentally stable, they replicate to very high titers in infected rabbits, and they are efficiently transmitted (*1*), suggesting that they would be relatively easy to disseminate inadvertently, analogous to the situation observed with the emergence of canine parvovirus (*48*).

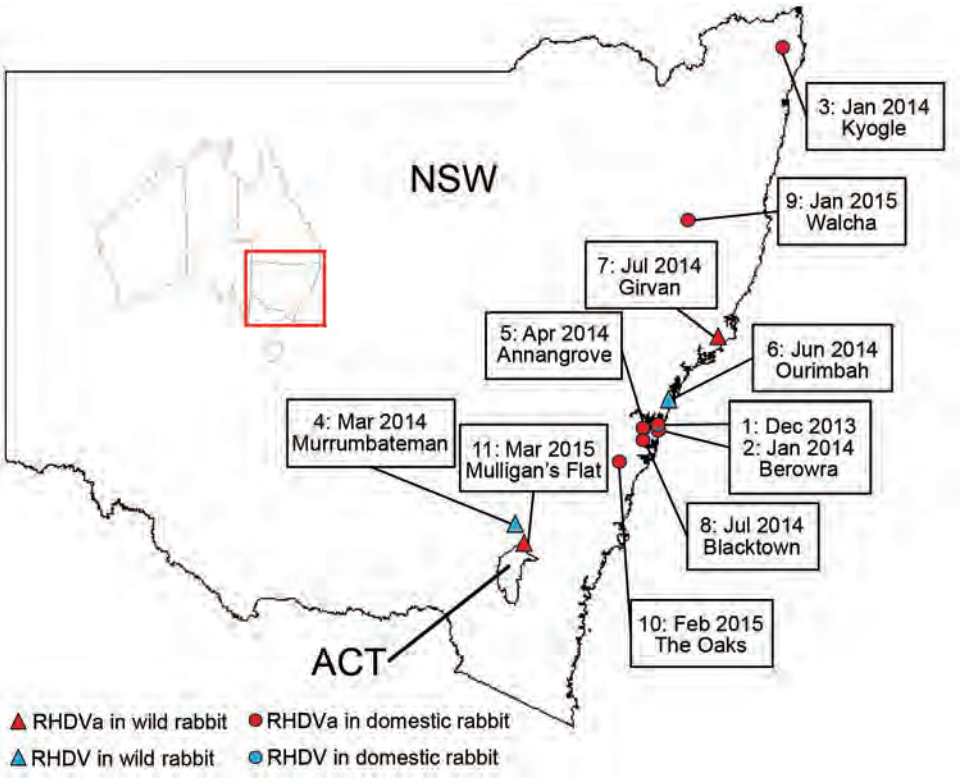

Figure 2. RHDV and RHDVa detections in eastern Australia, January 2014–March 2015. Sites where RHDVa and Australian RHDV field strains were detected are indicated on the map and numbered according to the order in which the outbreaks occurred. Inset shows location of NSW and ACT in Australia. ACT, Australian Capital Territory; NSW, New South Wales.

Unexpectedly, full-genome sequencing revealed that all RHDVa viruses in Australia had recombinant genomes, with the capsid gene closely related to a 2010 virus from China and nonstructural genes related to RCV-A1–like viruses (Figure 4). This finding highlights the importance of recombination in the generation of genetic diversity in this virus family. The emergence of this RCV-A1–like/RHDVa recombinant is of particular interest given the differences in tissue tropisms of RHDVa, which replicates in the liver, and nonpathogenic RCVs, which replicate in the small intestine (*1*,*35*). Recombinant viruses containing RCV-A1–like nonstructural genes and RHDV2 structural genes have also been reported (*46*). Although RHDV is widely disseminated in an infected animal, there is no evidence to suggest it can actively replicate in the small intestine. Conversely, RCV-A1 has only been shown to infect the columnar epithelial cells of the small intestine (*35*). Therefore, we hypothesize that the recombination event likely occurred during early viral replication in macrophages, which has been suggested for both RHDV (*1*) and RCV-A1 (T. Strive, unpub. data). Although the exact parental virus and source of the RCV-A1–like nonstructural genes were not identified, the distance between the nonstructural genes of RHDVa in Australia and currently sampled RCV-A1 nonstructural genes (83.3%–84.4% nt identity, Figure 4B) suggests either that the recombination event occurred before the virus arrived in Australia with an as-yet uncharacterized calicivirus or that recombination did occur in Australia but the parental virus belongs to a divergent RCV-A1 lineage that has not yet been identified.

The exotic RHDVa variant was first detected in Australia in samples from an RHD outbreak in December 2013 in domestic rabbits in a suburb of northern Sydney (*32*). During January 2014–March 2015, this virus was detected in a further 8 outbreaks. Detections were localized to southeastern Australia, predominantly in the Sydney basin. In contrast, the initial spread of the CAPM V-351 virus in 1995 was estimated to be 50 km/wk (*1*). Seven of the 9 documented RHDVa outbreaks were in domestic rabbits, although this probably reflects sampling bias, because very limited surveillance occurred in NSW and ACT in wild rabbits during the sampling period.

The seroprevalence of RHDV and RCV-A1 antibodies was shown to be very high in wild rabbit populations in southeastern Australia (*49*), which may limit RHDVa infection and transmission rates in wild rabbits compared with unvaccinated domestic rabbits, due to partial or complete immunological cross-protection from previous infection with RHDV, RCV-A1, or both. In addition, RHDV outbreaks are much more readily detected in domestic rabbit facilities where animals are checked daily, whereas outbreaks in wild rabbits often go unnoticed, particularly as carcasses are rapidly removed by scavengers. Movement of domestic rabbits for breeding and showing purposes may also have facilitated spread of the virus in this population within the Sydney basin. Nevertheless, RHDVa was

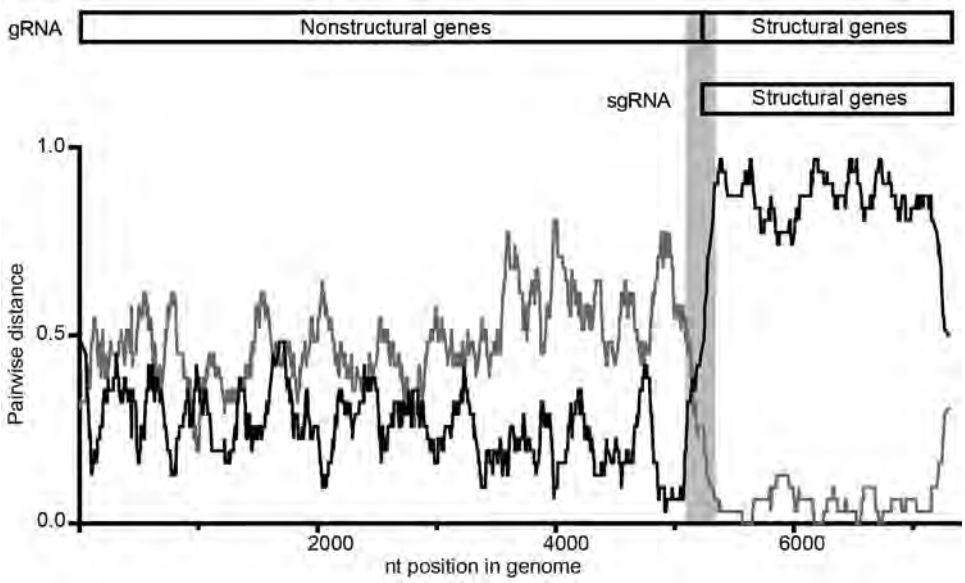

Figure 3. Recombination detection program plot (*42*) demonstrating recombination in a representative rabbit hemorraghic disease virus type a (RHDVa) strain from Australia. The pairwise identity of the recombinant, KYO-1, with the putative parental strains, RHDVa/AB300693.2/JPN/Hokkaido/2002 (black) and RCV-A1/EU871528.1/AUS/MIC-07(1–4)/2007 (dark gray), is plotted according to genome position (nt). A clear crossover event can be observed at the junction of RNA-dependent RNA polymerase and viral protein 60. The window size was set to 30. A schematic representation of the rabbit lagovirus gRNA and sgRNA is shown above the RDP plot to illustrate the genomic structure. The light gray bar shows the region where recombination was detected. gRNA, RNA genome; RCV, rabbit calicivirus; sgRNA, subgenomic RNA.

confirmed in 2 wild rabbit populations, in Girvan, NSW (outbreak 7), and Mulligan's Flat (outbreak 11; Table 4), suggesting infection and transmission of RHDVa was occurring in wild populations.

In Mulligan's Flat (outbreak 11), we recovered RHDVa RNA from a healthy shot rabbit following an observed decline in rabbit numbers. Subsequent serologic analyses from a sample of healthy shot rabbits suggests that high levels of cross-reacting isotype antibodies (IgA, IgM, and IgG), in combination with low or negative test results for specific competition ELISAs for RHDV and RCV-A1, may be used as a tool to infer previous exposure of a rabbit population to RHDVa. This particular serologic profile may prove useful for large-scale field epidemiology studies attempting to track the spread and impact of RHDVa in the Australian landscape, even in the absence of a specific serologic test. Furthermore, our study demonstrates that PCR analysis of bile in recovering, recently RHDV-infected (IgM-positive) rabbits can be an additional tool to determine to which virus a wild rabbit population had been exposed.

Several of the RHDVa cases in domestic rabbits were reported to have occurred in previously vaccinated rabbits, although details about the vaccination regimens and intervals were difficult to retrieve. The RHDVa variant JN165234/XA/China/2010, which closely resembles the VP60 sequence of the RHDVa from Australia we describe, was reported to be able to partially overcome protection induced by vaccination against classic RHDV (*14*). However, the vaccine used in that study was not a commercial preparation, and dose rates were not provided (*14*). The same study demonstrated that vaccination against classic RHDV induced 100% protection against another RHDVa virus from China (*14*). RHDV2 is considered to be a second lagovirus serotype, but the OIE Terrestrial Manual does not discriminate between RHDV and RHDVa with respect to vaccination and conservatively recommends that breeder rabbits be vaccinated every 6 months to ensure protection in commercial rabbitries (*11*). Australian authorities have issued updated vaccination recommendations subsequent to the detection of RHDV2 to reflect those of the OIE (*11*). Ultimately, a polyvalent vaccine effective against all viruses circulating in Australia (RHDV and RHDV2) would be preferred for domestic rabbits.

Before the detection of RHDVa, the deliberately released CAPM V-351 and its descendants were the only RHDV variants circulating in Australia, despite both RHDVa and RHDV2 circulating overseas since 1997 and 2010, respectively (*7*,*12*,*18*). As such, the RHDV antigen capture ELISA was routinely used for diagnostic purposes. Given that the RHDVa virus we report here shows little to no reactivity in this ELISA, it is recommended that the monoclonal antibodies used in diagnostic ELISAs be updated, as described in the OIE Terrestrial Manual, or that alternative detection methods such as RT-PCR be used for RHD diagnosis in Australia (*11*). At this stage, it is unclear what effects the presence of this new virus will have on Australia's wild rabbit populations and on biocontrol efforts using RHDV. With the recent (March 2017) release of another RHDVa virus (an RHDVa from South Korea), it is critical that ongoing monitoring and surveillance systems are in place to explore the interactions

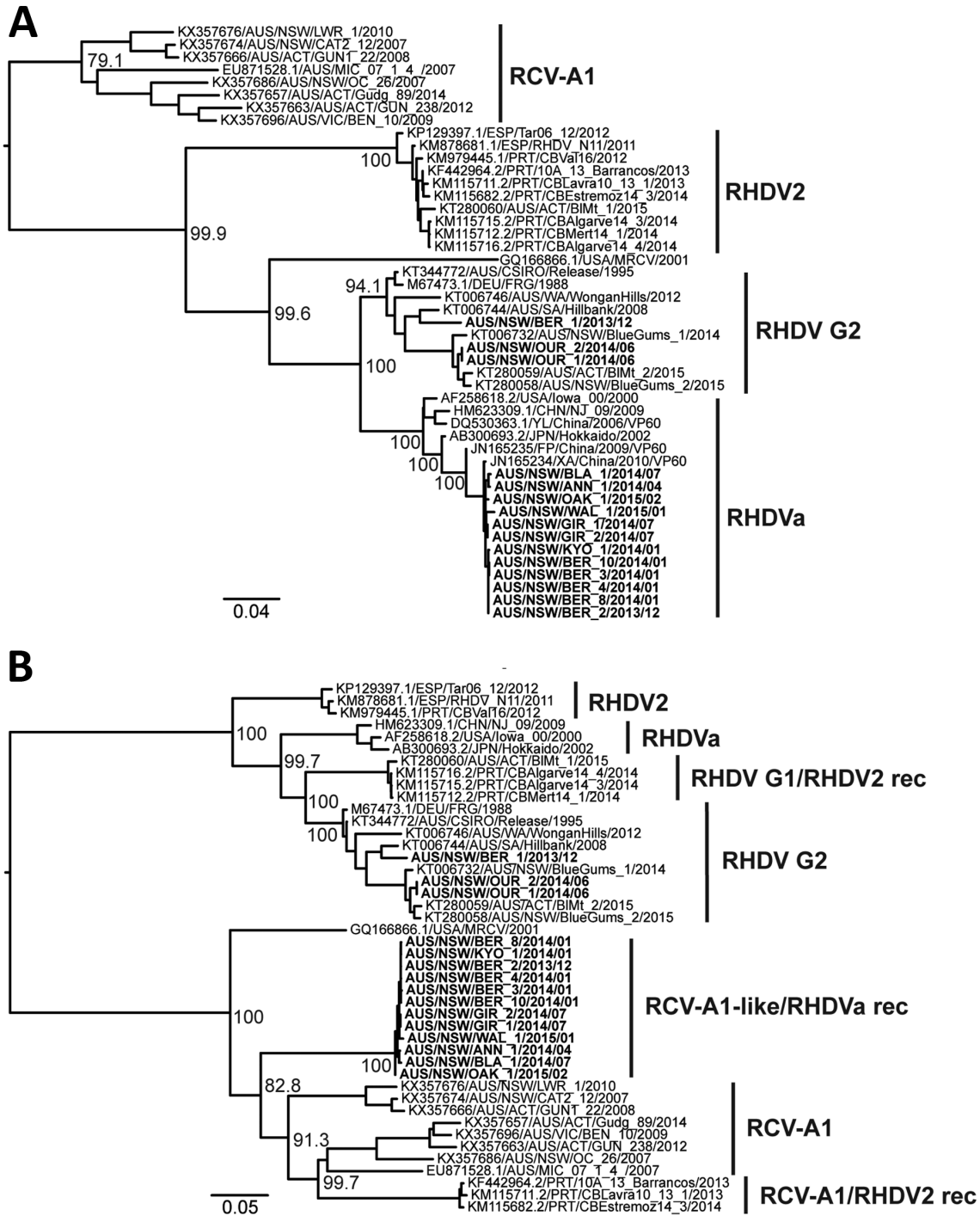

Figure 4. Phylogenetic analysis of viral protein 60 (VP60) capsid (n = 47) and nonstructural (n = 44) genes of RHDV strains from Australia and reference sequences. Maximum likelihood phylogenies of the A) VP60 capsid genes and B) nonstructural genes were prepared from an alignment of the newly sequenced RHDV samples (bold) along with published sequences (accession numbers of published sequences indicated in the taxa name). The JN165235/FP/China/2009 and JN165234/XA/China/2010 sequences were restricted to the capsid gene tree because nonstructural gene sequences are not available for these viruses. Variant names for each cluster are indicated. Recombinant (rec) variants are labeled as nonstructural/capsid gene type. Phylogenies were rooted using an early European brown hare syndrome virus isolate (not shown). Bootstrap support values are shown for the major nodes. Scale bars indicate nucleotide substitutions per site. RCV, rabbit calicivirus; RHDV, rabbit hemorrhagic disease virus.

of these new viruses as they establish under Australian field conditions (*50*). In this context, it will be particularly interesting to determine if the recombinant RHDVa described here persists in the face of the exotic incursion of RHDV2, which has reportedly replaced RHDV and RHDVa viruses in many parts of Europe (*24–26*). Once again, the epidemiology of these viruses demonstrates the utility of Australia's rabbit populations serving as a model system for disease emergence, viral competition, and evolution.

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Dr. Mahar is a postdoctoral associate at the University of Sydney and collaborates closely with CSIRO on a project that aims to understand the evolution of virulence in rabbit hemorrhagic disease virus. She has a general interest in molecular microbiology and is particularly interested in virus evolution and virus discovery.

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Geogenomic Segregation and Temporal Trends of Human Pathogenic *Escherichia coli* O157:H7, Washington, USA, 2005–2014[1]

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The often-noted and persistent increased incidence of *Escherichia coli* O157:H7 infections in rural areas is not well understood. We used a cohort of *E. coli* O157:H7 cases reported in Washington, USA, during 2005–2014, along with phylogenomic characterization of the infecting isolates, to identify geographic segregation of and temporal trends in specific phylogenetic lineages of *E. coli* O157:H7. Kernel estimation and generalized additive models demonstrated that pathogen lineages were spatially segregated during the period of analysis and identified a focus of segregation spanning multiple, predominantly rural, counties for each of the main clinical lineages, Ib, IIa, and IIb. These results suggest the existence of local reservoirs from which humans are infected. We also noted a secular increase in the proportion of lineage IIa and IIb isolates. Spatial segregation by phylogenetic lineage offers the potential to identify local reservoirs and intervene to prevent continued transmission.

Escherichia coli O157:H7 infections cause major public health challenges. Most *E. coli* O157:H7 infections occur sporadically, and the source of infection is often difficult to identify with certainty (*1*,*2*). Many reported infections are attributed to food vehicles (*1*), but studies have implicated other risk factors, and environmental transmission may be particularly notable in rural areas (*3*–*7*). Overall, the frequency of infections with *E. coli* O157:H7 has fallen in the United States, which is likely related to improved food safety (*8*), but it is not clear that rural incidence has also fallen.

Residing in a rural area confers increased risk for *E. coli* O157:H7 infection (*9*,*10*). *E. coli* O157:H7 can persist in certain locales, posing ongoing risk to humans. Multiple studies demonstrate that specific strains persist within cattle farms and spread to neighboring farms (*11*–*15*). The reservoirs enabling this persistence may include water, soil, and wild birds (*16*–*19*). It is, therefore, possible that humans incidentally acquire *E. coli* O157:H7 infections because they reside in a geographic region with a persistent reservoir. Using a generalizable population-based cohort, we sought to test the hypothesis that there are geographic foci of related *E. coli* O157:H7 infections, most likely of environmental origin, taking into account the genomic relatedness of different isolates (*20*,*21*) and the geographic, temporal, and secular attributes of their corresponding infections.

Methods

Study Population and Pathogen Characterization

We conducted a population-based retrospective cohort study of all culture-confirmed *E. coli* O157:H7 cases reported to the Washington State Department of Health (DOH; Shoreline, WA, USA) during 2005–2014. *E. coli* O157:H7 case reporting mandated by the Washington Administrative Code occurs primarily through diagnostic laboratories and healthcare providers. Local health jurisdictions use a standardized DOH case report form to abstract medical records; interview case-patients to obtain demographic information (including residence address), potential exposures, and details of the course of illness; and determine the most likely source of infection. For this study, case addresses were geocoded and census block groups determined. Case data were deidentified for analysis. This study was deemed exempt by the Washington State Institutional Review Board.

All *E. coli* O157:H7 isolates are sent to DOH for microbiologic confirmation and XbaI pulsed-field gel electrophoresis (PFGE) typing. We obtained isolates from

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DOH and determined their lineage according to the phylogenetic tree developed by Bono et al. (*20*) and expanded by Jung et al., who identified some lineages as clinical and others as bovine-biased (*21*). We used the Jung et al. 48-plex single-nucleotide polymorphism (SNP) assay to type a subset of isolates (*21*). We assumed that all isolates with a given PFGE pattern would be SNP typed to the same lineage. Thus, we typed ≥1 isolate from each PFGE pattern in the dataset and inferred the lineage of nontyped isolates. Concordance among isolates with identical PFGE profiles was confirmed (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-0851-Techapp1.pdf). We analyzed the clinically common lineages Ib, IIa, and IIb separately and analyzed the bovine-biased and remaining sparsely represented lineages (*21*) as a clinically rare group.

Phylogenetic Lineage Spatial Segregation

Spatial segregation is the ecologic concept that one species or species type is more likely to be surrounded by like than by nonlike individuals (*22*). We used Diggle's kernel estimation method (*23*) and spatialkernel package (*24*) in R (*25*) to test spatial segregation of *E. coli* O157:H7 by phylogenetic lineage (online Technical Appendix). In brief, we first estimated a smoothed probability surface for each lineage by comparing the distance between cases infected with the same lineage to the distance between cases infected with different lineages. A peak in the lineage-specific probability surface indicates an area with a high probability of that lineage, relative to the distribution of the other lineages. For example, if 80% of cases in a given proximity are infected with lineage Ib but in all other areas lineage Ib causes only 50% of cases, we would observe a peak in the lineage Ib-specific probability surface, suggesting spatial segregation. To determine overall spatial segregation, the probability surfaces were compared with a null distribution in which the proportion of infections caused by each lineage is constant across space.

We next sought to account for potential confounders and to detect geographic trends. To do so, we modeled the risk surface using a multinomial generalized additive model (GAM). We estimated the effect of a bivariate thin plate regression spline smooth of latitude and longitude on the odds of infection with a given lineage compared with the most common lineage. This smoothing technique produces a risk surface that can vary flexibly across both horizontal and vertical coordinates. In this analysis, we compared lineages IIa and IIb and the group of clinically rare lineages separately with lineage Ib, which served as the reference (most common) lineage. The model was adjusted for sex and age group (<5, 5–9, 10–19, 20–59, and ≥60 years); isolates from cases of unknown age (n = 1) or sex (n = 10) were excluded from analysis. We estimated parameters using restricted maximum likelihood and the mgcv package in R (*26*,*27*). We further conducted a series of sensitivity analyses to determine the robustness of our results by seeking to confirm our results with 2 independent methods: Dixon's nearest-neighbor test (*22*) and multinomial spatial scan statistics (*28*) (online Technical Appendix).

Temporal Variation in Spatial Segregation

To determine whether spatial segregation of lineages varied over time, we replicated our spatial segregation analyses incorporating time. To do so, we split the years of analysis into 3 intervals (2005–2007, 2008–2010, and 2011–2014) and calculated a kernel-based estimate of spatial segregation for each. We evaluated the effect of time in the multinomial GAM by adding year to the model as a continuous variable, testing the effect of year as both a linear term and as a smoothed term using a thin plate spline. The thin plate spline allows the association between lineage and year to smoothly change in magnitude and direction.

Exploratory Risk Factor Analysis

We explored potential drivers of segregation by testing the association of risk factors included on the DOH case report form with each lineage compared with the reference lineage Ib. Using multinomial GAMs adjusting for sex, age, year, and latitude and longitude as a thin plate spline bivariate smoother, we tested each risk factor (online Technical Appendix Table 1). In addition to the statewide analyses, region-specific analyses were conducted for the 3 regions with the highest *E. coli* O157:H7 incidence to determine locally key associations. Regions were defined based on major population centers, areas of increased agricultural intensity, and observed segregation clusters, and models were adjusted for sex, age, and year.

Results

During the study period, 1,160 *E. coli* O157:H7 cases were reported to DOH. Of these, 33 isolates, representing 31 PFGE types, were not available for typing (online Technical Appendix), and isolates from 6 cases were excluded as biochemically atypical *E. coli* O157:H7 (online Technical Appendix Figure 1). We SNP typed 793 isolates and, by extension, matched another 328 to a known lineage using PFGE, enabling us to assign a specific lineage of *E. coli* O157:H7 to isolates from 1,121 cases. Ten cases lacked address data and were excluded, leaving 1,111 cases for analysis.

Lineages Ib, IIa, and IIb, in descending order, were the most common lineages (Table). Twelve clinically rare lineages were identified, including 2 not previously described, encompassing 45 unique PFGE types (online Technical Appendix Figure 1). Lineage Ib comprised 210 PFGE types, whereas lineage IIa comprised only 38 PFGE types

Table. *Escherichia coli* O157:H7 lineage frequency by case characteristic among culture-confirmed human cases reported in Washington, USA, 2005–2014*

Variable	Lineage Ib	Lineage IIa	Lineage IIb	Rare lineages†
Total	586 (52.7)	260 (23.4)	199 (17.9)	66 (5.9)
Mean isolates per PFGE type (SD)‡	2.8 (5.3)	6.8 (14.3)	7.7 (24.7)	1.5 (1.7)
Sex				
F	333 (56.8)	163 (62.7)	105 (52.8)	33 (50.0)
M	244 (41.6)	97 (37.3)	94 (47.2)	32 (48.5)
Unknown	9 (1.5)	0	0	1 (1.5)
Age group, y				
<5	119 (20.3)	72 (27.7)	63 (31.7)	10 (15.2)
5–9	81 (13.8)	32 (12.3)	33 (16.6)	12 (18.2)
10–19	97 (16.6)	51 (19.6)	31 (15.6)	6 (9.1)
20–59	207 (35.3)	81 (31.2)	49 (24.6)	29 (43.9)
≥60	81 (13.8)	24 (9.2)	23 (11.6)	9 (13.6)
Unknown	1 (0.2)	0	0	0
HUS				
Yes	37 (6.3)	18 (6.9)	20 (10.0)	0
No	526 (89.2)	236 (90.1)	173 (86.1)	67 (98.5)
Unknown	27 (4.6)	8 (3.1)	8 (4.0)	1 (1.5)

*Values are no. (%) except as indicated. HUS, hemolytic uremic syndrome; PFGE, pulsed-field gel electrophoresis.
†Twelve clinically rare lineages.
‡PFGE type percentages indicate the proportion of PFGE types with an assigned lineage (n = 355) belonging to each lineage.

and lineage IIb 26 PFGE types (online Technical Appendix Figure 1). Lineage IIa contained an average of 7 (SD 14) and IIb an average of 8 (SD 25) isolates per PFGE type, compared with 3 (SD 5) for lineage Ib and 1 (SD 2) for the clinically rare lineages (Table).

Distribution of cases by sex, age group, and hemolytic uremic syndrome (HUS) status varied by lineage (Table). Lineage IIa and IIb isolates originated disproportionately from children <5 years of age compared with isolates in lineage Ib. Patients infected with lineage IIb bacteria also had higher frequencies of HUS (10%) than other patients (6%). None of the patients with infections caused by isolates from the clinically rare lineages developed HUS.

Spatial Segregation

The result of Diggle's kernel estimation test was statistically significant ($p = 0.001$), suggesting spatial segregation. Lineage-specific probability surfaces showed separate, distinct peaks for lineages Ib, IIa, and IIb (Figure 1). The southwest region of Washington was marked by segregation of lineage IIb isolates and correspondingly lower probability of isolating lineage Ib from cases. Spatial segregation was observed for lineage Ib isolates in northwest Washington and for lineage IIa isolates in the south-central region. There was low probability of lineage IIb isolates in both these areas. Sensitivity analysis corroborated these results (online Technical Appendix).

Consistent with the kernel regression results, the adjusted GAM risk surface of lineage IIb varied significantly from that of Ib ($p<0.001$), providing additional support of the spatial segregation. The frequency of lineage IIb isolation was greater than the frequency of Ib in the southwest region, but this imbalance diminished as latitude and longitude increased (Figure 2), that is, in areas northward and eastward. This spatial pattern was also observed in the kernel estimation map of lineage IIb (Figure 1). The risk surfaces of lineage IIa and the clinically rare lineage group did not differ significantly from that of Ib (online Technical Appendix Table 2). In sensitivity analyses designed to gauge the robustness of results to model assumptions, the spatial risk surface of lineage IIb consistently varied significantly from the risk surface of lineage Ib (online Technical Appendix Table 2). The spatial risk surface of lineage IIa also varied significantly from the risk surface of lineage Ib in some sensitivity analyses, similar to the spatial distribution in the kernel estimation lineage IIa–specific probability surface.

We also found significant differences in lineage by age of infected patients, independent of geography. The likelihood of being an adult (age ranges 20–59 and ≥60 years of age) versus being a toddler (<5 years of age) was lower among IIa-infected patients than among Ib-infected patients (20–59 years odds ratio [OR] 0.65, 95% CI 0.44–0.96; ≥60 years OR 0.49, 95% CI 0.28–0.85). The odds of being 20–59 years of age versus <5 years were also lower among IIb-infected patients than among Ib-infected patients (OR 0.44, 95% CI 0.28–0.69). Thus, adults comprised a smaller proportion of patients infected with lineage IIa or IIb *E. coli* O157:H7 than of those infected with lineage Ib. We found no significant differences by sex.

Temporal Variation

The incidence of *E. coli* O157:H7 averaged 1.73/100,000 population during the study period. Although incidence fluctuated from a low of 1.37/100,000 population in 2014 to a maximum of 2.28/100,000 population in 2013, we found no discernible trend in overall incidence. However,

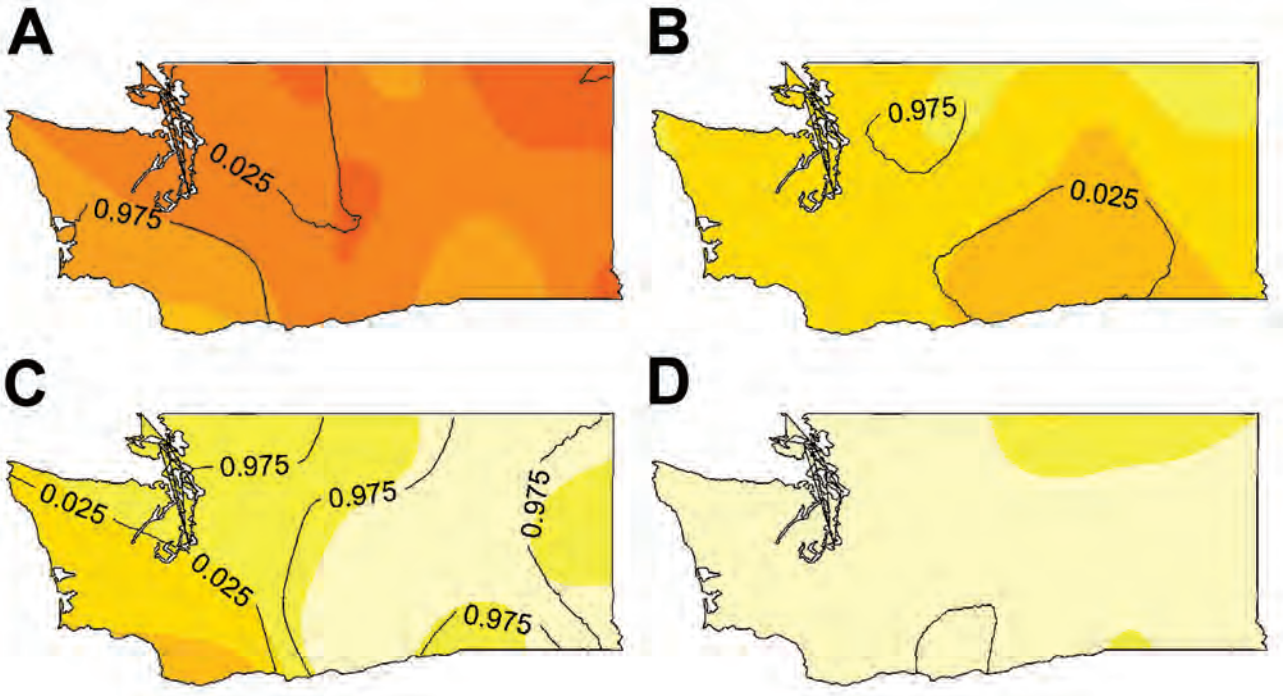

Figure 1. *Escherichia coli* O157:H7 lineage frequency among culture-confirmed human cases reported in Washington, USA, 2005–2014. A) Lineage Ib; B) lineage IIa; C) lineage IIb; D) rare lineages (12 different clinically rare lineages). Lineage-specific probability surfaces were determined by kernel-based estimation of spatial segregation. Darker shading indicates higher risk for that lineage. Contour lines marked 0.025 define areas in which there is a high probability of cases being caused by a given lineage, suggesting spatial segregation. Contour lines marked 0.975 define areas in which there is a low probability of cases being caused by the given lineage.

the composition of the *E. coli* O157:H7 population shifted over time (Figure 3). In the GAM analysis including year as a linear term, incidence relative to lineage Ib increased over time for lineage IIa (OR 1.26, 95% CI 1.19–1.34), lineage IIb (OR 1.10, 95% CI 1.03–1.17), and clinically rare lineages (OR 1.13, 95% CI 1.02–1.26).

We observed a peak of lineage IIb incidence during the middle of the study period in southwest Washington and the Seattle–Tacoma region (Figure 3). Using kernel regression, we identified statistically significant temporal variation in spatial segregation across intervals ($p = 0.001$). We observed statistically significant overall spatial segregation only during the 2008–2010 interval ($p = 0.001$). Some portion of the southwest region of the state showed increased probability of lineage IIb isolation during all intervals, and lineages Ib and IIa were segregated during 2008–2010 and 2011–2014 (Video, https://wwwnc.cdc.gov/EID/article/24/1/17-0851-V1.htm). Cross-validated log-likelihood bandwidths used in these analyses ranged from 0.73 to 1.0. In sensitivity analysis, a lower bandwidth yielded statistically significant spatial segregation during all periods (online Technical Appendix). Latitude and longitude remained significant predictors of Ib in GAMs that included year (online Technical Appendix Table 2).

Sensitivity Analysis

Alternate analytic approaches confirmed the results of our primary analyses. Dixon's test for spatial segregation identified statistically significant spatial segregation overall, as well as for lineages Ib, IIa, and IIb (online Technical Appendix Tables 3, 4). Three clusters identified using multinomial spatial scan statistics paralleled areas of segregation found in the kernel regression analysis and were consistent with the southwest trend toward proportionally greater IIb observed in the multinomial GAM (online Technical Appendix Figures 3, 4).

To focus on potential local reservoirs, which are not likely to be human, we also conducted the analysis without cases due to presumptive person-to-person transmission (online Technical Appendix). We used the most likely source of infection documented on the DOH case report form to exclude patients most likely infected by other persons. After discounting secondary transmission, we observed spatial segregation using the kernel estimation method ($p = 0.002$). The risk surface of lineage IIb still varied significantly from that of Ib ($p<0.001$). The trend toward greater IIb relative to Ib risk in southwest Washington was consistent with the analysis of all cases, but relative IIb risk was substantially lower in the northeast region than that observed in the primary analysis.

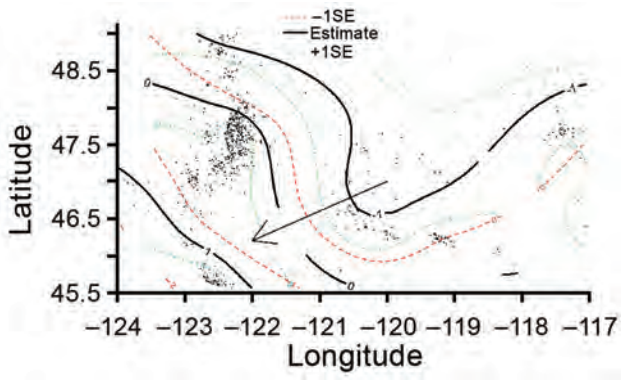

Figure 2. Risk surface of *Escherichia coli* O157:H7 lineage IIb relative to lineage Ib using a multinomial generalized additive model and a bivariate thin plate smooth function for longitude and latitude for culture-confirmed human cases reported in Washington, USA, 2005–2014. The black contour lines show the mean effect estimate for lineage IIb relative to Ib as latitude and longitude change. The 0-marked black line indicates no effect. The 1-marked black line indicates greater proportional incidence of lineage IIb toward the southwest corner of the area as compared to lineage Ib ($p<0.001$). The arrow indicates the general direction of the trend from higher Ib risk to higher IIb risk. Dashed red lines show the effect estimate 1 standard error (SE) below (to the south and west) the mean estimate. Dotted green lines show the effect estimate 1 standard error above (to the north and east) the mean estimate.

This pattern suggests that lineage IIb infections in northeast, but not southwest, Washington may be disproportionately attributed to secondary transmission compared with Ib infections. Finally, we found no evidence of case ascertainment bias that could independently explain our results (online Technical Appendix).

Exploratory Risk Factor Analysis

Statewide, patients infected with lineage IIa *E. coli* O157:H7 were more likely to have reported raw fruit or vegetable consumption than those infected with lineage Ib pathogens (OR 1.81, 95% CI 1.05–3.11). Patients infected with lineage IIb *E. coli* O157:H7 were more likely to have reported raw milk consumption than those infected with lineage Ib pathogens (OR 2.46, 95% CI 1.15–5.28). All examined risk factors and associations are summarized in online Technical Appendix Table 1.

Discussion

The geographic differences and temporal trends in the relative frequencies of lineages of *E. coli* O157:H7 from cases in Washington demonstrate that, in addition to genomic variation reported at the national level (*29*,*30*), persistent geogenomic variation exists at the regional level. Several geospatial associations warrant elaboration. In all analyses, lineage IIb cases were segregated in the southwest region of the state. Southwest Washington includes Olympia, the state capital, as well as suburbs of Portland, Oregon, north of the Columbia River; however 27% of the population in the 12 southwest region counties is considered rural, compared with 16% of the state as a whole (*31*). Small farms are common. The southwest region is home to >20% of the state's farms but accounts for only 7.1% of its cattle and 6.3% of farm acreage (*32*). Roosevelt elk roam the southwest region, and elk elsewhere in the country have been identified as Shiga toxin–producing *E. coli* carriers (*33*). Water is also a potential factor in *E. coli* O157:H7 epidemiology in the southwest region, which has abundant coastal and river exposures. The largest recognized IIb outbreak in this region accounted for only 11 cases linked to a particular daycare center (out of 77 IIb infections in the region), so the observed segregation is unlikely due to a single point source. Notably, lineages IIa and IIb have the greatest overlap with the putatively hypervirulent clade 8 (*34*), making their segregation of particular concern.

Lineage IIb isolates were relatively uncommon in the northwest and south-central regions of Washington, both major cattle-production regions. Lineage Ib showed segregation in the northwest and IIa in the south-central region in some analyses, although their adjusted risk surfaces did not differ significantly, suggesting overlap. More research is needed to clarify why lineage IIb has not yet also established itself in areas with abundant cattle.

The presence of spatially segregated lineages indicates local environmental reservoirs producing infections above and beyond those caused by widely distributed exogenous sources such as food. We propose that persistent spatial segregation of a lineage could reflect a founder effect, in which an ancestral pathogen has become established in a region, persisted, and expanded and occasionally crosses into the human population. Such a dynamic would result in phylogenetically similar bacteria being isolated in the same general geographic region separated by months or years, as we have observed in this study. A possible precedent exists in a report of 2 cases from Webster County, Missouri, USA (*35*). Our findings are also consistent with those of Jaros et al., who found that geography explains some variation in *E. coli* O157:H7 strains in New Zealand (*36*). In addition, prior work from Washington demonstrated shifts over time in the Shiga toxin genotypes of *E. coli* O157:H7 (*37*).

The clinical infections in our study were dominated by *E. coli* O157:H7 in lineages Ib, IIa, and IIb, consistent with the results of Jung et al. (*21*). Our work is also consistent with a national study showing that lineage Ib *E. coli* O157:H7 causes most clinical cases in the United States (*30*). Relative to lineage Ib, Washington experienced statistically significant increases in the other clinically common lineages during the study period. The increase is most dramatic for lineage IIa, which appears to have emerged in most regions in the latter half of the study period (Figure 3).

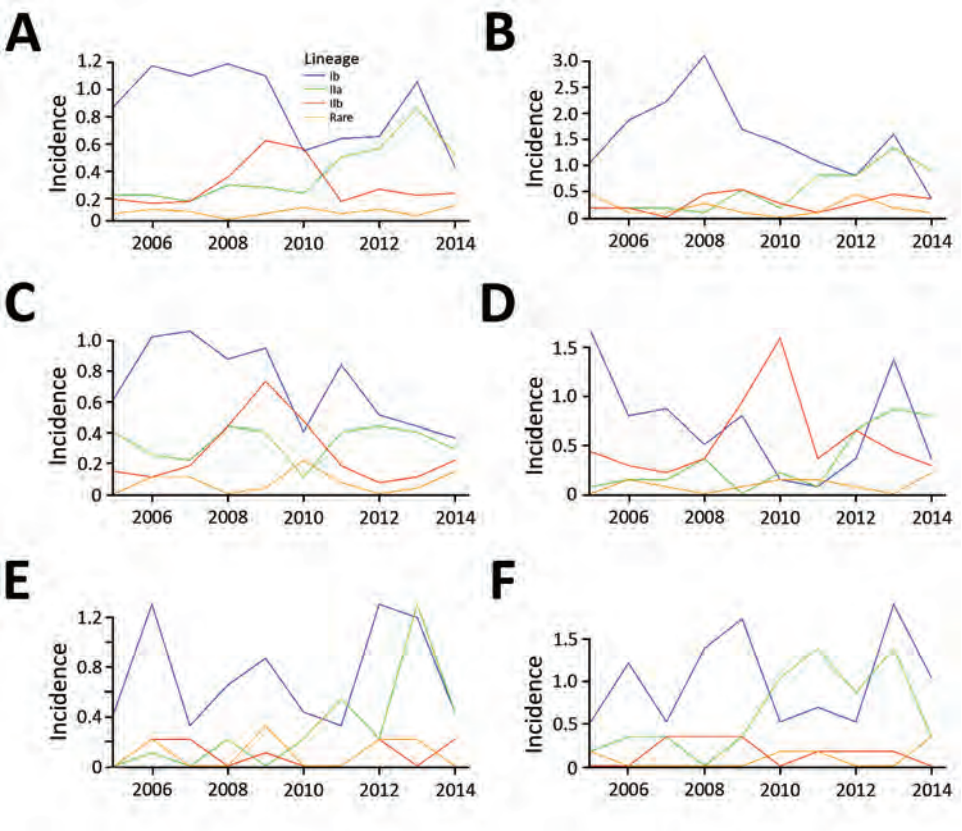

Figure 3. Annual incidence (per 100,000 population) of reported *Escherichia coli* O157:H7 cases by phylogenetic lineage, Washington, USA, 2005–2014. A) Statewide; B) northwest region; C) Seattle–Tacoma region; D) southwest region; E) northeast region; F) south-central region. Regions were defined according to major demographic characteristics and patterns of segregation observed in analyses for the whole period. The northwest region experienced the highest peak incidence. The Seattle–Tacoma region and the northeast region experienced the lowest incidences. "Rare" indicates 12 different clinically rare lineages. **Video.** Lineage-specific probability surfaces for *Escherichia coli* O157:H7 from culture-confirmed human cases reported in Washington, USA, 2005–2014. Probabilities were determined by kernel-based estimation of spatial segregation for 3 intervals: 2005–2007 (n = 305, bandwidth = 1.0000); 2008–2010 (n = 367, bandwidth = 0.7256); and 2011–2014 (n = 439, bandwidth = 0.9314). Overall spatial segregation was not statistically significant for the 2005–2007 interval (p = 0.769) or 2011–2014 interval (p = 0.138) but was statistically significant for the 2008–2010 interval (p = 0.001). Circles indicate case locations. Darker hues indicate higher risk. Contour lines marked 0.025 define areas in which there is a high probability of cases being caused by a given lineage, suggesting spatial segregation. There is an area of statistically significant spatial segregation for lineage IIb in all 3 intervals. Contour lines marked 0.975 define areas in which there is a low probability of cases being caused by the given lineage.

This difference could reflect the changing epidemiology of *E. coli* O157:H7 discussed by Rivas et al., owing to changes in food sources and consumption, or, possibly, pathogen evolution (*38*). Lineage IIa *E. coli* O157:H7 has emerged as a major cause of disease across the state, suggesting a disseminated driver of infections for this lineage overall. Lineage IIa's observed association with raw fruit and vegetable consumption, as compared with that for lineage Ib, is consistent with this hypothesis. The south-central region of Washington, identified in some analyses as an area of IIa segregation, experienced an uptick in IIa infections earlier than in other regions. This area includes the Yakima Valley, an area of higher agricultural intensity; a local IIa reservoir in this region could produce the observed segregation independent of statewide trends.

Our findings suggest exposures that may be preferentially associated with particular lineages. Specifically, we observed associations of lineage IIb with drinking untreated/unchlorinated water and raw milk in the southwest region, where this lineage is segregated (online Technical Appendix Table 1). There may be a lineage IIb reservoir in animals producing raw milk in this area, or bacteria from environmental reservoirs in the area may spill over into these animals and local water sources. Only 1 small, recognized raw milk outbreak in 2005 was noted on the DOH case report forms, making it unlikely that a single source is responsible for the association we found over time. It is possible that some *E. coli* O157:H7 lineages may be especially successful in surviving in particular vehicles or environments, such as raw produce or unpasteurized milk or water. Secular changes might also be the result of shifting environmental exposure risk if, for example, contact between a reservoir and humans varies over time. Better knowledge of small-intermediate area transmission patterns will open opportunities for intervention if reservoirs can be identified.

Our study is limited by its reliance on SNP data to define phylogenetic lineages. Whole-genome sequencing would have supported finer resolution of relatedness, particularly among isolates that were segregated in time and space, and enabled us to trace the history of segregated clusters. Such an analysis would not necessarily alter our conclusions, however, because evolution of specific clades of *E. coli* O157:H7 within a region, and the identification of different sublineages, would still be consistent with a founder effect. In fact, the precise delineation of the chromosomal architecture in these pathogens might actually confirm a common progenitor, as demonstrated from worldwide analyses of *E. coli* O157:H7 (*39*). Our use of phylogenetic lineages rather than PFGE profiles is also a strength of the work, because PFGE does not put differences into evolutionary perspective (*39*). By basing the analysis on phylogenetic lineages, we captured relatedness among strains and indicate the level of *E. coli* O157:H7 diversity as it circulates through its host populations. We also used multiple analytic techniques to provide confidence that our results were not due to assumptions made by any particular method.

In summary, clusters of spatial segregation by phylogenetic lineage in Washington suggest local reservoirs that perennially cause human disease. Further exploration of land use, human movements, and social–behavioral factors could elucidate within-region drivers of spatial segregation. We see comparison of lineage-specific spatial patterns with distributions of these and other factors as an essential next step in understanding *E. coli* O157:H7 spatial segregation. Environmental risk assessment and longitudinal studies based on our findings would also provide valuable information by identifying pathogen reservoirs that have not been identified by traditional public health surveillance and that could be mitigated by public health or environmental measures. The makeup of the *E. coli* O157:H7 population in the state is also shifting. To manage emerging lineages, attention is needed to the heterogeneity in risk factors across the phylogenetic tree. Greater knowledge of the most likely sources of infection for particular lineages has the potential to focus both outbreak investigations and efforts to identify persistent reservoirs.

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Drug-Resistant Polymorphisms and Copy Numbers in *Plasmodium falciparum*, Mozambique, 2015

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One of the fundamental steps toward malaria control is the use of antimalarial drugs. The success of antimalarial treatment can be affected by the presence of drug-resistant populations of *Plasmodium falciparum*. To assess resistance, we used molecular methods to examine 351 *P. falciparum* isolates collected from 4 sentinel sites in Mozambique for *K13*, *pfmdr1*, *pfcrt*, and *pfdhps* polymorphisms and for *plasmepsin2* (*pfpm2*) and *pfmdr1* copy numbers. We found multiple copies of *pfpm2* in 1.1% of isolates. All isolates carried *K13* wild-type alleles (3D7-like), except 4 novel polymorphisms (Leu619Leu, Phe656Ile, Val666Val, Gly690Gly). Prevalence of isolates with *pfcrt* mutant (K76T) allele was low (2.3%). Prevalence of isolates with *pfdhps* mutant alleles (A437G and K540E) was >80%, indicating persistence of sulfadoxine/pyrimethamine resistance; however, markers of artemisinin were absent, and markers of piperaquine resistance were low. Piperaquine resistance isolates may spread in Mozambique as dihydroartemisinin/piperaquine drug pressure increases.

During the past decade, malaria control strategies have substantially reduced the malaria burden worldwide; several countries are advancing toward malaria elimination (*1*,*2*). A fundamental pillar for contributing to the reduction of the malaria burden has been artemisinin-based combination therapy. Unfortunately, the effectiveness of antimalarial drugs used for malaria treatment and chemoprevention during pregnancy has been threatened by the emergence of drug-resistant parasite populations (*2*–*5*).

The emergence of artemisinin resistance in *Plasmodium falciparum*, with reduced in vivo susceptibility to artesunate, was reported in Southeast Asia (*3*,*6*). Detectable polymorphisms in the *Kelch 13* (*K13*) propeller domain in *P. falciparum* associated with artemisinin resistance have subsequently provided an additional tool for monitoring resistance to antimalarial drugs (*7*,*8*). In Cambodia, polymorphisms in the *K13* propeller domain (mainly Y493H, R539T, I543T, and C580Y) were associated with in vitro prolonged parasite survival rates and in vivo delayed parasite clearance rates (*8*,*9*). Recently, *plasmepsin 2* (*pfpm2*) copy number and *pfcrt* C101F polymorphism have been associated with piperaquine resistance (*10*–*12*). In addition, increased *pfmdr1* copies have been associated with resistance to mefloquine (in vivo, in vitro, or both) and partially to lumefantrine (*13*–*18*). Specific point polymorphisms (at codons 86, 184, 1034, 1042, and 1246) of the *pfmdr1* gene have also been linked to resistance to antimalarial drugs (*19*,*20*). In field isolates tested in vitro as well as in laboratory lines, N86Y polymorphism was associated with chloroquine resistance (*21*). Further, polymorphisms in the *pfcrt* gene have also been shown to affect parasite susceptibility to chloroquine (*22*), amodiaquine (*23*,*24*), and artemether/lumefantrine (*25*). Recently, a nonsynonymous polymorphism in the *pfcrt* gene was shown to be prevalent in the genetic background of *K13* mutant artemisinin-resistant isolates (*26*). In addition, polymorphisms in *pfdhfr* and *pfdhps* genes, specifically the quintuple mutant, including the *pfdhfr* substitutions N51I, C59R, and S108N, as well as the *pfdhps* substitutions A437G and K540E, have been associated with a failure of sulfadoxine/pyrimethamine treatment against uncomplicated *P. falciparum* malaria (*27*). In Africa, the *pfdhps* K540E polymorphism has been considered a useful epidemiologic marker of the quintuple mutations (*28*).

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The development of drug resistance could be influenced by multiple factors such as polymorphism rate, fitness costs, overall parasite load, strength of drug selection, treatment compliance, transmission intensity, host immunity, and erythrocyte disorders (*29–31*). Naturally acquired immunity plays a major role in the emergence and clearance of artemisinin-resistant parasites (*32*). Because of increasing concern over the effectiveness of the nationally recommended antimalarial drugs, the Mozambique Ministry of Health has made several changes in antimalarial drug policy. In 2002, chloroquine monotherapy was replaced with sulfadoxine/pyrimethamine/amodiaquine as the first line of treatment against uncomplicated malaria (*33*); 2 years later, this combination was replaced with artesunate/sulfadoxine/pyrimethamine (*33*). In 2008, artemether/lumefantrine was introduced to replace artesunate/sulfadoxine/pyrimethamine (*34*). Molecular markers for antimalarial drug resistance have been considered useful for confirming parasite resistance, a major factor causing treatment failure. To determine whether parasites carrying these polymorphisms or gene amplifications exist in Mozambique, we conducted molecular surveillance targeting *K13*, *pfmdr1*, *pfcrt*, and *pfdhps* polymorphisms and *pfpm2* and *pfmdr1* copy numbers in field isolates collected from 4 sentinel sites.

Materials and Methods

Study Sites and Population

We performed a descriptive observational study on blood samples collected before artemether/lumefantrine treatment (on day 0) in 2015 from 352 symptomatic children at 4 sentinel sites in Mozambique (Figure 1): 1) Hospital Rural de Montepuez in Cabo Delgado Province (northern region), 2) Centro de Saúde de Dondo in Sofala Province (central region), 3) Hospital Provincial de Moatize in Tete Province (central region), and 4) Hospital Rural de Chokwe in Gaza Province (southern region). In Mozambique, transmission usually peaks during the rainy season (November–April). Transmission intensity in southern Mozambique is generally low, although areas of high transmission may still occur (*35*). To determine molecular markers of drug resistance, we analyzed samples collected during a clinical trial conducted in 2015 (registration no. ACTRN12616001680459); the trial aimed to assess the efficacy and safety of artemether/lumefantrine for treatment of uncomplicated *P. falciparum* malaria in children <5 years of age. The National Mozambican Ethical Review Committee (Mozambique) and Hospital Clínic (Barcelona, Spain) ethics review committees approved the study, and signed written informed consent was obtained from all participants' guardian or parent.

Molecular Procedures

We extracted DNA from half of a 50-μL dried blood drop on Whatman 3-mm filter paper by using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. We used an ABI PRISM 7500 HT Real-Time System (Applied Biosystems, Foster City, CA, USA) to amplify purified parasite DNA templates, following a previously described method (*36*,*37*). A standard curve was prepared from an in vitro culture of 3D7 strain containing known numbers of ring-infected erythrocytes.

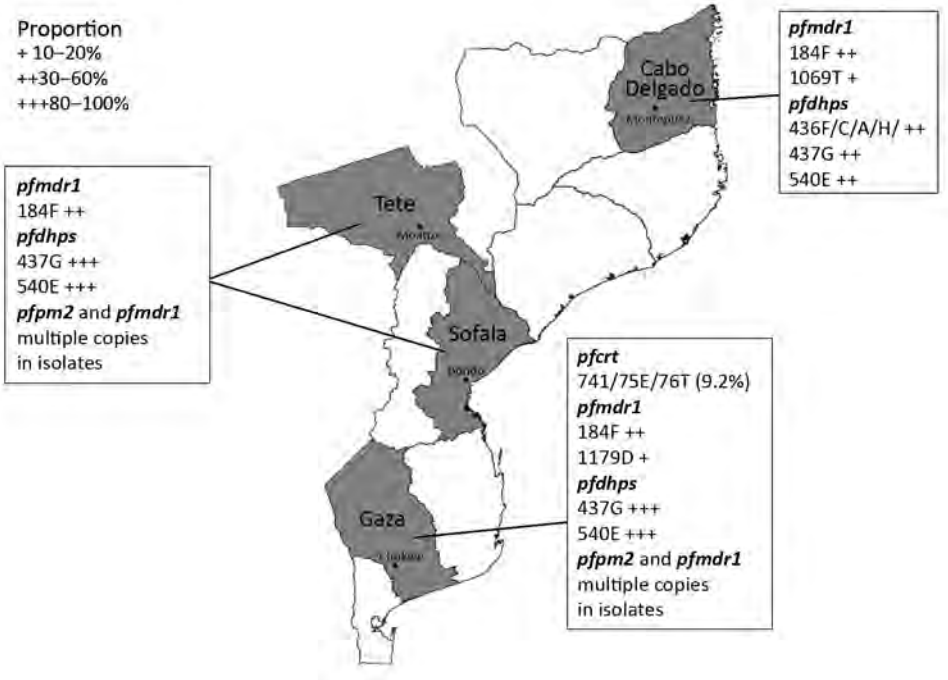

Figure 1. Location of sampling sites and distribution of resistance markers of *Plasmodium falciparum* in Mozambique, 2015.

The standard curve was run in triplicate for each test with 5 serially diluted points. Parasitemia in the clinical samples was quantified by extrapolation against the standard curve.

To assess polymorphisms in the *K13*, *pfcrt*, *pfmdr1*, and *pfdhps* genes, we amplified purified DNA templates by using a 2720 Thermal Cycler (Applied Biosystems), following protocols described for *K13* PCR (*38*) and *pfcrt* PCR (*35*). To genotype polymorphisms in *pfmdr1* and *pfdhps* genes, we designed new assays by using Sanger sequencing and restriction fragment length polymorphisms (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-0864-Techapp1.pdf). A total of 6 positive controls with known *K13* alleles, provided by the Institut Pasteur in Cambodia, and 4 parasite lines (3D7, 7G8, Dd2, and V1/S) with known *pfcrt* and *pfmdr1* alleles, available in the laboratory, were also processed, amplified, and sequenced at the same time as the studied samples (PCR characteristics in online Technical Appendix Table). To determine the detection limit of Sanger sequencing, we used artificially mixed DNA samples of *P. falciparum* laboratory strains containing various known proportions of wild- and mutant-type alleles of *pfcrt* (K76T) and *pfmdr1* (Y184F and S1034C) genes. To estimate polymorphism frequency, we considered isolates with mixed alleles to be mutated.

We assessed copy numbers of *pfpm2* and *pfmdr1* genes as described elsewhere (*11*) with minor changes (online Technical Appendix) by using quantitative PCR (qPCR). We performed amplification in 20-μL reaction mixtures for *pfpm2*, *pfmdr1*, and *pfβ-tubulin* genes, separately. We used the *pfβ*-tubulin gene as an endogenous control. All samples with estimated copy numbers >1.5 were defined as containing multiple copies and repeated for confirmation. The estimated copy numbers were the average of the copy number of each clone in the isolate.

Data Analyses

We calculated the proportion of the mutant alleles and isolates with multiple copies of *pfpm2* and *pfmdr1* genes on the basis of the number of samples with wild- and mutant-type alleles as well as isolates with single and multiple copies of the gene from *P. falciparum* isolates from each study site. To compare continuous data and categorical data between sites, respectively, we performed analyses of variance and χ^2 tests. We defined statistical significance as $p<0.05$.

Results

Demographics and *P. falciparum* Infection

Among the 352 blood samples collected before artemether/lumefantrine treatment (on day 0) during 2015, and followed up as part of the clinical trial, 351 (99.7%) were *P. falciparum*–infection positive according to 18SrRNA qPCR. The mean (± SD) parasitemia (by qPCR) was 100,229 ± 325,214 parasites/μL. Among participants, 159 (45.2%) were female, mean (± SD) age was 2.8 ± 1.3 y, mean body temperature was 38.1 ± 1.1°C, and mean hemoglobin level was 9.2 ± 1.9 g/dL. We also compared demographic data and parasite densities according to study site (Table 1). Efficacy of artemether/lumefantrine in the in vivo study was high, and for nearly all patients (349 [99.4%] of 351), parasitemia reverted to 0 in the first 3 days; however, for 2 patients, parasites were still detectable by microscopy: 1 from Moatize (514 parasites/μL) and 1 from Chokwe (3,763 parasites/μL). PCRs targeting *msp1*, *msp2*, and *glurp* genes were used to differentiate recrudescence (same parasite strain) and reinfection (different parasite strain). We noted recrudescence of *P. falciparum* infections for 5 children (1 in Chokwe, 3 in Moatize, and 1 in Montepuez) on days 21 and 28 after artemether/lumefantrine administration and reinfection for 7 children (3 in Moatize and 4 in Montepuez); 3 were reinfected on day 21 and 4 on day 28 (*39*).

The polymorphism analyses of *K13*, *pfmdr1*, *pfcrt*, and *pfdhps* genes were successful for 98.3% to 100% isolates. Because no amplifications were noticed in negative controls (with water and human genomic DNA), PCR assays were specific to *P. falciparum* genomic DNA only.

Detection Limit of Mixed Samples by Sanger Sequencing

We identified ¨A¨ alleles of *pfcrt* (K76T) and *pfmdr1* (Y184F) codons in artificially mixed samples by using Sanger sequencing when the proportion of target DNA was ≥10%. However, we identified ¨C¨ and ¨T¨ alleles of *pfcrt* (K76T) and *pfmdr1* (Y184F) polymorphisms, respectively, in mixed samples when their proportion was ≥20% (online Technical Appendix Figure 1). For *pfmdr1* (S1034C) polymorphism, the minor allele was detected when its proportion was ≥20% in a mixed sample (online Technical

Table 1. Characteristics of study participants with *Plasmodium falciparum* malaria, by site, Mozambique, 2015

Characteristic	Montepuez, n = 87	Dondo, n = 88	Moatize, n = 89	Chokwe, n = 88	p value
Female, no. (%)	36 (41.4)	40 (45.5)	41 (46.1)	42 (47.7)	0.63
Age, y, mean ± SD	2.4 ± 1.1	2.7 ± 1.1	2.7 ± 1.1	3.1 ± 1.1	0.0002
Temperature, °C, mean ± SD	37.9 ± 0.9	38.5 ± 1.1	38.1 ± 1.0	37.9 ± 1.5	0.0043
Parasite density, parasites/μL, mean ± SD*	$1.2 \times 10^5 \pm 1.3 \times 10^5$	$3.7 \times 10^4 \pm 4.4 \times 10^4$	$1.2 \times 10^5 \pm 6.1 \times 10^5$	$1.2 \times 10^5 \pm 1.8 \times 10^5$	<0.0001
Hemoglobin, g/dL, mean ± SD	8.9 ± 2.2	8.7 ± 1.8	9.8 ± 1.7	9.2 ± 2.0	0.0018

*By quantitative PCR.

Appendix Figure 1, panel C). For positive controls, we used several parasite lines with known *K13*, *pfmdr1*, and *pfcrt* alleles. As expected, sequencing analysis of all positive controls revealed wild- and mutant-type alleles of *K13*, *pfmdr1*, and *pfcrt* polymorphisms.

Copy Numbers for *pfpm2* and *pfmdr1*

We successfully analyzed 351 (100%) samples for copy number variation in the *pfpm2* and *pfmdr1* genes. PCR efficiencies were 98.4% for *pfpm2*, 97.2% for *pfmdr1*, and 99.2% for *pfß-tubulin* genes. As expected, the estimated *pfpm2* and *pfmdr1* copy numbers for the positive controls were 3–4 copies. The estimated mean (interquartile range) copy numbers were 3.51 (3.37–3.62) for *pfpm2* and 3.62 (3.51–3.79) for *pfmdr1* positive controls. When we used a copy number threshold of 1.5 to define multiple copies, only 4 (1.1%) and 5 (1.4%) of the 351 isolates had multiple copies of *pfpm2* and *pfmdr1*, respectively (Table 2; Figure 2). The range of estimated *pfpm2* copy numbers was 0.59–1.79 and of *pfmdr1* was 0.58–1.88. The copy number of *pfpm2* and *pfmdr1* genes did not significantly differ between isolates from different sites. The proportion of isolates with multiple copies of the *pfpm2* gene was the highest at Chokwe (2 [2.3%] of 87). Only 1 (1.1%) of 88 samples from Dondo had multiple copies of *pfpm2* and *pfmdr1* genes.

K13 Polymorphisms

We successfully achieved *K13* PCR and sequencing for all 351 isolates. None of the isolates analyzed contained the polymorphisms most frequently found in isolates from Cambodia (*8*). However, we observed 4 novel polymorphisms at nt 1725147 (codon 619; 0.28% [1/351]); 1725032 (codon 656; 0.28% [1/351]); 1725000 (codon 666; 0.57% [2/351]); and 1724927 (codon 690; 0.85% [3/351]) of the *K13* gene. All polymorphisms were synonymous except for 1 at codon 656, which led to a change from phenylalanine to isoleucine. When we compared frequencies of new polymorphisms between sites, we found no significant differences. We also observed the polymorphism Cys469Cys, previously described in *P. falciparum* field isolates from Ghana (*40*), in 3 (0.85%) of the 351 isolates. Isolates from patients with parasitemia on day 3 and recrudescence contained wild-type *K13* gene polymorphisms.

pfcrt Polymorphisms

We successfully amplified all 351 samples for *pfcrt* and sequenced the amplification products; mutant alleles were found at codons M74I, N75E, and K76T only in 8 (2.3%) samples. The mutant alleles (M74I, N75E, and K76T) were present only in isolates collected from Chokwe (8 [9.2%] of 87). When we compared frequencies of mutant alleles between sites, the difference was significant ($p<0.0001$). In the studied isolates, the mutant (F) allele at codon 101 was absent. Isolates from patients with parasitemia on day 3 and recrudescence contained wild-type *pfcrt* gene polymorphisms.

pfmdr1 Polymorphisms

We successfully amplified and sequenced *pfmdr1*_f1 for 351 (100%) samples and *pfmdr1*_f2 fragments for 350 (99.7%) samples. We identified 15 polymorphisms all across the *pfmdr1* gene, including 5 (33.3%) with nonsynonymous polymorphisms and 10 (66.7%) with synonymous polymorphisms. Among nonsynonymous polymorphisms, 3 (T1192A, F1194S, and Y1197N) were newly identified and 2 (N86Y and Y184F) had been previously reported (*41*). Among synonymous polymorphisms, 7 (L1030L, D1061D, D1127D, S1137S, L1174L, D1179D, and N1189N) were newly identified and 3 (G102G, G182G, and T1069T) had been previously reported (*41*). Among the 351 isolates, we found 11 (3.1%) N86Y and 164 (46.7%) Y184F mutant alleles (Table 3). All newly identified nonsynonymous mutant alleles were present only once, except for Y1197N, which was found twice (0.6% [2/350]). The frequency of polymorphisms (N86Y and D1179D) differed significantly between isolates from the 4 sites (Table 3). The proportion of N86Y and D1179D polymorphisms was highest in isolates from Chokwe. We observed none of the other most frequent polymorphisms (S1034C, N1042D, and D1246Y) of the *pfmdr1* gene among the analyzed samples. Isolates from patients with parasitemia on day 3 and recrudescence contained wild-type *pfmdr1* gene polymorphisms.

pfdhps Polymorphisms

Polymorphism analysis by PCR followed by sequencing for S436F and A437G polymorphisms was successful for 345 (98.3%) samples and analysis by PCR–restricted fragment length polymorphism for K540E polymorphism for 348 (99.1%) samples. Among all isolates, 10 (2.9%) of 345

Table 2. *Plasmodium falciparum* isolates with increased *pfpm2* and *pfmdr1* gene copy numbers, 4 sentinel sites, Mozambique, 2015

	pfpm2				*pfmdr1*			
	No. (%)				No. (%)			
Site	≤1.2	1.2–1.5	≥1.5	Mean ± SD	≤1.2	1.2–1.5	≥1.5	Mean ± SD
Montepuez, n = 87	82 (94.3)	5 (5.7)	0	0.85 ± 0.2	87 (100.0)	0	0	0.93 ± 0.1
Dondo, n = 88	66 (75.0)	21 (23.9)	1 (1.1)	1.02 ± 0.2	68 (77.3)	18 (20.4)	2 (2.3)	0.99 ± 0.3
Moatize, n = 89	73 (82.0)	15 (16.9)	1 (1.1)	1.01 ± 0.2	81 (91.0)	7 (7.9)	1 (1.1)	0.99 ± 0.2
Chokwe, n = 87	77 (88.5)	8 (9.2)	2 (2.3)	0.94 ± 0.2	70 (80.5)	15 (17.2)	2 (2.3)	0.98 ± 0.2

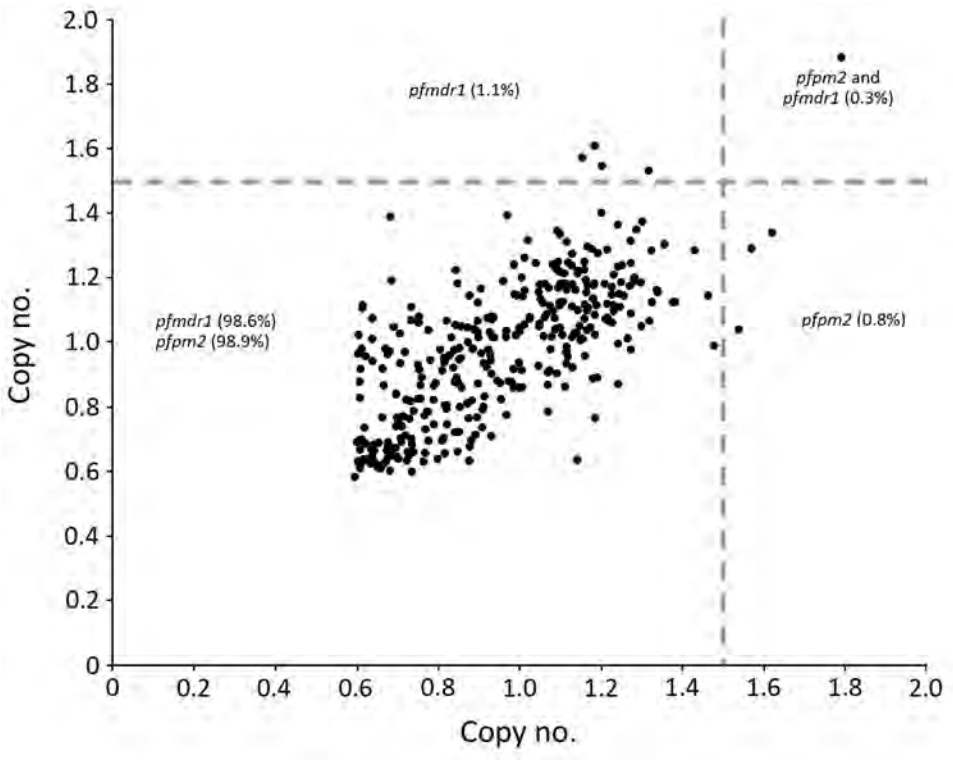

Figure 2. *pfpm2* and *pfmdr1* copy numbers of *Plasmodium falciparum* isolates from 4 sentinel sites, Mozambique, 2015. Multiple copies of *pfpm2* and *pfmdr1* genes have been associated with resistance to piperaquine and mefloquine, respectively.

contained S436F, 289 (83.8%) of 345 contained A437G, and 286 (82.2%) of 348 contained K540E mutant alleles. At codon 436, we also found 3 mutant alleles: S436C (0.9%), S436A (4.9%), and S436H (0.6%). When we compared frequencies of 3 single-nucleotide polymorphisms at different sites, we noted significant differences (Table 4). The proportion of isolates with A437G and K540E polymorphisms was the highest at Moatize, and the proportion with S436F, S436C, S436A, and S436H alleles was highest at Montepuez.

Discussion

We provide evidence for the presence of multiple copies of *pfpm2* in 4 (1.1%) of 351 *P. falciparum* isolates circulating in southern Mozambique despite the absence of piperaquine drug pressure. Thus, with adequate drug pressure, isolates resistant to piperaquine may spread in Mozambique, as occurred in Southeast Asia (*10*,*42*,*43*). In selected areas of Cambodia in 2008, piperaquine was introduced as a partner drug of artemisinin (*44*). Soon after its introduction, as early as 2010, piperaquine resistance in western Cambodia emerged at an alarming rate (*45*). Subsequent reports confirmed a rapid increase in failure of dihydroartemisinin/piperaquine in other parts of Cambodia (*42*,*46*,*47*). The most frequent *K13* mutants associated with artemisinin resistance were absent in the isolates from Mozambique. We also determined that prevalence of *pfcrt* (K76T) and *pfmdr1* (N86Y) markers of resistance are low, supporting previous evidence for the return of parasites carrying *pfcrt* wild-type alleles in Mozambique (*35*), in contrast to persistence of *pfdhps* (A437G [83.8%]) and K540E [82.2%]) polymorphisms, markers of sulfadoxine/pyrimethamine resistance (*34*). The well-characterized polymorphism in *pfmdr1* (Y184F [46.7%]) was also prevalent in Mozambique.

We found very low prevalence (<1%) for 4 new polymorphisms (Leu619Leu, Phe656Ile, Val666Val, and Gly690Gly) in the *K13* gene of *P. falciparum* isolates from Mozambique. All polymorphisms except Phe656Ile were synonymous. Previously, V494I *K13* nonsynonymous polymorphism has also been reported in Mozambique (*48*). In Africa, *K13* nonsynonymous polymorphisms have also been reported at low frequencies in isolates from Cameroon, Central African Republic, Democratic Republic of the Congo, Gabon, The Gambia, Kenya, Madagascar, Malawi, Mali, Rwanda, Togo, Uganda, Zambia, and Equatorial Guinea (*38*,*40*,*49*–*50*; references *51*,*52* in online Technical Appendix). The association of nonsynonymous polymorphisms with delayed parasite clearance has only recently been identified in Africa (reference *52* in online Technical Appendix).

Resistance to both chloroquine and amodiaquine has been mainly associated with a single K76T mutant allele in the *pfcrt* gene (*22*–*24*). In our study, its prevalence in 8 (2.3%) of 351 samples was significantly lower than that found in previous studies in Mozambique (*33*,*34*;

Table 3. Distribution of *Plasmodium falciparum pfmdr1* polymorphism (mutated allele) frequencies among 4 sentinel sites, Mozambique, 2015*

SNP	Montepuez, no. (%)	Chokwe, no. (%)	Moatize, no. (%)	Dondo, no. (%)	p value
N86Y	1 (1.1)	6 (6.9)	0	4 (4.5)	0.03
G102G	1 (1.1)	2 (2.3)	2 (2.2)	1 (1.1)	0.88
G182G	4 (4.6)	1 (1.1)	3 (3.4)	1 (1.1)	0.37
Y184F	45 (51.7)	39 (44.8)	42 (47.2)	38 (43.2)	0.69
L1030L	0	0	1 (1.1)	0	0.40
D1061D	0	1 (1.1)	0	0	0.40
T1069T	10 (11.5)	5 (5.7)	4 (4.5)	8 (9.2)	0.28
D1127D	1 (1.1)	0	2 (2.2)	0	0.30
S1137S	2 (2.3)	0	0	2 (2.3)	0.25
L1174L	1 (1.1)	0	0	0	0.40
D1179D	0	9 (10.3)	2 (2.2)	0	0.0001
N1189N	0	0	1 (1.1)	1 (1.1)	0.57
T1192A	0	1 (1.1)	0	0	0.40
F1194S	1 (1.1)	0	0	0	0.40
Y1197N	1 (1.1)	0	0	1 (1.1)	0.57

*SNP, single-nucleotide polymorphism.

reference *53* in online Technical Appendix). Our *pfcrt* data align with previous evidence for the return of parasites carrying *pfcrt* wild-type alleles in Mozambique (*35*) and in other countries in Africa, such as Ethiopia (reference *54* in online Technical Appendix), Malawi (reference *55* in online Technical Appendix), and Cameroon (reference *56* in online Technical Appendix). The selective disadvantage of mutant parasites in the absence of drug pressure has been proposed as the leading factor contributing to the reemergence of chloroquine-susceptible parasites (reference *57* in online Technical Appendix). Because artemether/lumefantrine has been shown to select for the wild-type *pfcrt* 76K allele (*25*), this reemergence might be accelerated because of the increased use of artemether/lumefantrine as a first-line treatment for uncomplicated malaria in Mozambique (reference *53* in online Technical Appendix).

Our study also provides evidence for the presence of few *P. falciparum* isolates with multiple copies of the *pfmdr1* gene (5 [1.4%] of 351) circulating in southern Mozambique (*34*). Increased *pfmdr1* copies have been associated with resistance to mefloquine and partial resistance to lumefantrine (*13–18*). Our study found that prevalence of the *pfmdr1* N86Y mutant allele has decreased and the Y184F mutant allele has increased over time, in contrast with findings of other studies from Mozambique (*34*; references *55*,*58* in online Technical Appendix). We identified 10 new polymorphisms (L1030L, D1061D, D1127D, S1137S, L1174L, D1179D, N1189N, T1192A, F1194S, and Y1197N) that had not been previously described for the *pfmdr1* gene. Among the 15 polymorphisms identified in the *pfmdr1* gene, we observed significant differences between sites for the N86Y and D1179D polymorphisms only.

Of 351 children who had received adequate treatment with artemether/lumefantrine (6 doses), 2 were still positive for parasitemia on day 3 (*39*). These isolates contained wild-type *K13* gene polymorphisms. *P. falciparum*–positive patients for whom artemether/lumefantrine treatment failed had parasites that carried wild-type *pfcrt* and *pfmdr1* polymorphisms. This observation suggests that in vivo artemether/lumefantrine resistance may be caused not only by variations in the *pfcrt* and *pfmdr1* genes but possibly by parasite selection of variations in other genes; however, drug bioavailability issues may also have contributed.

A high proportion of the *P. falciparum* isolates from Mozambique contained K540E (82.2%) and A437G (83.8%) mutant alleles. These mutant alleles may still not jeopardize the effectiveness of sulfadoxine/pyrimethamine for malaria prevention in Mozambique; recent findings suggest that only >90% prevalence of a *pfdhps* K540E polymorphism could reduce the effectiveness of intermittent preventive therapy to clear peripheral parasites and prevent new infections during pregnancy (reference *59* in online Technical Appendix). Therefore, sulfadoxine/pyrimethamine remains effective for intermittent preventive therapy during pregnancy, despite the high frequency of quintuple mutants; thus, the World Health Organization continues to recommend the use of intermittent preventive therapy to prevent malaria during pregnancy (references *60–62* in online Technical Appendix).

Table 4. Distribution of *Plasmodium falciparum pfdhps* gene polymorphism (mutated allele) frequencies among 4 sentinel sites, Mozambique, 2015*

SNP	Montepuez, no. (%)	Chokwe, no. (%)	Moatize, no. (%)	Dondo, no. (%)	p value
S436F/C/A/H	30 (34.5)	2 (2.4)	0	0	<0.0001
A437G	51 (58.6)	79 (94.1)	82 (93.2)	77 (89.5)	<0.0001
K540E	50 (57.5)	77 (90.6)	83 (93.3)	76 (87.4)	<0.0001
A437G + K540E	47 (54.1)	77 (91.7)	80 (90.9)	76 (88.4)	<0.0001

*SNP, single-nucleotide polymorphism.

However, alternative antimalarial drugs for intermittent preventive therapy during pregnancy are needed because the prevalence of the K540E polymorphism in Mozambique is close to the threshold.

In conclusion, we report that prevalence of isolates with multiple copies of *pfpm2* is lower than that found by previous studies in Cambodia (34.3%) and Vietnam (54.3%) (*10*,*43*), and we report the absence of *K13* polymorphisms known to be associated with artemisinin resistance. We also report the return of parasites carrying *pfcrt* wild-type alleles (except in Chokwe) and persistence of parasites with *pfdhps* mutations associated with sulfadoxine/pyrimethamine resistance in Mozambique. Sulfadoxine/pyrimethamine–resistant isolates may be maintained by the constant use of intermittent preventive therapy during pregnancy, use of drug outside of hospitals, the very common use of co-trimoxazole (as prophylaxis for HIV-infected persons), and the low fitness cost of the polymorphisms (*33*; references *63*,*64* in online Technical Appendix). In contrast, the fitness cost of the *pfcrt* mutant allele seems to be high, probably accounting for the return of parasites carrying *pfcrt* wild-type alleles in Mozambique (reference *57* in online Technical Appendix). Current regional elimination efforts, as part of the G8 Malaria Elimination Initiative, may lead to more aggressive strategies involving population-wide distribution of antimalarial drugs, such as dihydroartemisinin/piperaquine, resulting in significantly increased drug pressure. Our findings might provide baseline prevalence data that enable us to directly determine the effects that increasing malaria control efforts or elimination programs will have on resistance evolution.

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Japanese Encephalitis Virus Transmitted Via Blood Transfusion, Hong Kong, China

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Japanese encephalitis virus (JEV) is a mosquitoborne virus endemic to China and Southeast Asia that causes severe encephalitis in <1% of infected persons. Transmission of JEV via blood transfusion has not been reported. We report transmission of JEV via blood donation products from an asymptomatic viremic donor to 2 immunocompromised recipients. One recipient on high-dose immunosuppressive drugs received JEV-positive packed red blood cells after a double lung transplant; severe encephalitis and a poor clinical outcome resulted. JEV RNA was detected in serum, cerebrospinal fluid, and bronchoalveolar lavage fluid specimens. The second recipient had leukemia and received platelets after undergoing chemotherapy. This patient was asymptomatic; JEV infection was confirmed in this person by IgM seroconversion. This study illustrates that, consistent with other pathogenic flaviviruses, JEV can be transmitted via blood products. Targeted donor screening and pathogen reduction technologies could be used to prevent transfusion-transmitted JEV infection in highly JEV-endemic areas.

Japanese encephalitis virus (JEV) is a member of the genus *Flavivirus* and is the eponymous member of the JEV antigenic complex of viruses that also includes West Nile virus (WNV). JEV is endemic to Southeast Asia and China, where ≈67,900 cases occur every year (*1*). The virus is maintained in a complex enzootic cycle involving pigs and birds; humans are infected via the bite of infected *Culex* spp. mosquitoes (particularly *C. tritaeniorhynchus*) (*2*). Humans infected with JEV have low viremia levels and are considered to be dead-end hosts (*3*). Although neurologic manifestations are observed in <1% of infected patients, encephalitis caused by JEV is a devastating condition with a mortality rate of 20%–30%. Survivors often suffer permanent neurologic sequelae. No treatment has been proven to be effective for Japanese encephalitis in clinical trials (*4*).

Arthropodborne viruses are an emerging threat to the blood supply. Transmission via blood transfusion has been described for 4 arthropodborne viruses: dengue virus, WNV, tick-borne encephalitis virus, and Zika virus (*5*–*10*). Furthermore, chikungunya virus and Usutu virus have also been found in blood donor samples, indicating a risk for transmission via this route as well (*11,12*). In contrast, transfusion-related JEV transmission has not been reported in the literature, although the potential for this type of transmission has been recognized (*13*). In this study, we describe a case of nosocomial Japanese encephalitis in an immunocompromised lung transplant recipient. An outbreak investigation was conducted to ascertain the source of the infection and if other patients were at risk for Japanese encephalitis.

Materials and Methods

Setting

Queen Mary Hospital is a 1,700-bed, university-affiliated, tertiary referral center in Hong Kong that has a lung transplantation service. After lung transplantation, patients are transferred for extended care to Grantham Hospital, a 388-bed specialized respiratory care hospital in the healthcare network of Hong Kong West Cluster.

Virologic Investigations

Virologic investigations for JEV were performed at the Public Health Laboratory Services Branch, Centre for Health Protection, of the Hong Kong Department of Health and the microbiology laboratory at Queen Mary Hospital. We performed JEV IgM testing on serum and cerebrospinal fluid (CSF) using the JE Detect IgM Antibody Capture ELISA (InBiOS, Seattle, WA, USA). We amplified flavivirus RNA in clinical specimens using a conventional

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panflavivirus heminested reverse transcription PCR (RT-PCR) with primers targeting the nonstructural protein 5 (NS5) gene as described previously (*14*). We detected RT-PCR products using gel electrophoresis after the first and second rounds of amplification. We performed real-time RT-PCR specific for JEV using an in-house–developed assay with primers targeting the NS5 gene (forward primer 5′-GGAGCTGGATGGAATGTGAA-3′, reverse primer 5′-TCCCTCCGATGGAAGTATAGAA-3′, probe 6-FAM-CCAAAGCGTATGCACAGATGTGGC-BBQ-650; online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/24/1/17-1297-Techapp1.pdf). We tested for other pathogens that can cause encephalitis, including herpes simplex virus, varicella zoster virus, enterovirus, adenovirus, cytomegalovirus, erythroparvovirus B19, rabies virus, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, and *Toxoplasma gondii*, using in-house–developed PCR and RT-PCR assays (*15*).

Sequencing and Phylogenetic Analysis

Using the inner primer pairs of the panflavivirus heminested RT-PCR, we performed Sanger sequencing of flavivirus NS5 gene amplicons with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We compared the resulting sequences to the sequences in GenBank using BLAST (*16*) and performed phylogenetic analysis using MEGA 6.06 (http://www.megasoftware.net/). We performed multiple alignment with the NS5 gene sequences obtained from this study (167 nt long) and those of other JEV strains using ClustalW, followed by phylogenetic tree construction using a Kimura 2-parameter substitution model plus invariant site and the maximum-likelihood method as previously described (*17–19*).

Case Definitions

Patients were defined as having JEV infection if any 1 of 2 laboratory criteria was met: detection of JEV-specific IgM in CSF or serum specimens, or detection of JEV RNA in blood or CSF specimens. Patients were defined as having a confirmed transfusion-transmitted JEV infection if they met the above laboratory criteria for JEV infection and received a blood product transfusion from a donor with JEV viremia during the 3 weeks before illness onset. We performed phylogenetic analysis to compare the blood donor and recipient JEV sequences whenever sequences were available.

Outbreak Investigation

Clinical details of the index patient, including date of transplantation, dates of blood transfusions, immunosuppressant dosages, and laboratory investigation results, were retrieved from the electronic patient record system. Archived clinical specimens from the index patient, including serum, CSF, bronchoalveolar lavage fluid, feces, urine, and saliva samples, were retrieved from the microbiology laboratory at Queen Mary Hospital and tested using the panflavivirus heminested PCR assay.

We retrieved details on the organ donor and other transplant recipients from the organ donor registry. We retrieved the remaining blood products of persons who donated blood that was transfused into the index patient during the 3 weeks before illness onset from the Hong Kong Red Cross Blood Transfusion Service and tested these samples using the panflavivirus RT-PCR and JEV-specific RT-PCR described previously.

Because the index patient had resided in Grantham Hospital for the entire JEV incubation period, mosquito presence in Grantham Hospital was directly assessed by interviewing healthcare workers, as described in our previous outbreak investigations (*20–23*). *Culex* vector surveillance is performed by the Food and Environmental Hygiene Department in selected areas of Hong Kong. We were able to retrieve the monthly surveillance data from 1 of their surveillance sites near Grantham Hospital (2.5 km away). This study was approved by the institutional review board of the University of Hong Kong and Hospital Authority Hong Kong West Cluster.

Results

Index Patient

A 52-year-old man with advanced chronic obstructive pulmonary disease underwent double lung transplantation on May 10, 2017, in Queen Mary Hospital. After transplantation, the patient's clinical course was complicated by nosocomial pneumonia; *Burkholderia cepacia* and methicillin-resistant *Staphylococcus aureus* were isolated from the patient's sputum specimens. The infection required a prolonged course of broad-spectrum antimicrobial drugs and ventilator support. Antirejection prophylaxis included tacrolimus (0.5 mg/d), mycophenolate mofetil (250 mg 2×/d), and a tapering course of prednisolone. He was transferred multiple times between Queen Mary Hospital and Grantham Hospital (Figure 1). The patient required transfusions of packed red blood cells for anemia on June 20, 22, and 25 (1 unit/d) in Grantham Hospital.

On July 6 (57 days after transplantation and 11–16 days after transfusions), he had a transient maculopapular rash and fever. Blood tests showed leukocyte (8.99×10^9 cells/L), neutrophil (7.17×10^9 cells/L), and lymphocyte (1.18×10^9 cells/L) counts within reference ranges. Plasma sodium decreased to 128 mmol/L, and liver and renal function test results were unremarkable. His conscious level progressively deteriorated, and he developed myoclonic jerks 2 days after the onset of rash. Serial blood tests showed transient lymphopenia (0.44×10^9 cells/L) and a drop in platelet count (33×10^9/L).

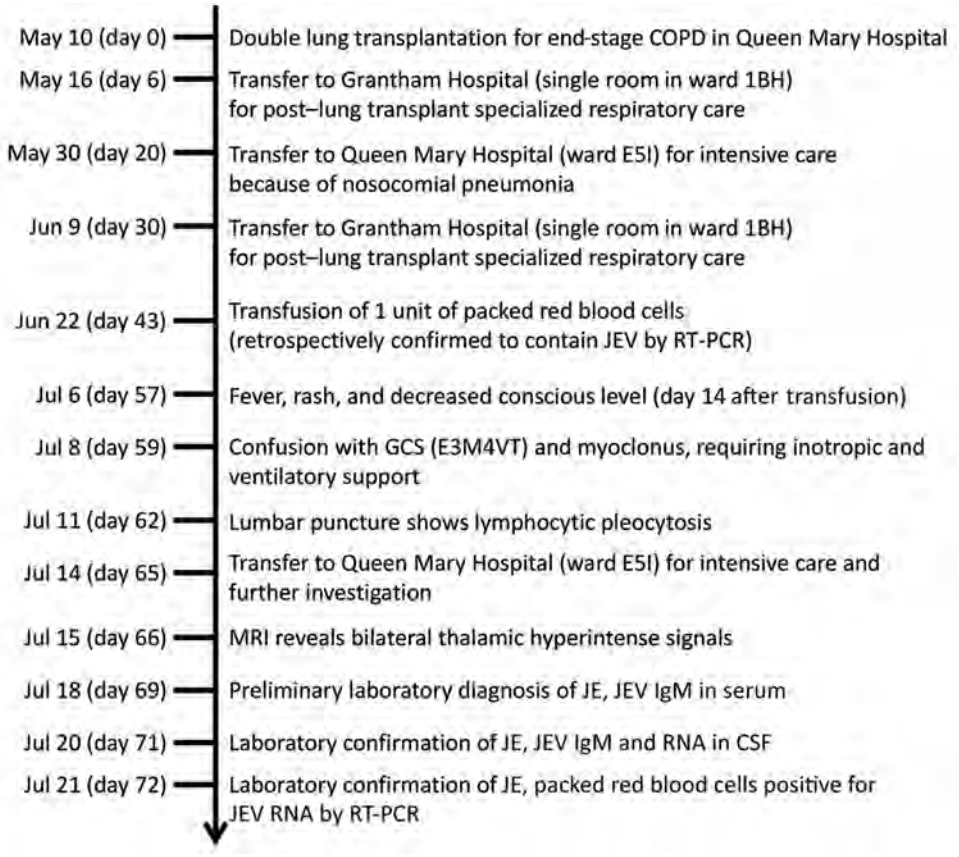

Figure 1. Timeline of index patient with transfusion-transmitted JEV infection, Hong Kong, China, May–July 2017. Day counts indicate the number of days after double lung transplant, unless specified otherwise. COPD, chronic obstructive pulmonary disease; CSF, cerebrospinal fluid; GCS, Glasgow Coma Scale; JEV, Japanese encephalitis virus; MRI, magnetic resonance imaging; RT-PCR, reverse transcription PCR.

A lumbar puncture was performed 6 days after symptom onset. The opening pressure was 13.4 cm H_2O (reference range 6–20 cm H_2O), and the CSF had a total cell count of 20×10^6 cells/L, with a predominance of monocytes (58%) and lymphocytes (35%). CSF protein was elevated (1.61 g/L). Magnetic resonance imaging (MRI) of the brain performed 10 days after symptom onset showed symmetric hyperintensities in the bilateral thalami, substantia nigra, and medial temporal lobes (Figure 2).

In view of the CSF pleocytosis and elevated protein levels, an extensive workup was performed for infectious causes of meningoencephalitis. Gram and Ziehl-Neelsen stains, bacterial and fungal cultures, and the cryptococcal antigen test all gave negative results. PCR for herpes simplex virus, varicella zoster virus, cytomegalovirus, Epstein-Barr virus, enterovirus, rabies virus, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, and *Toxoplasma gondii* were all negative. Serum PCR screening for adenovirus and erythroparvovirus B19 were also negative.

Because of the characteristic distribution of MRI abnormalities involving the basal ganglia, the possibility of flaviviral encephalitis was considered. The patient's CSF was positive for JEV IgM. Subsequent serum samples collected July 15 and 18 were also positive for JEV IgM, but the archived serum specimen obtained May 31, 2017, was negative (Table). Panflavivirus heminested PCR performed on CSF yielded a positive result, with sequencing of the amplicon confirming the presence of JEV (genotype 1). Archived clinical specimens obtained from the patient during the 2 weeks before symptom onset were retrospectively tested using the panflavivirus heminested RT-PCR. Two serum specimens collected June 26 and 28 (10 and 8 days, respectively, before symptom onset) and a bronchoalveolar lavage fluid specimen collected day 5 after symptom onset also tested positive for flavivirus RNA and were confirmed to be JEV by sequencing (GenBank accession no. MF594404). The patient died on October 1, 2017.

Outbreak Investigation

In response to this case of nosocomial acquisition of JEV, the hospital infection control team launched an outbreak investigation in Queen Mary Hospital and Grantham Hospital by tracing the placement of the patient during his entire hospitalization. The index patient was treated in air-conditioned rooms in Queen Mary Hospital and Grantham Hospital throughout his hospitalization. Therefore, mosquitoborne transmission was considered unlikely. Furthermore, a vector surveillance point surveyed by the Food and Environmental Hygiene Department, located 2.5 km from Grantham Hospital, showed that *C. tritaeniorhynchus*

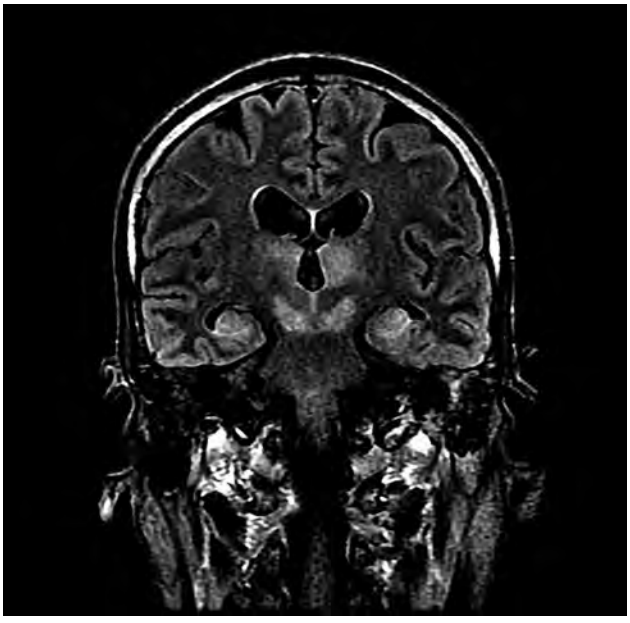

Figure 2. Magnetic resonance imaging of brain of index patient 66 days after double lung transplantation, Hong Kong, China. Coronal FLAIR (FLuid Attenuation Inversion Recovery sequence) image of the head at the level of the lateral ventricles, thalamus, and midbrain shows high signal at bilateral thalamus, midbrain, and medial temporal lobes.

mosquitoes had not been detected since April 2017. Therefore, alternative sources of infection, including blood transfusion, sharps injury with contaminated blood, and organ transplantation, were considered.

The onset of symptoms in the index patient was 57 days after the transplantation date, longer than the usual 5–15-day incubation period for JEV. The organ donor was a 70-year-old road traffic accident victim. Urgent contact tracing of the recipients who received organs (1 liver and 2 kidneys) from this donor revealed that the recipients were asymptomatic. JEV IgM testing of serum samples from these transplant recipients on day 71 (for the liver transplant recipient), day 76 (for renal transplant recipient 1), and day 83 (for renal transplant recipient 2) after transplantation were all negative. A serum sample from the organ donor was not available for JEV testing.

Because the index patient had received 3 units of packed red blood cells from 3 different donors on 3 different days during the 3 weeks before illness onset (i.e., 1 unit each on June 20, June 22, and June 25), the remaining blood products from all 3 donors were traced from the Hong Kong Red Cross Blood Transfusion Service and samples were taken. Only the sample from the transfusion on June 22 tested positive by both real-time RT-PCR for JEV and conventional heminested PCR for flavivirus. Sequencing of the amplicons obtained by conventional PCR yielded a sequence identical to that obtained from the index patient (Figure 3). Phylogenetic comparison with other JEV sequences from GenBank showed that the index case and blood donor isolates belonged to JEV genotype 1, a circulating genotype common in southern China (*24*). The blood donor was a 46-year-old man who resided in Tin Shui Wai of the Yuen Long District in the New Territories region of Hong Kong. He had been asymptomatic at the time of blood donation (May 29, 2017) and had no recent travel to JEV-endemic regions outside of Hong Kong. He did not develop any symptoms of Japanese encephalitis after the blood donation. On July 22, a serum sample from the donor tested positive for JEV IgM. He was deferred from donating blood for 1 year.

Evaluation for Secondary Cases

After confirmation that a blood donor was the source of JEV infection in the index patient, we urgently traced other patients who received blood products from this donor and identified 2 recipients (Table). One was a 61-year-old man with newly diagnosed acute myeloid leukemia who had received induction chemotherapy with cytarabine and daunorubicin during May 8–14, 2017; he was transfused with platelets obtained from the viremic blood donor on June 2, 2017. His white blood cell count on the day of platelet transfusion was 3.39×10^9 cells/L, and he had severe lymphopenia (0.24×10^9 cells/L). His lymphocyte counts steadily rose to 0.95×10^9 cells/L 10 days after the transfusion and normalized by the end of June. He was asymptomatic at follow-up 2 months after transfusion. Upon recognition that this patient received a potentially JEV-infected blood product, staff retrieved his archived serum specimens collected before transfusion and 33 and 50 days after transfusion to test for JEV IgM. The serum specimens collected before transfusion tested negative for JEV IgM, but those collected after tested positive, confirming a recent asymptomatic infection probably contracted from the platelet transfusion. JEV RNA was not detected in a urine specimen collected 50 days after or a plasma sample collected 53 days after the transfusion.

The other blood product recipient was a 64-year-old man who was admitted for intracranial hemorrhage. He received a plasma transfusion June 20, 2017, and died July 4, 2017, due to respiratory failure. No serum specimens were available for serologic testing from this patient, and an autopsy was not performed.

Discussion

In our study, we report nosocomial transmission of JEV through blood transfusion products from an asymptomatic viremic donor to 2 immunocompromised persons, resulting in 1 case of severe encephalitis and another asymptomatic infection with seroconversion. JEV has been documented to be exclusively transmitted via the bite of *Culex* mosquitoes. However, when we diagnosed JEV infection in a

Table. Demographics, investigation results, and clinical details of asymptomatic JEV-infected blood donor and recipients, Hong Kong, China, May–July 2017*

Variable	Donor	Packed red blood cell recipient (index patient)	Platelet recipient	Plasma recipient
Underlying disease	No history of disease, no recent travel to JEV-endemic regions outside Hong Kong; resides in JEV-endemic area Tin Shui Wai, Yuen Long	End-stage chronic obstructive pulmonary disease with lung transplantation, May 10	Acute myeloid leukemia postinduction chemotherapy with cytarabine and daunorubicin	Intracranial hemorrhage
Blood donation or transfusion type, date	Blood donation, May 29	Packed red blood cell transfusion, June 22	Platelet transfusion, June 2	Plasma transfusion, June 20
Pretransfusion IgM serology result, date	Specimen not available	Serum negative, May 31	Serum negative, May 25	Specimen not available
Posttransfusion IgM serology result, date	Serum positive, July 22	CSF positive, July 11; serum positive, July 15 and 18	Serum positive, July 5 and 22	Specimen not available
JEV nucleic acid test, specimen type and result, date	Positive archived blood specimen	Positive blood sample, June 26 and 28; positive bronchoalveolar lavage, July 10	Negative plasma sample, July 22; negative urine sample, July 25	Specimen not available
Clinical symptoms	Asymptomatic	Fever and rash July 6 (14 d after transfusion), followed by decreased consciousness and myoclonic jerks; MRI showed typical appearance of T2 hyperintensity of bilateral thalami, substantia nigra, and medial temporal lobes	Asymptomatic	Unknown
Outcome	Full recovery	Died October 1, 2017	Full recovery	Died from respiratory failure 14 d after transfusion

*CSF, cerebrospinal fluid; JEV, Japanese encephalitis virus; MRI, magnetic resonance imaging.

patient who had been hospitalized for a prolonged period of time, mosquitoborne transmission was considered unlikely because of several factors. First, the index patient was treated in an air-conditioned facility with closed doors and windows during the entire incubation period. Second, rigorous integrated pest management programs (including weekly inspections by dedicated staff and regular monthly pest control services) were implemented in public hospitals and clinics in Hong Kong in 2014 in response to the increasing threat of mosquitoborne infectious diseases, such as dengue in nearby Guangzhou Province, China (*25*). Furthermore, although real-time information on *Culex* spp. breeding density in the district was not available, a *C. tritaeniorhynchus* mosquito distribution survey in Hong Kong showed that adult vector prevalence was low in the Aberdeen area, where Grantham Hospital is located (*26*).

Because of these circumstances, other sources of infection were considered. Transmission via organ transplantation, which has been reported for WNV, was considered (*27*), but serologic screening of other organ recipients from the same donor did not reveal any evidence of JEV infection. Blood product transfusion was then considered as a possible source of infection. JEV RNA was detected in a blood donation sample from 1 donor, and phylogenetic analysis showed 100% sequence identity between the isolates in the donor and recipient, confirming transfusion-transmitted JEV.

The blood donor resided in Tin Shui Wai of the Yuen Long District, an area that has been shown to have a high *C. tritaeniorhynchus* vector density (*26*). From January 2003 through June 2017, seventeen cases of locally acquired JEV infection in Hong Kong were recorded by the Department of Health, and 11 (64.7%) of the 17 patients resided in Yuen Long District. Therefore, the donor was likely to have been infected in Tin Shui Wai. This report illustrates that localized pockets of high JEV endemicity can facilitate the transmission of JEV via unconventional routes.

Arboviruses pose unique threats to the blood supply (*28–30*). Compromise of the blood supply by arboviruses has been well documented during explosive outbreaks of Zika, dengue, WNV, and chikungunya virus infections in recent years (*11*,*31*). Our findings have major implications for JEV-endemic areas, where undetected transmission of JEV by blood transfusion might be widespread because of high rates of asymptomatic infection in both donors and recipients. In the case we report, 24 days elapsed between the time of blood donation and transfusion to the index patient, indicating that the virus can remain viable in packed red blood cells over a prolonged period of time. Risk assessment of the presence of JEV in the blood supply in JEV-endemic areas should be undertaken.

Mitigation of such transmission is difficult because standard measures to protect the blood supply during

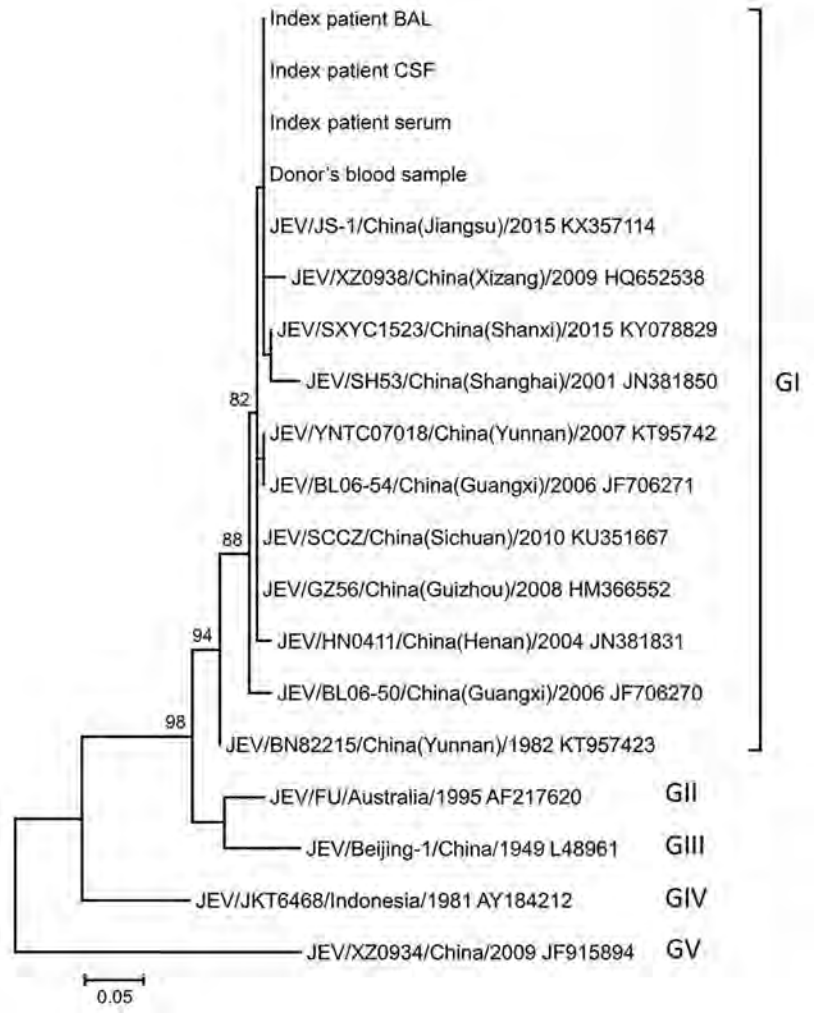

Figure 3. Phylogenetic tree constructed by using partial nonstructural protein 5 (NS5) sequences of JEV isolates detected in index patient and donor blood samples, Hong Kong, China, and other JEV reference strains available in GenBank (accession numbers shown). The tree was inferred from data by using the maximum-likelihood method with bootstrap values calculated from 1,000 trees. Only bootstrap values >70% are shown. A 167-nt fragment of NS5 from each virus was used in this analysis. Labels at right indicate JEV genotypes (GI–V): JEV from patient and donor samples grouped with GI strains. Scale bar indicates estimated number of nucleotide substitutions per 20 nt. BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; JEV, Japanese encephalitis virus.

arboviral outbreaks, such as donor symptom and travel questionnaires (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/1/17-1297-Techapp2.pdf), are not useful in the case of JEV, which has a high rate of subclinical infection and low incidence in Hong Kong. Two approaches to minimize the risk for transfusion-transmitted JEV infection are donor JEV screening and pathogen reduction technology (PRT). Both approaches have limitations. Donor screening would involve nucleic acid testing (NAT) of either individual donors or multiple donors by using minipools, as has been described for WNV and Zika virus (*32*–*34*). The biggest challenge for implementing screening is the lack of well-established and licensed JEV NAT for use in the blood donor setting. The use of clinical diagnostic NAT for donation screening is associated with the potential for false-positive results by cross-contamination caused by amplicon carryover. Individual donor NAT is likely to be prohibitively expensive, and minipool NAT is difficult to standardize for JEV, given the lack of data on viral loads in asymptomatic persons. Although historically most Japanese encephalitis cases in Hong Kong have occurred during the rainy summer season (May–August), even limited seasonal screening of residents in high-risk areas of Hong Kong might be impractical because of the highly mobile population and imprecise delineation of seasons due to climate change. Although donor screening might not be cost-effective for universal application, a more selective application of blood products used for immunocompromised persons might be considered. However, JEV can cause life-threatening disease even in immunocompetent persons, and the correlation between immunosuppression and disease severity is not clear. A range of novel PRTs for blood products that involve the combined use of ultraviolet light and reagents such as psoralens or riboflavin are available (*35*,*36*). Examples include the INTERCEPT Blood System for platelets and plasma (Cerus Corporation, Concord, CA, USA); Mirasol Pathogen Reduction Technology for plasma, platelets, and whole blood (Terumo BCT, Lakewood, CO, USA); and the THERAFLEX platform for

plasma and platelets (Macopharma, Tourcoing, France). These methods require individual component processing, are not suitable for all blood components, and are expensive to implement. No direct evidence indicates that these products are efficient at reducing JEV infectivity, although such efficacy could be extrapolated from studies on PRT for Zika and dengue viruses (*37*,*38*). Also, leukocyte depletion of blood products might theoretically reduce the risk for JEV transmission, but this method also requires validation. Implementation of a JEV vaccination program with high coverage in areas with high *C. tritaeniorhynchus* mosquito breeding density might also be considered to definitively eliminate this virus from the blood supply. If transfusion-transmitted JEV is confirmed to be a significant threat to the blood supply in highly endemic regions, a combination of these methods might be required to prevent transfusion-transmitted JEV infection.

Transmission of WNV, another member of the JEV antigenic serocomplex, to immunocompromised patients via blood transfusion has been reported (*7*). Immunocompromised patients infected with WNV tended to have longer incubation periods (≥10 days) and higher rates of severe illness (*39*). In our study, the index patient had symptom onset 14 days after the transfusion and overt encephalitis 2 days later, a relatively long incubation period, comparable with the observations made for WNV. Clinical specimens from immunocompetent patients with JEV infection are typically PCR negative at symptom onset, reflecting immune-mediated pathogenesis of the disease. However, in our immunocompromised patient, JEV RNA was detected in a serum specimen from 10 days before symptom onset and in bronchoalveolar fluid and CSF samples from days 5 and 6, respectively, after symptom onset, reflecting the inability of this immunocompromised patient to effectively clear the virus. The detection of JEV in lower respiratory tract specimens has not been reported previously. However, WNV has been reported to cause pneumonia in immunocompromised transplant recipients (*40*). The clinical significance of JEV in the respiratory tract of our patient is unclear; the patient had extensive consolidative changes over both lung fields on chest radiograph, but culture of the bronchoalveolar fluid specimen yielded *B. cepacia*, suggesting a component of bacterial pneumonia. However, pulmonary involvement caused by disseminated JEV infection in this immunocompromised patient cannot be excluded.

We identified a second patient who had evidence of JEV seroconversion after receiving platelets from the viremic donor. This patient was immunocompromised at the time of platelet transfusion, which was 19 days after completion of induction chemotherapy for acute leukemia. His relatively asymptomatic clinical course was probably related to the recovery of bone marrow function during the incubation period, in contrast to the index patient, who was subjected to continuous lymphocyte-depleting immunosuppression from the antirejection prophylaxis administered during the incubation period. However, other factors might be responsible, including differences in virus inoculum (the JEV envelope protein has hemagglutinating properties, which could have resulted in a higher accumulation of virus in the packed red blood cell packet); blood product storage conditions (platelet concentrates are stored at higher temperatures, which could have lowered JEV viability in this blood product); and other subtle differences in host-pathogen interactions.

In summary, this study illustrates that JEV can be transmitted via transfusion of cellular blood components to immunocompromised persons and could cause severe outcomes. Enhanced understanding of the prevalence of JEV in the blood supply, the incidence of transfusion-transmitted JEV, and measures for risk mitigation in JEV-endemic areas are urgently needed.

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Increased Severity and Spread of *Mycobacterium ulcerans*, Southeastern Australia

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Reported cases of *Mycobacterium ulcerans* disease (Buruli ulcer) have been increasing in southeastern Australia and spreading into new geographic areas. We analyzed 426 cases of *M. ulcerans* disease during January 1998–May 2017 in the established disease-endemic region of the Bellarine Peninsula and the emerging endemic region of the Mornington Peninsula. A total of 20.4% of case-patients had severe disease. Over time, there has been an increase in the number of cases managed per year and the proportion associated with severe disease. Risk factors associated with severe disease included age, time period (range of years of diagnosis), and location of lesions over a joint. We highlight the changing epidemiology and pathogenicity of *M. ulcerans* disease in Australia. Further research, including genomic studies of emergent strains with increased pathogenicity, is urgently needed to improve the understanding of this disease to facilitate implementation of effective public health measures to halt its spread.

Mycobacterium ulcerans causes a necrotizing disease of skin and soft tissue known as Bairnsdale or Daintree ulcer in Australia and Buruli ulcer worldwide. The pathogenesis of *M. ulcerans* is driven by production of mycolactone, a polyketide-derived macrolide that triggers apoptotic cell death (*1*). The clinical spectrum of *M. ulcerans* disease ranges from usually painless nodules or ulcers on the limbs, to more severe forms of the disease, including edematous lesions (*2*). More severe disease has major implications for patients in terms of increased illness and long-term deformities, more complicated and prolonged treatments, and increased treatment costs (*3*,*4*). The World Health Organization (WHO) classification system classifies *M. ulcerans* disease by severity: category 1 represents mild disease, and categories 2 and 3 represent more severe disease (*5*). The disease is classified as a WHO neglected tropical disease and has become a major public health issue in sub-Saharan Africa and Australia.

In the state of Victoria in Australia, *M. ulcerans* disease was first observed in the Bairnsdale District in the 1930s and is now established on the Bellarine Peninsula (*6*). In recent years, the epidemiology of *M. ulcerans* disease in southern Victoria has noticeably changed, with rapidly increasing numbers of human cases reported per year and expansion into new geographic areas, including the Mornington Peninsula, an adjacent area with previously few cases (*7*). The reasons for this expansion are unknown but might be related to changing climate, population expansion, human activities, or a complex zoonotic cycle involving possums (*8*,*9*).

Clinicians from Barwon Health, a tertiary hospital in Geelong, Victoria, Australia, which is adjacent to the Bellarine Peninsula, manage a large proportion of reported case-patients in Victoria (*10*), and have recently observed an increasing number of severe cases of *M. ulcerans* disease with devastating consequences for patients. If true, this increase might suggest emergence of more pathogenic strains of *M. ulcerans* among other factors putting humans at risk. Therefore, the purpose of this study was to describe the epidemiology and pathogenesis of severe *M. ulcerans* disease, assess risk factors for its development, and clarify the evolution of severe disease in this region. Our findings might facilitate development of effective public health interventions to reduce illness and the costs of this disease.

Methods

Case-Patient Identification

All patients with confirmed *M. ulcerans* disease managed at Barwon Health during January 1998–May 2017 were included in this study. A confirmed *M. ulcerans* case was defined as presence of a lesion clinically suggestive of *M. ulcerans* infection plus 1 of the following: culturing of *M. ulcerans* from a lesion, a positive PCR result for IS2404 (*11*) for swab or biopsy specimens from a lesion (performed at the Victorian Infectious Diseases Reference Laboratory, Melbourne, Victoria, Australia), or histopathologic findings for an excised lesion showing a necrotic ulcer and presence of acid-fast bacilli.

Data Collection

We prospectively collected clinical and demographic data by using Epi Info version 6 (Centers for Disease Control

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and Prevention, Atlanta, GA, USA). These data were information regarding age of patient; duration of symptoms at diagnosis; patient sex; geographic location of the case; concurrent conditions, such as diabetes mellitus and immune suppression; and type, site, and WHO category of lesions. We determined lesion size by measuring the extent of induration associated with the lesion with a ruler.

Definitions

We defined severe disease as any lesion classified as WHO category 2 or 3 at diagnosis. Using WHO criteria, we defined category 1 lesions as single lesions <5 cm in diameter, category 2 lesions as single lesions 5–15 cm in diameter, and category 3 lesions as single lesions >15 cm in diameter; multiple lesions, lesions at a critical site (eye, breast, genitalia), or osteomyelitis (*5*). Plaque lesions were firm, painless, elevated lesions >3 cm in diameter with ill-defined edges. Edematous lesions were diffuse and usually nonpitting swelling with ill-defined margins involving part or all of a limb or other body part. A lesion over a joint was defined as a lesion overlying 1 of the following large joints: ankle, elbow, knee, wrist, or shoulder. Immune suppression was defined as current treatment with immunosuppressive medication (e.g., prednisolone) or an active malignancy. We classified cases by geographic location as acquired from either of the disease-endemic areas of the Bellarine or Mornington Peninsulas (Figure 1). Calendar years were categorized as time periods (1998–2004, 2005–2010, and 2011–2017) and included as a variable in analyses to assess whether time periods were associated with disease severity.

Statistical Analysis

We analyzed data by using SPSS version 24 (IBM, Armonk, NY, USA). We compared categorical variables by using the Fisher exact test, χ^2 test, or χ^2 test for trend and

Figure 1. Locations (circles) of 426 cases of *Mycobacterium ulcerans* disease in Bellarine and Mornington Peninsulas, Barwon Health Cohort, Geelong, Victoria, Australia, January 1998–May 2017. Size of circles indicates number of cases, given in parentheses. Box in inset shows study region in southeastern Australia.

categorical and numerical variables by using the Independent Student *t*-test, as appropriate between groups. A p value <0.05 indicated statistically significant differences.

We constructed a logistic regression model to assess the association of variables with severe disease. We obtained crude odds ratios (ORs) by performing univariate analysis and then performed multivariable analysis for age and sex a priori and all other variables showing an association with severe disease by univariate analysis (assessed by p<0.20): geographic location, position of lesion over a joint, time period (range of years of diagnosis), and diabetes mellitus. We determined p values for assessing the strength of the association of each variable with disease severity, which were controlled for all other variables in the multivariable model, by using the likelihood ratio test. In addition, we individually compared the association of severe disease at 5 body sites (ankle, elbow, hand, forearm, and knee) with severe disease at all other body sites combined, except for these 5 sites, by using univariate logistic regression.

Ethics Approval

The study was approved by Barwon Health Human Research and Ethics Committee. All data were deidentified before analysis.

Results

A total of 446 case-patients with *M. ulcerans* disease were managed at Barwon Health during the study period. Twenty (4.5%) patients did not have WHO disease category recorded and were excluded. Thus, we included 426 case-patients in the study (Figure 1). We determined the number of cases included per calendar year stratified by disease severity (Figure 2).

Baseline Characteristics

Median age for the 426 patients included in the analysis was 58 years (range 38–74 years), and 230 (54%) patients were male. Thirty-four (9.2%) cases were diagnosed during 1998–2014, 97 (24.2%) during 2005–2010, and 295 (66.6%) during 2011–2017. Median duration of symptoms before diagnosis was 42 days (interquartile range [IQR] 28–75 days).

We determined lesion type at diagnosis: 358 (84.0%) of lesions were ulcers, 27 (6.3%) were nodules, 36 (8.5%) were edematous lesions, and 4 (0.9%) were plaques. Diagnoses were made on the basis of a positive PCR result for 398 (93.4%) patients, positive histopathologic results for 23 (5.4%), and positive culture for 5 (1.2%). We determined that the WHO category of lesions was category 1 for 335 (78.6%) patients, category 2 for 46 (10.8%), and category 3 for 45 (10.6%), which resulted in 335 (78.6%) patients classified as having nonsevere disease and 91 (20.4%) as having severe disease. Lesions categorized as severe had an increased likelihood of being edematous (OR 19.52, 95% CI 8.83–43.17; p<0.001), and 21 (23.1%) of case-patients with severe disease had >1 lesion at presentation.

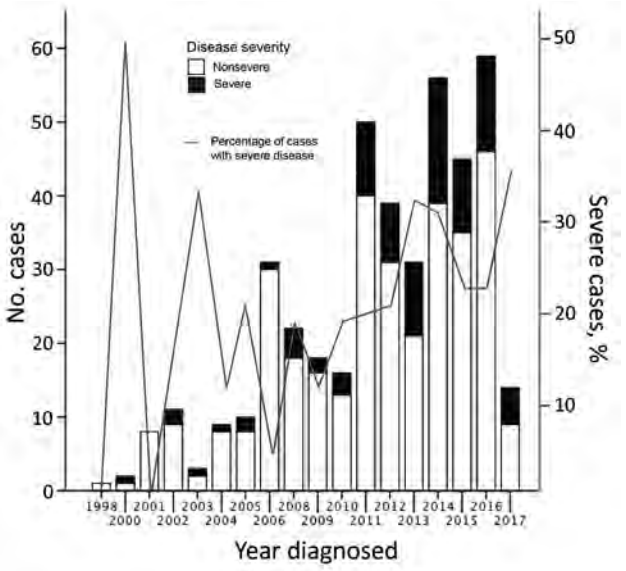

Figure 2. Proportion of severe and nonsevere cases of *Mycobacterium ulcerans* disease, Barwon Health Cohort, Geelong, Victoria, Australia, January 1998–May 2017.

We determined sites of lesions and the proportion of severe disease per site (Table 1). When compared with all other lesions on the body combined apart from the ankle, elbow, forearm, hand, and knee, we found that there was a significantly higher likelihood of severe disease if lesions were located on the ankle (OR 3.99, 95% CI 2.12–7.50; p<0.001), elbow (OR 3.12, 95% CI 1.52–6.40; p = 0.002), or knee (OR 2.60, 95% CI 1.10–6.13; p = 0.029) (Table 1). We also identified additional baseline characteristics stratified by disease severity (Table 2).

Patients with Severe Disease

Patients with severe disease were significantly older (median age 68 years, IQR 44–82 years) than patients without severe disease (median age 56 years, IQR 35–70 years; p<0.001). Univariate analysis showed that age (p = 0.004), geographic location (the Mornington Peninsula compared with the Bellarine Peninsula; p = 0.03), lesion located over a joint (p<0.001), time period (p = 0.01), and presence of diabetes mellitus (p = 0.06) were strongly associated with disease severity (Table 2). Univariate analysis showed that sex, duration of symptoms before diagnosis, and immune suppression were not associated with disease severity (Table 2). Multivariate logistic regression analysis adjusting for age, sex, geographic location, position of lesion over a joint, time period, and diabetes mellitus showed that only the position of lesion over a joint (p<0.001), age (p = 0.006), and

Table 1. Association between lesions at body sites and proportion of persons with severe *Mycobacterium ulcerans* disease, Barwon Health Cohort, Geelong, Victoria, Australia, January 1998–May 2017*

Site	Nonsevere disease, no. (%)	Severe disease, no. (%)	Crude odds ratio (95% CI)	p value
Ankle	40 (62.5)	26 (37.5)	3.99 (2.12–7.50)	<0.001
Elbow	32 (68.1)	15 (31.9)	3.12 (1.52–6.40)	0.002
Hand	16 (72.7)	6 (27.3)	2.49 (0.91–6.85)	0.077
Forearm	18 (75.0)	6 (25.0)	2.21 (0.82–6.01)	0.118
Knee	23 (71.9)	9 (28.1)	2.60 (1.10–6.13)	0.029
Arm	22 (88.0)	3 (12.0)	1	ND
Buttock	2 (66.7)	1 (33.3)	1	ND
Foot	17 (85.0)	3 (15.0)	1	ND
Leg	127 (86.4)	20 (13.6)	1	ND
Head	4 (80.0)	1 (20.0)	1	ND
Shoulder	3 (100.0)	0	1	ND
Thigh	18 (90.0)	2 (10.0)	1	ND
Trunk	4 (100.0)	0	1	ND
Wrist	9 (90.0)	1 (10.0)	1	ND

*ND, not determined.

time period (p = 0.03) were significantly associated with severe disease (Table 2).

Discussion

The findings of our study suggest a serious change in the epidemiology of *M. ulcerans* disease in southeastern Australia. There appears to be an increase in the proportion of severe cases in recent years, with a near doubling compared with earlier time periods to 25% of all case-patients who came to our health service during 2011–2017. More severe disease has major implications: case-patients with severe disease frequently require surgical treatment with tissue reconstruction (*2*) and hospitalization, and often have long-term deformities (*4*). Severe disease also causes major increases in cost of treatment (*3*). This finding is in contrast to nonsevere disease, which can usually be managed with oral antimicrobial drugs; outpatients have shown good outcomes (*12–14*). We also report a large proportion of severe cases that affected 1 of 5 patients in our cohort. However, these rates are lower than those reported for cohorts in Africa, in which the proportion of cases with severe disease (WHO categories 2 and 3 combined) was >70% (*15–17*). It has been postulated that strains in Africa are more virulent because of production of increased quantities and more potent forms of mycolactone (*18*).

The reason for the increasing proportion of severe cases in recent years is not clear but might be related to evolution of a more pathogenic strain of *M. ulcerans* in the region. In the area of *M. ulcerans* research, utility of whole-genome sequencing has been studied from an ecologic and epidemiologic perspective. Studies indicate a strong relationship between the genotype of an isolate and the geographic origin of disease at a national and regional level (*19–21*). However, there is no information available on the relationship between the genotype of an isolate and the clinical severity of disease in affected patients. In the related field of *M. tuberculosis* research, there has been increasing interest in using genomic information of individual disease isolates to predict clinical severity of disease in humans (phenotype), especially for *M. tuberculosis* strains with increased drug resistance (*22*). We advocate that genomic studies be conducted to explore whether particular *M. ulcerans* strains are associated with more severe disease, and if strains in disease-endemic regions are evolving over time to become more pathogenic. These studies might provide useful information for public health policy.

Other possible explanations for the increased proportion of severe cases of *M. ulcerans* disease could include environmental or climatic changes, which lead to a higher inoculum of organisms. In addition, there might have been a change in population dynamics or characteristics that make humans more susceptible to severe disease (*23*). Although human populations in disease-endemic regions are steadily increasing, these increases are not sufficient to explain the rapid increase in reported cases, and additional studies of these factors are needed.

Our analysis suggested that there was no major difference in the proportion of severe cases of *M. ulcerans* disease reported between the 2 peninsulas. This finding is consistent with our understanding of restricted genetic diversity of *M. ulcerans* from the same geographic location (*20*,*24*). A recent study of isolates from 11 disease-endemic countries in Africa identified only 2 specific *M. ulcerans* lineages; these lineages were subdivided into 4 major clusters, and most of these isolates were in cluster 1 (*25*). The spread of disease across both peninsulas in our study region, given their close geographic proximity, probably resulted from clonal expansion of the same genotype. This hypothesis is consistent with findings of a study of isolates from Australia in which 1 isolate from Frankston (Mornington Peninsula) had genomic sequences similar to those for isolates from Point Lonsdale and St. Leonards (Bellarine Peninsula) (*20*).

Apart from time period, we have identified 2 risk factors for severe *M. ulcerans* disease: a lesion situated over a

Table 2. Logistic regression analysis of adjusted and unadjusted associations between patient characteristics and severe *Mycobacterium ulcerans* disease, Barwon Health Cohort, Geelong, Victoria, Australia, January 1998–May 2017*

Characteristic	Nonsevere disease, no. (%)	Severe disease, no. (%)	Crude odds ratio (95% CI)	p value	Adjusted odds ratio (95% CI)	p value
Age, y						
≤15	31 (77.5)	9 (22.5)	1.62 (0.71–3.71)	0. 004	1.73 (0.73–4.12)	0.006
16–64	184 (84.8)	33 (15.2)	1		1	
≥65	120 (71.0)	49 (29.0)	2.28 (1.38–3.75)		2.34 (1.38–3.98)	
Sex						
F	156 (79.6)	40 (20.4)	1	0.66	1	0.51
M	179 (77.8)	51 (22.2)	1.11 (0.70–1.77		1.18 (0.72–1.94)	
Location						
Mornington Peninsula	72 (70.6)	30 (29.4)	1	0.03	1	0.24
Bellarine Peninsula	263 (81.2)	61 (18.8)	0.56 (0.33–0.93)		0.71 (0.40–1.26)	
Lesion over a joint						
No	228 (84.4)	42 (15.6)	1	<0.001	1	<0.001
Yes	107 (68.6)	49 (31.4)	2.67 (1.64–4.36)		2.71 (1.65–4.43)	
Time period						
1998–2004	29 (85.3)	5 (14.7)	1	0.01	1	0.03
2005–2010	85 (87.6)	12 (12.4)	0.82 (0.27–2.52)		0.88 (0.28–2.80)	
2011–2017	221 (74.9)	74 (25.1)	1.94 (0.73–5.20)		2.11 (0.74–5.99)	
Duration of symptoms before diagnosis, d						
≤75	241 (77.7)	69 (22.3)	1	0.76	NA	NA
>75	81 (81.0)	19 (19.0)	0.82 (0.46–1.44)			
Missing	13 (81.3)	3(18.8)	0.81 (0.22–2.91)			
Diabetes mellitus						
No	312 (79.8)	79 (20.2)	1	0.06	1	0.14
Yes	23 (65.7)	12 (34.3)	2.06 (0.98–4.32)		1.86 (0.82–4.23)	
Immune suppression						
No	310 (79.3)	81 (20.7)	1	0.31	NA	NA
Yes	25 (71.4)	10 (28.6)	1.50 (0.69–3.27)			

*Adjusted for age, sex, location, position of lesion over a joint, time period, and diabetes mellitus. NA, not applicable.

joint and age. The reason for more severe disease occurring over joints is not clear. Reduced skin and subcutaneous tissue and the absence of muscle compared with adjacent regions might lead to lower tissue temperatures and increased growth of organisms (*26*,*27*). In addition, these factors might facilitate spread across tissue planes, and a reduced blood flow in the absence of muscle might relatively reduce local immune function. In contrast, increased tissue movement and physical stress over joints might increase lymph flow in the region and enhance spread of the infection, such as that hypothesized for hand, foot and mouth disease (*28*,*29*).

Our finding of older (≥65 years) age as a risk factor for severe *M. ulcerans* disease is consistent with our previous studies that demonstrated that these patients were more likely to have multiple lesions or be categorized into WHO category 3 (*30*). This finding might be related to reduced immunity in patients in older age groups. Reduced immunity inhibits control of the mycobacterium and results in larger, more severe forms of *M. ulcerans* disease, which is similar to immunosuppressive effects of HIV (*31*). Reduced immunity in elderly persons might also be related to an increased prevalence of other concurrent conditions. Likewise, our study findings suggest that children might be at increased risk for severe disease, which might be related to relative immune suppression in an immune system that is still developing.

We found that, similar to findings from Africa (*32*), more prolonged signs and symptoms before diagnosis were not associated with severe disease. Therefore, severe disease is not directly related to a delay in diagnosis, as is often believed.

With the increasing number and severity of cases of *M. ulcerans* disease in Victoria, public health measures to prevent the disease are needed, although these measured are hampered because the environmental reservoir and mode of transmission of *M. ulcerans* are unknown. Current recommendations involve mosquito and insect bite preventive strategies, given some evidence that mosquitoes or another vector might be responsible for transmission (*33*,*34*). There is evidence of reduced risk for disease for persons outdoors in disease-endemic areas who reported regular use of insect repellent (*35*). In addition, sleeping under bed nets in Africa has been associated with a reduced incidence of disease (*36*). Possums have been postulated as a potential environmental reservoir in Australia (*8*,*9*), and more research is needed on the role of possums in the epidemiology of human *M. ulcerans* disease.

There were some limitations to this study. First, because we conducted an observational study, other unmeasured confounders associated with severe disease could affect the results. Second, there might be referral bias for case-patients managed at Barwon Health and increased

numbers of severe cases referred over time because of the increasing expertise of clinicians in managing *M. ulcerans* disease. However, Barwon Health is the only tertiary referral center on the Bellarine Peninsula, and case-patients who are residents of this peninsula are seen by this health service regardless of disease severity. Therefore we believe that referral bias accounting for increasing disease severity over time is unlikely.

The epidemiology and pathogenicity of *M. ulcerans* disease in southeastern Australia is changing rapidly; we observed increases in numbers of cases per year and disease severity. Risk factors for severe disease include age, time period, and lesions located over a joint. Reasons for the changing epidemiology and pathogenicity are unknown but these factors urgently need to be identified and addressed to halt spread of this increasingly devastating disease.

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Emergence of Vaccine-Derived Polioviruses during Ebola Virus Disease Outbreak, Guinea, 2014–2015

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During the 2014–2015 outbreak of Ebola virus disease in Guinea, 13 type 2 circulating vaccine-derived polioviruses (cVDPVs) were isolated from 6 polio patients and 7 healthy contacts. To clarify the genetic properties of cVDPVs and their emergence, we combined epidemiologic and virologic data for polio cases in Guinea. Deviation of public health resources to the Ebola outbreak disrupted polio vaccination programs and surveillance activities, which fueled the spread of neurovirulent VDPVs in an area of low vaccination coverage and immunity. Genetic properties of cVDPVs were consistent with their capacity to cause paralytic disease in humans and capacity for sustained person-to-person transmission. Circulation ceased when coverage of oral polio vaccine increased. A polio outbreak in the context of the Ebola virus disease outbreak highlights the need to consider risks for polio emergence and spread during complex emergencies and urges awareness of the challenges in polio surveillance, vaccination, and diagnosis.

Poliovirus, the etiologic agent of paralytic poliomyelitis, can cause acute flaccid paralysis (AFP) (*1*). Three serotypes of poliovirus (1, 2, and 3) belong to *Enterovirus species C* (family *Picornaviridae*, genus *Enterovirus*). Since the World Health Organization (WHO) and partners launched the Global Polio Eradication Initiative in 1988, the widespread use of live-attenuated oral poliovirus vaccine (OPV) has been crucial for reducing polio cases >99% (*2*). Although OPV has many advantages (easy administration by mouth, low cost, effective intestinal immunity, and durable humoral immunity), it has the disadvantage of genetic instability. Because of the plasticity and rapid evolution of poliovirus genomes and selective pressures during replication in the human intestine, vaccine poliovirus can lose key genetic determinants of attenuation through mutation or recombination with closely related polio and nonpolio enterovirus strains, acquiring the neurovirulence and infectivity characteristics of wild-type poliovirus (WPV) (*3*). Because of this genetic instability, in settings where a substantial proportion of the population is susceptible to poliovirus, OPV use can lead to poliovirus emergence and sustained person-to-person transmission and spread in the community of genetically divergent circulating vaccine-derived polioviruses (cVDPVs). cVDPVs (as well as VDPVs excreted by immunodeficient persons) are defined as those with >1% nt sequence divergence (for polioviruses types 1 and 3) or >0.6% (for poliovirus type 2) in the major viral capsid protein coding region 1 (VP1) of the corresponding OPV strain (*3*).

The first known outbreak of cVDPV infection was reported in Hispaniola in 2000–2001 (*4*), although retrospective analyses of poliovirus isolates from Egypt during the 1960s suggest that this phenomenon might have been more common than anticipated. Although cVDPV outbreaks have been associated with all 3 components of trivalent OPV, most (97%) VDPV isolates that have emerged from OPV use are type 2 (*5*). Since 2005, type 2 cVDPV outbreaks have occurred in 17 countries of Africa and Asia, causing ≈600 cases of paralytic polio (*5*–*7*).

In Guinea, AFP case surveillance was established in 1997 as part of the Global Polio Eradication Initiative; fecal samples were tested at the WHO Reference Polio Laboratory in Senegal. AFP surveillance and vaccination with OPV led to the interruption of WPV transmission in Guinea, where the last known indigenous WPV type 1 dates back to 2009, and the last case of wild-type paralytic poliomyelitis

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was detected in August 2011 after importation of a type 3 WPV from Côte d'Ivoire.

We report the emergence and spread of type 2 cVDPVs in Guinea in 2014–2015 during the Ebola virus disease (EVD) epidemic. In 2014, WHO declared that EVD and polio were Public Health Emergencies of International Concern (PHEICs). We describe the occurrence of a PHEIC within a PHEIC. The occurrence of a polio outbreak in the context of the Ebola emergency highlights the need to evaluate the challenges in polio surveillance, vaccination, and diagnosis during complex emergencies to improve prevention and response strategies for future outbreaks in high-risk areas. To clarify the genetic properties of cVDPVs and to reconstruct the events leading to their emergence and spread, as well as the subsequent public health responses used to stop the polio epidemic, we analyzed epidemiologic and virologic data of polio cases in Guinea.

Materials and Methods

Surveillance of AFP Cases

We identified patients with paralytic poliomyelitis through the Guinea AFP surveillance system according to WHO guidelines (*8*). Through this system, district health officers routinely investigate AFP cases reported by health centers and hospitals, collect fecal samples, investigate clinical histories, record the total number of OPV doses received, and assess residual paralysis 60 days after onset. During September 2015–December 2016, additional fecal samples were collected from contacts of most AFP patients. According to the outcome of laboratory investigations, AFP cases were classified as confirmed polio or discarded as non–polio-associated AFP (*8*).

Epidemiologic Outbreak Investigation

To assess epidemiologic factors associated with the outbreak, during December 17–28, 2015, we conducted field investigations in Siguiri and Kankan Prefectures, Guinea. The investigation team was composed of trained healthcare workers from the Ministry of Health, WHO, and UNICEF. The workers interviewed parents/caregivers of 5 children from whom laboratory-confirmed type 2 cVDPV had been isolated (3 case-patients [nos. 15-078, 15-114, and 15-115] and 2 contacts [15-115-C4, 15-115-C5]) and parents/caregivers of potential contacts in the community. Data were collected through face-to-face interviews with the parents/caregivers by use of a structured questionnaire. The specific questions included vaccination history (routine and supplementary), travel history, household demographics, living conditions, water supply, and sanitary conditions. Vaccination status was checked from immunization cards (where available) or by a convincing history of vaccination from the parent/caregiver. Finally, the team conducted active searches in the communities for new AFP cases, collected fecal samples from contacts, raised awareness in families to promote poliomyelitis vaccination, and assessed 5 health centers in both prefectures.

Assessment of Vaccination Coverage

Supplementary immunization activities (SIAs) are mass vaccination campaigns conducted in a short period, during which a dose of OPV is administered to all children <5 years of age, regardless of previous vaccination history (*9*). Data on routine vaccination coverage with 3 doses of OPV (OPV3) were available from administrative reports of routine vaccination.

Virus Isolation and Molecular Typing

Polioviruses were isolated from fecal samples according to WHO standard procedures (*10*) and subjected to intratypic differentiation by reverse transcription PCR targeting the VP1 region, according to Centers for Disease Control and Prevention protocol (*11*). During 2013–2015, typing of nonpolio enterovirus isolates was performed as previously described (*12*).

Sequencing and Sequence Analysis

We sent isolates with discordant intratypic differentiation results to the National Institute for Communicable Diseases, Johannesburg, South Africa, for entire VP1 sequencing according to WHO guidelines (*10*). To determine VP1 genetic diversity, we compared all complete VP1 sequences of type 2 cVDPV isolates from AFP patients and their contacts with the sequence of the Sabin-2 OPV reference strain (GenBank accession no. AY184220). We aligned sequences by using the ClustalW alignment program within the BioEdit Sequence Alignment Editor package version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nearly complete genome of type 2 cVDPV isolate 2015-114-C6 was sequenced by deep sequencing. Random PCR and poliovirus-specific PCR products were generated and sequenced independently. We performed sequence amplification and analysis for the random approach according to Mee et al. (*13*) and for poliovirus-specific primers as described by Stern et al. (*14*). We used Geneious R10 software (https://www.geneious.com/) for deep-sequencing data analysis. The nucleotide sequence of type 2 cVDPV isolate 2015-114-C6 has been deposited in GenBank under accession no. MF346171.

Phylogenetic and Recombination Analysis

For phylogenetic analyses, we used MEGA version 5.0 (http://www.megasoftware.net/). We determined sequence divergence by calculating mean pairwise distances within groups. To analyze its possible recombination structure, we

compared the full-length genome sequence of isolate 2015-114-C6 with those of Sabin and WPV strains of the 3 serotypes and with prototype strains of 21 species of enteroviruses by using SimPlot version 3.5.1 (https://sray.med.som.jhmi.edu/SCRoftware/simplot/).

Results

Epidemiology

During December 2013–May 2016, a total of 3,351 laboratory-confirmed cases of EVD occurred in Guinea, resulting in 2,083 deaths and reaching a peak of 509 confirmed cases in October 2014 (Figure 1, panel A). In September 2014, a case of laboratory-confirmed type 2 cVDPV infection was identified in Guinea. The case-patient (no. 14-120) was a 4-year-old boy in Siguiri Prefecture. On August 30, fever and bilateral paralysis of lower extremities developed; the patient was hospitalized 3 days later with AFP, and type 2 cVDPV strains were isolated from fecal samples on September 6 and 7.

During October 2014–March 2015, collection of fecal samples from AFP patients in Guinea was interrupted because of the outbreak of EVD (Figure 1, panel A). On September 4, 2015, type 2 cVDPV was isolated from a fecal sample from a child from the Kankan region of Guinea; the child had become paralyzed on July 20 and was transported to Mali for treatment (*16*). Subsequently, type 2 cVDPV isolates were recovered from 5 AFP patients and 7 healthy contacts; dates of AFP onset were September–December 2015 (Figure 1, panel A). The 7 healthy type 2 cVDPV–positive contacts were epidemiologically linked to 3 of the AFP case-patients. Most (12/13) poliovirus-positive case-patients were incompletely vaccinated children (Table 1). All 13 type 2 cVDPV strains were isolated from persons in the Kankan region in eastern Guinea, near the border with Mali (Figure 1, panel B); most (12/13) persons were from Siguiri Prefecture. During the first semester of 2015, the coverage of routine OPV3 vaccination in Siguiri Prefecture was 31%. In 2014, the official national OPV3 routine coverage in Guinea was 42%.

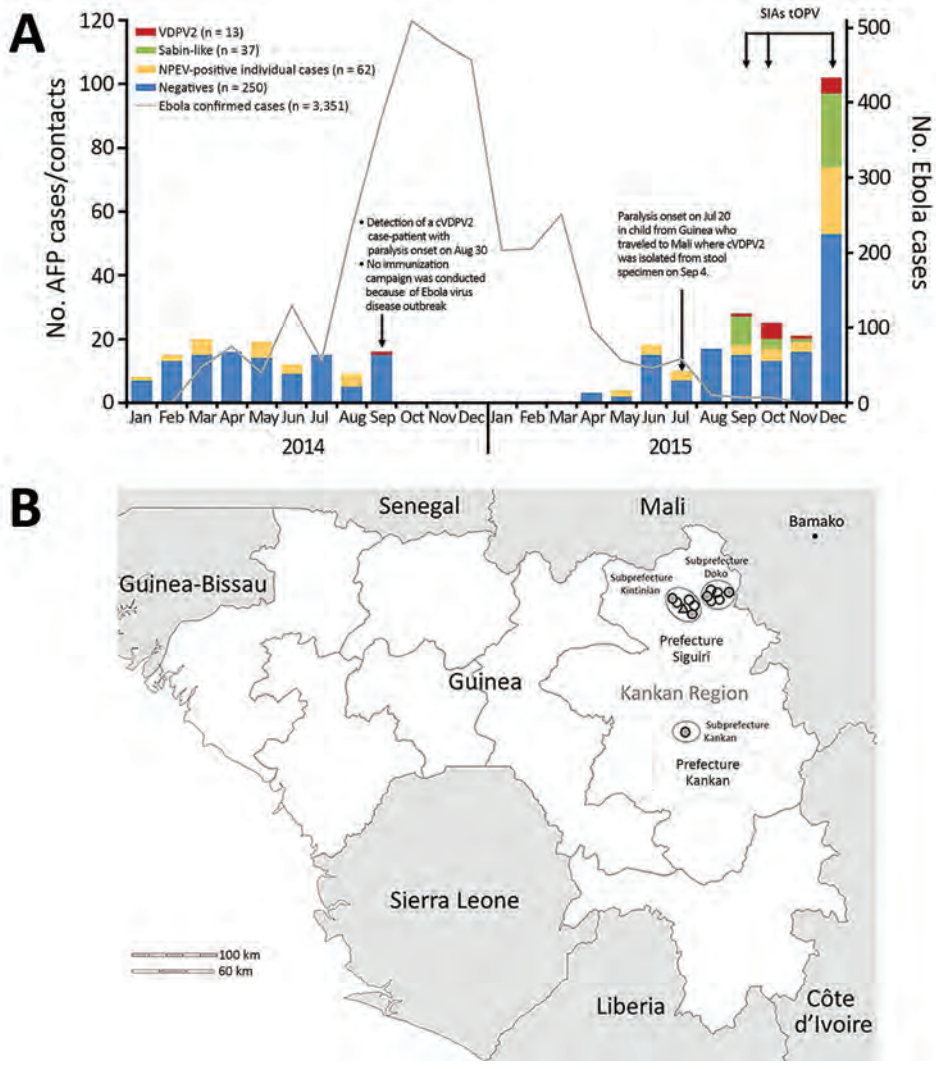

Figure 1. Epidemiologic context for emergence of vaccine-derived polioviruses during Ebola virus disease outbreak, Guinea, 2014–2015. A) Distribution of AFP cases (n = 132 in 2014; n = 113 in 2015) and contacts (n = 0 in 2014; n = 119 in 2015) for each month according to date of first fecal sample collection. Data for Ebola cases accessed at (*15*). B) Geographic distribution of case-patients (n = 6) and contacts (n = 7) with laboratory-confirmed VDPV2 infection. Outer circles indicate subprefectures; gray circles represent case-patients with paralysis onset in 2015; white circles represent laboratory-confirmed contacts; triangle represents case-patient with paralysis onset in 2014. AFP, acute flaccid paralysis; NPEV, nonpolio enterovirus; OPV, oral polio vaccine; tOPV, trivalent OPV; SIAs, supplementary polio immunization activities; VDPV2, type 2 vaccine-derived poliovirus.

Table 1. Clinical and virologic features for case-patients and contacts from whom laboratory-confirmed type 2 cVDPVs were isolated from fecal samples, Guinea, 2014–2015*

Name†	Subpref‡	Paralysis onset date	Date of first sample	Paralysis within 3 d	Asymm. paralysis	No. OPV doses	Date of last OPV	No. VP1 nt changes vs. Sabin-2	% Match Sabin-2	GenBank accesion no.
Case-patient no., age, y/sex										
14-120, 4/M	Kintinian	2014 Aug 30	2014 Sep 6	Yes	No	1	2013 Oct 25	12	98.67	MF322697
15-078, 3/F	Kankan	2015 Sep 7	2015 Sep 10	No	No	2	2015 Jun 5	22	97.56	MF322698
15-114, 3/M	Kintinian	2015 Sep 30	2015 Oct 9	Yes	No	1	2012 May 12	20	97.78	MF322699
15-115, 1/M	Doko	2015 Oct 2	2015 Oct 18	No	No	1	2015 Sep 18	24	97.34	MF322700
15-136, 4/M	Kintinian	2015 Oct 10	2015 Nov 10	Yes	No	2	2015 Oct 1	27	97	MF322701
15-170, 1/M	Doko	2015 Dec 14	2015 Dec 18	No	Yes	0	NR	23	97.45	MF322702
Contact no., age, y/sex										
15-114-C6, 1/F	Kintinian	NA	2015 Dec 25	NA	NA	3	2015 Dec 6	22	97.56	MF322703
15-115-C4, 1/F	Doko	NA	2015 Oct 22	NA	NA	2	2015 Sep 28	22	97.56	MF322704
15-115-C5, 3/F	Doko	NA	2015 Oct 22	NA	NA	2	2015 Oct 29	22	97.56	MF322705
15-115-C8, 4/M	Doko	NA	2015 O t 23	NA	NA	2	2015 Sep 29	20	97.78	MF322706
15-115-C10, 0.58/F	Doko	NA	2015 Dec 23	NA	NA	1	2015 Dec 6	23	97.45	MF322707
15-136-C1, 1/F	Kintinian	NA	2015 Dec 11	NA	NA	1	2015 Dec 9	24	97.34	MF322708
15-136-C2, 0.5/F	Kintinian	NA	2015 Dec 10	NA	NA	1	2015 Dec 9	25	97.23	MF322709

*AFP, acute flaccid paralysis; Asymm., asymmetrical; cVDPV, circulating vaccine-derived poliovirus; NA, not applicable; NR, vaccine not received; OPV, oral polio vaccine; subpref, subprefecture; VP, capsid viral protein.
†All case-patients had AFP and fever at onset of paralysis. No contact had AFP.
‡All case-patients and contacts were from Siguiri Prefecture, except 15-078 (Doko Prefecture).

Households of the 5 children from whom laboratory-confirmed type 2 cVDPV was isolated (3 case-patients and 2 contacts) were investigated. All used man-made wells as the primary source of water and reported sharing a latrine with neighbors. None of the 5 households had been visited by children with AFP in the 3 months before paralysis onset. Parents all panned or mined for gold for a living and knew about polio but took no control measures to prevent infection and transmission. They all said they understood the value of vaccination and confirmed their commitment to vaccination activities.

We identified 59 potential contacts. All were incompletely vaccinated children. Of these 59 families, most (38 [64.4%]) were reluctant to allow fecal sample collection.

In the 5 health centers investigated, we found nonfunctional refrigeration equipment and insufficient kits for sample collection and transportation. Frequent lack of polio vaccine stocks was also reported.

Public Health Response and Vaccination Coverage

Since the first detection of a case of type 2 cVDPV infection in 2015 in Guinea, during 2015 and 2016, the Guinea Department of Health has conducted nationwide national immunization days (NIDs) and districtwide subnational immunization days (SNIDs) (Table 2). Assessments of vaccination coverage during the 3 campaigns in 2015 were conducted in Siguiri Prefecture. A total of 1,997 children were investigated (431 in SNIDs 1, 639 in SNIDs 2, and 927 in NIDs 1). The survey found that 181 (9%) of the 1,997 children remained unvaccinated. The most frequently reported reasons for not receiving OPV were absence at the time of vaccination (62.5%) and vaccination refusal (16.7%). The estimated OPV3 coverage in Siguiri Prefecture after the 3 SIAs of 2015 was 94%. Guinea's official national OPV3 routine coverage for 2015 was 87%. Moreover, in late 2015 and in 2016 during SIAs, AFP surveillance activities were strengthened throughout the country. During 2016, a total of 2,585 fecal specimens from 1,298 children with AFP and 995 specimens from their 507 contacts were collected throughout Guinea. All specimens from 2016 were confirmed negative for VDPVs. Table 3 describes the evolution of the most representative AFP surveillance performance indicators in Guinea before, during, and after the polio outbreak.

Isolates, Molecular Types, and VP1 Sequences

All 13 VDPV isolates showed discordant intratypic differentiation results and were further characterized by sequencing

Table 2. Supplementary polio immunization activities, Guinea, 2015–2016*

Dates	Type	Regions	Target population	No. vaccinated children	Vaccination coverage, %	Vaccine type
2015						
Sep 16–19	SNID	Faranah, Kankan, Nzérékoré, Labé	1,142,259	1,175,963	102.95	tOPV
Sep 28–Oct 1	SNID	Faranah, Kankan, Nzérékoré, Labé	1,175,963	1,224,364	104.12	tOPV
Dec 5–8	NID	Nationwide	2,523,431	2,497,033	98.95	tOPV
2016						
Jan 28–31	NID	Nationwide	2,611,902	2,738,818	104.86	tOPV
Mar 3–6	NID	Nationwide	2,611,902	2,883,669	110.40	tOPV
Apr 7–10	NID	Nationwide	2,880,679	3,066,638	106.46	tOPV
Apr 25–28	SNID	Faranah, Kankan	787,399	908,092	115.33	tOPV
Oct 6–9	NID	Nationwide	3,187,032	3,330,472	105.00	bOPV
Dec 2–5	NID	Nationwide	3,348,132	3,706,752	110.70	bOPV

*Vaccination coverage rates >100% result from vaccination of children who are not part of the initial target because of the movement of the population.
bOPV, bivalent oral polio vaccine; NID, national immunization days; SNID, subnational immunization days; tOPV, trivalent oral polio vaccine.

the VP1 capsid coding region (Table 4). All isolates diverged >0.6% nt from the type 2 OPV strain, which confirmed their classification as type 2 VDPVs (Table 1). All VDPV study isolates shared 8 nt substitutions, suggesting that all were derived from a common infection. The key determinant of Sabin-2 attenuated phenotype at nt 2909 of VP1 (*17*) reverted in all cVDPV strains (U_{2909}→C resulting in an Ile_{143}→Thr substitution) (Table 5).

Phylogeny

All 13 type 2 cVDPV strains from Guinea clustered in a monophyletic group supported by a high (96%) bootstrap value (Figure 2, panel A). The close genetic relationship between the type 2 cVDPV strains from Guinea (97.8%–98.4% sequence similarity for nucleotides and 98.7%–99.3% for amino acids) supported the conclusion that all had a common precursor. The VP1 sequences segregated in 2 genetic lineages, which we designated I and II (with bootstrap values of 82% and 100%, respectively) and which probably correspond to different chains of transmission (Figure 2, panel A). A notable observation was the geographic segregation of type 2 cVDPV strains corresponding with the 2 observed genetic lineages. Strains isolated in the Kintinian subprefecture clustered in lineage I; those isolated in the Doko subprefecture clustered in lineage II (Figure 2, panel A). Estimates of average evolutionary divergence between study VDPV sequence pairs and the Sabin-2 OPV strain are shown in Table 6.

Estimated Time of OPV Dose Initiation

We estimated the time of OPV dose initiation from the VP1 sequence divergence from Sabin-2 shown by the cVDPV isolates (Figure 2, panel B) and extrapolated the regression line for the evolution rate of nucleotide substitutions back to 0 in the Sabin-2 VP1. This date was estimated to be April 27, 2013 (95% CI February 10, 2012–December 7, 2013). When estimates were obtained for all VP1 sequences individually, with an assumed VP1 nucleotide substitution rate of 1% (*20*), the average date for initial OPV infection was April 20, 2013 (range November 13, 2012–July 18, 2013), remarkably similar to the above date estimated by linear regression. This finding suggests that all cVDPV isolates were derived from a single infection event.

Whole-Genome Sequence of an Outbreak Type 2 cVDPV Strain

We subsequently obtained an almost-complete genomic sequence of type 2 VDPV isolate 2015-114-C6, from nt 33 to nt 7434 (Sabin-2 numbering) by deep sequencing and compared the sequence to that of the Sabin-2 vaccine reference strain. Consensus sequences obtained from both random and poliovirus-specific PCR products were identical and 100% similar to the original VP1 sequence determined by Sanger sequencing. Strain 2015-114-C6 contained an A-to-G nt substitution at 481 in the 5′-untranslated region (UTR), which represents the reversion of a major attenuation determinant of Sabin-2. This isolate differed from

Table 3. AFP surveillance quality indicators in Guinea, 2012–2016*

Indicator	Target	2012	2013	2014	2015	2016
AFP cases reported, no.	NA	186	221	132	113	1,298
AFP cases with 2 fecal specimens collected within 14 d of onset of paralysis, %	80	92.4	92.3	95.7	71.7	89.6
Fecal specimens arriving at national level within 3 d of being sent, %	80	83.4	87.9	87.8	79	58.8
Fecal specimens arriving at laboratory in good condition, %	90	63.7	54.6	86.4	94.3	94.2
Fecal specimens for which laboratory results were sent within 14 d of receipt at lab, %	80	100	100	99.2	93.1	62.5
Fecal specimens from which nonpolio enterovirus was isolated, %	10	12.8	8.5	12.4	7.5	16.1
VDPVs, no.	NA	0	0	1	12	0
Sabin virus, no.	NA	12	29	1	37	182

*AFP, acute flaccid paralysis; NA, not applicable; VDPV, vaccine-derived poliovirus.

Table 4. Frequency of samples, AFP cases, contacts, cVDPVs isolated, NPEV cases, and Sabin-like poliovirus cases in Guinea, 2012–2016*

Year	No. fecal samples received	No. AFP cases	No. contacts	No. cVDPVs isolated from case-patients	No. cVDPVs isolated from contacts	No. NPEV-positive cases	No. Sabin-like polioviruses
2012	366	185	0	0	0	23	12
2013	446	223	0	0	0	21	29
2014	258	132	0	1	0	22	1
2015	453	113	119	5	7	40	37
2016	3,580	1,298	507	0	0	363	182

*AFP, acute flaccid paralysis; cVDPV, circulating vaccine-derived poliovirus; NPEV, nonpolio enterovirus.

Sabin-2 at 807 nt and 65 aa substitutions in the open reading frame. Among all 65 aa changes, 30 represented reversions to amino acid residues found in MEF-1, a laboratory reference type-2 WPV strain. The outbreak isolate was a vaccine/nonvaccine recombinant; although 5′-UTR and P1 capsid genomic sequences were homologous to those of Sabin-2 (2.9% nt divergence), the noncapsid region (P2 + P3) and 3′-UTR sequences were dissimilar (18.2% nt divergence). The substitutions in the vaccine part of the VDPV genome are shown in Table 5. We estimated the crossover recombination point to be somewhere between nt 3262 and 3443. Similarity plot analysis revealed that nucleotide sequences in the 3′ half of the genome were dissimilar to those of other WPVs, vaccine poliovirus strains,

Table 5. Nucleotide and amino acid substitutions in the vaccine part of the genome of vaccine-derived polioviruses, Guinea, 2014–2015*

Genomic region, nt or aa position	Sabin-2	15-114-C6	14-120	15-078	15-114	15-115	15-136	15-170	15-115-C4	15-115-C5	15-115-C8	15-115-C10	15-136-C1	15-136-C2	Phenotype
5′-UTR															
187	A	G													
202	C	T													
216	T	C													
290	G	A													
398	T	C													
438	A	G													
481	**A**	**G**													Attenuation
653	G	C													
654	G	A													
655	T	C													
696	T	C													
710	A	G													
713	C	T													
718	A	G													
725	G	A													
736	A	G													
VP4															
47	Ala	Thr													
VP3															
73	Ser	Asn													NAg site 3
75	Thr	Ala													NAg site 3
78	Ser	Thr													NAg site 3
VP1															
4	Asp	–	–	Asn	–	–	Ser	–	–	Gly	–	–	Gly	Gly	
10	Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	
19	Val	Ala	–	–	–	Ala	Ala	–	–	–	–	–	Ala	Ala	
23	Ser	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	
24	Thr	Ala	–	–	–	–	–	–	–	–	–	–	–	–	
26	Ser	–	–	Gly	–	–	–	–	–	–	–	–	–	–	
30	Thr	Ile	Ile	–	–	–	Ile	–	–	–	–	–	Ile	Ile	
31	Lys	–	–	–	Arg	Arg	–	Arg	Arg	Arg	Arg	Arg	–	–	
43	Ala	–	–	–	–	–	–	–	–	–	–	Val	–	–	
103	Arg	Lys	–	–	–	–	–	–	–	–	–	–	–	Lys	NAg site 1
143	**Ile**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	**Thr**	Attenuation
171	Asn	–	–	–	–	–	Asp	–	–	–	–	–	Asp	Asp	NAg site 1

*In the 5′-UTR, all nucleotide positions differentiating VDPV 15-114-C6 from Sabin-2 are shown. In the capsid proteins region (VP4–VP1), only amino acid changes are shown. In the VP4-VP3 capsid protein regions, all amino acid positions differentiating VDPV 15-114-C6 from Sabin-2 are shown. For the VP1 capsid protein region, all amino acid positions differentiating all VDPVs from Guinea isolated in this study are shown. NAg, neutralizing antigen; UTR, untranslated region; VDPV, vaccine-derived poliovirus; VP, viral capsid protein. Boldface indicates relevant nucleotide or amino acid sites in the Sabin-2 genome described as being involved in Sabin-2 attenuation (*16*) or those predicted NAg sites important for immune recognition (*18,19*); italics indicate amino acid positions; – indicates no amino acid change; blank cells indicate no sequencing data.

or nonpolio species C enterovirus isolates (Figure 3). No isolates with sequence identity >84% to isolate 2015-114-C6 in the 3′ half of the genome were found in public sequence databases. We looked for circulating enteroviruses in samples from AFP cases that occurred in Guinea during 2013–2015. We found no enteroviruses of species C or D species (data not shown).

Discussion

We isolated and characterized type 2 cVDPV strains during the 2014–2015 outbreak of EVD in Guinea. The origin and spread of the cVDPVs probably resulted from a combination of factors known to be associated with increased risk for cVDPV emergence and spread: insufficient population immunity against poliovirus and lack of high-quality and sensitive AFP surveillance (*3*). The low population immunity against poliovirus in Guinea resulted from low rates of routine OPV coverage and absence of indigenous circulation of the corresponding WPV serotype. According to administrative data, nationwide routine OPV3 coverage for 2014 was low (42%). During the first half of 2015, just before detection of the polio outbreak, routine OPV3 coverage in the affected prefecture of Siguiri was even lower (31%). An additional risk factor was poor

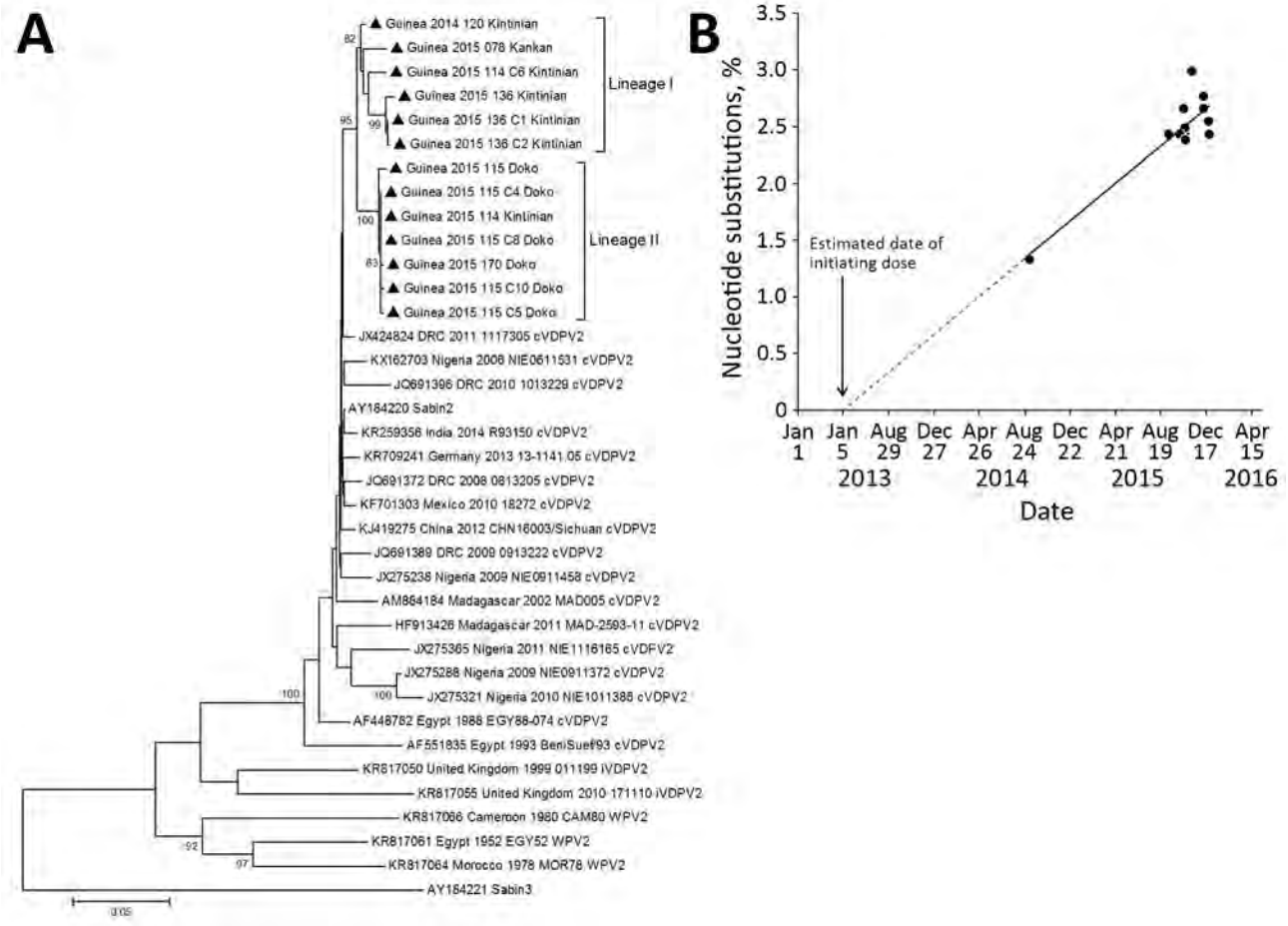

Figure 2. VP1 sequence analysis of cVDPV2 isolated from case-patients (n = 6) and contacts (n = 7) in Guinea, 2014–2015. A) Phylogenetic tree inferred with the complete VP1 region nucleotide sequences (903 bp). Our data were compared with a representative global set of 23 isolates representing type 2 VDPV strains from immunodeficient persons, Sabin-2 strain, cVDPV2s, and wild-type polioviruses identified by GenBank search. The Sabin-3 poliovirus sequence was introduced as an outgroup. The neighbor-joining tree was constructed by using MEGA 5.0 (http://www.megasoftware.net/). Distances were computed by using the Kimura 2-parameter model after excluding positions containing gaps and missing data from the alignments. The robustness of the nodes was tested by 1,000 bootstrap replications. Bootstrap support values >80 are shown in nodes. Triangles indicate the strains from this study. Study strains are indicated by the country, year of isolation, laboratory code, and subprefecture from which isolated. GenBank accession numbers for published sequences are shown in the tree. B) Estimation of the date of initial vaccination with Sabin-2, showing the proportion of VP1 nucleotide changes in the 13 cVDPVs isolates from Guinea with respect to the Sabin-2 reference vaccine strain (AY184220). The data were adjusted to a linear function for the accumulation of nucleotide substitutions ($y = 0.0028*x + 1.372$; $R^2 = 0.80$). Date of initial OPV infection was calculated by extrapolating the line for the evolution rate of nucleotide substitutions backward to 0% substitutions. Scale bar represents nucleotide substitutions per site. cVDPV2, type-2 circulating vaccine-derived poliovirus; OPV, oral polio vaccine; VP, viral capsid protein.

Table 6. Estimates of average evolutionary divergence over type-2 vaccine-derived poliovirus sequence pairs and the Sabin-2 oral poliovirus strain*

Location	nt p-distance value	SE	aa p-distance value	SE
Within lineage 1	0.017	± 0.003	0.009	± 0.004
Within lineage II	0.002	± 0.001	0.003	± 0.002
Between lineages I and II	0.028	± 0.004	0.013	± 0.005
Between lineage I – Sabin 2	0.025	± 0.005	0.021	± 0.007
Between lineage II – Sabin 2	0.024	± 0.005	0.015	± 0.006

*No. base substitutions/site or amino acid (aa) differences from averaging over all sequence pairs within or between each lineage are shown. Analyses were conducted by using the Kimura 2-parameter model and the pairwise distance at the nucleotide (nt) and aa levels, respectively. All positions containing gaps and missing data were eliminated. SEs were obtained by a bootstrap procedure (1,000 replicates).

household sanitation conditions linked to nonhygienic fecal disposal as revealed by our field investigations.

The concurrent EVD outbreak brought several challenges for poliovirus surveillance, vaccination, and diagnosis activities because most public health resources were used to detect and control EVD cases. First, AFP surveillance activities were interrupted from October 2014 through March 2015, just after detection of the first type 2 cVDPV case, which must have favored the undetectable spread of cVDPVs. Second, SIAs were cancelled and the quality of routine OPV vaccination services declined (*21*). Because the type 2 cVDPV detected in 2014 might represent hundreds to thousands of asymptomatic cVDPV infections (*7*), the fact that no follow-up SIAs were conducted might have fueled further spread. Similarly, emergence of other vaccine-preventable diseases, in particular measles, resulting from disruption of vaccination campaigns during the EVD outbreak have also been reported (*21*,*22*). Third, the EVD epidemic affected the processing of samples for polio diagnosis in the laboratory because of

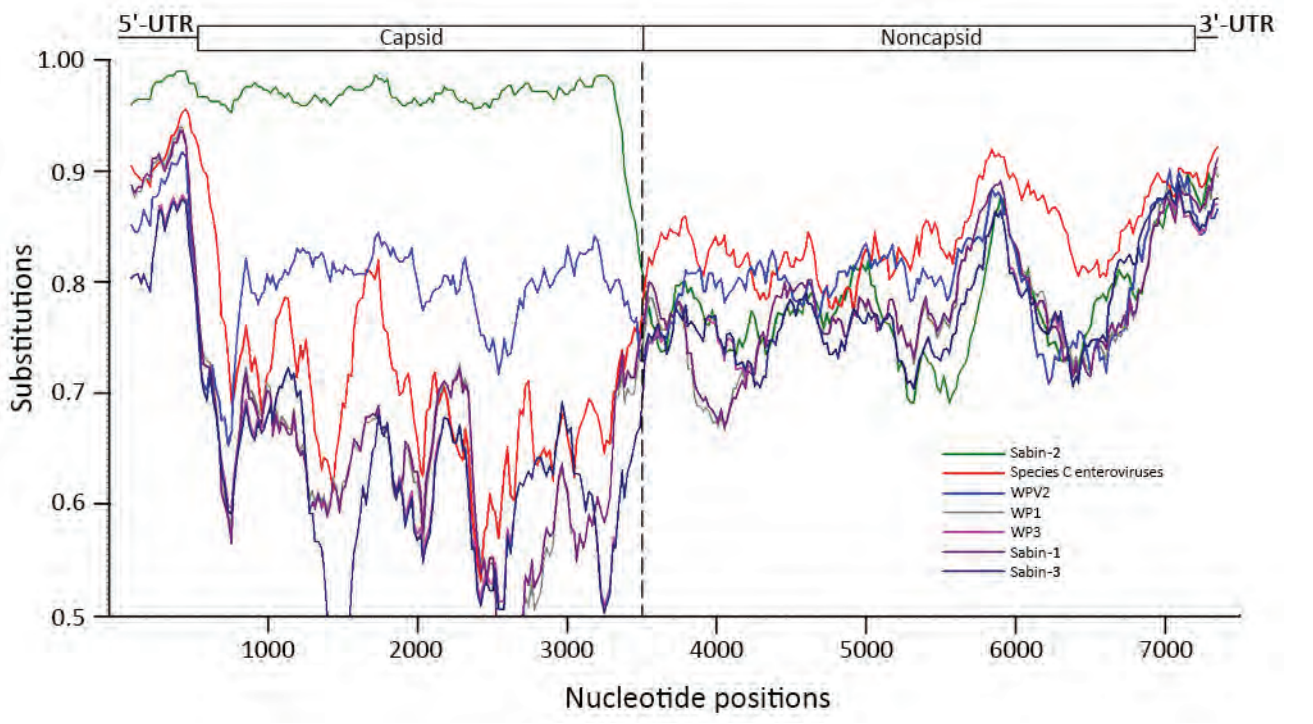

Figure 3. Whole-genome sequence analysis, showing similarity between cVDPV2 isolate 2015-114-C6 (query), 21 prototypes of human enterovirus species C, 3 WPVs, and 3 Sabin poliovirus strains. Approximate nucleotide positions in the poliovirus genome are indicated. The enterovirus genetic map is shown at top. Analyses were calculated by using SimPlot version 3.5 (https://sray.med.som.jhmi.edu/SCRoftware/simplot/). Similarity was calculated in each window of 300 bp by using the Kimura 2-parameter method with a transition:transversion ratio of 2. The window was advanced along the genome alignment in 20-bp increments with 1,000 resamplings. GenBank accession numbers for viruses tested: poliovirus type 1 (V01149), poliovirus type 2 (AY238473 and M12197), poliovirus type 3 (K01392); Sabin-1 (AY184219), Sabin-2 (AY184220), Sabin-3 (AY184221); coxsackie virus 1 (AF499635), coxsackie virus 11 (AF499636), coxsackie virus 13 (AF465511), coxsackie virus 17 (AF499639), coxsackie virus 19 (AF499641), coxsackie virus 20 (AF499642, EF015012, and EF015019), coxsackie virus 21 (AF465515 and D00538), coxsackie virus 22 (AF499643), coxsackie virus 24 (EF026081); enterovirus C96 (HQ415759), enterovirus C99 (EF555644), enterovirus C102 (EF555645), enterovirus C104 (JX982259), enterovirus C105 (KM880098), enterovirus C109 (GQ865517), enterovirus C117 (JX262382), enterovirus C116 (JX514942), and enterovirus C118 (JX961709). cVDPV2, type 2 circulating vaccine-derived poliovirus; UTR, untranslated region; WPV, wild-type poliovirus.

increased biosafety requirements. Samples were first tested for Ebola virus in a mobile laboratory in Conakry and then sent in batches of hundreds to the WHO Polio Reference Laboratory in Dakar. The shipping and processing of samples at 2 laboratories meant an exceptional workload in both laboratories and a shortage of supplies for diagnosis. These challenges for polio surveillance, vaccination, and diagnosis argue for the development of contingency plans during complex emergencies to ensure completion and maintenance of global polio eradication.

Vaccination activities conducted in 2015 and 2016 most likely contributed to the disruption of VDPV transmission because, despite an intensive search, no polio cases were detected in Guinea after December 2015. However, the country remains vulnerable to polio importation because gaps in AFP surveillance continue to occur in some areas and routine OPV coverage recorded for 2015 (87%) and 2016 (86%) remained suboptimal.

The area where the outbreak occurred is a gold mining region. Field investigations revealed that all parents of the investigated case-patients were gold miners or panners. There is a probable epidemiologic link between cases because of the frequent migratory movements in these gold mining areas, especially in the subprefectures of Kintinian and Doko. Gold panning sites are areas of high risk because of precarious living conditions; attention should be focused on strengthening and prioritizing vaccination efforts (routine and SIAs) in these specific populations. Field investigations also revealed hesitancy by some families to vaccinate and reluctance of some contacts' families to collect fecal samples from their children. These findings call for social mobilization efforts to increase vaccination trust and polio surveillance adherence in these communities.

We found that the clinical characteristics of VDPV infections were similar to those associated with WPV infection. Available clinical data showed that all cVDPV case-patients had residual paralysis after 60 days, typical of poliomyelitis.

Genetic analysis of cVDPVs isolates was consistent with the 2 most serious biological properties of cVDPVs: capacity for sustained person-to-person transmission and capacity to cause paralytic disease in humans. Effectively, the findings that the VDPV isolates had high genetic divergence from the Sabin-2 vaccine strain in the VP1 coding region (1.3%–3% nt differences) and a vaccine/nonvaccine recombinant genome are indications of sustained circulation of VDPVs. Moreover, our finding that the 2 key genetic determinants of the attenuated phenotype of the Sabin-2 strain (A481G in the 5′-UTR and I143T in the VP1 [*17*,*23*]) have reverted points to the cause of the paralysis. Recombination of vaccine strains with other species C enteroviruses and replacement of the 2 key attenuating substitutions of the Sabin-2 strain are features frequently seen among type 2 VDPVs reported in previous outbreaks (*18*,*24*–*27*).

Phylogenetic analysis of VP1 sequences suggests that all type-2 cVDPV strains diverged from a common OPV precursor and circulated along 2 chains of transmission that gave rise to 2 descending lineages. Each lineage was restricted to a limited geographic region, Kintinian or Doko. Our results suggest that viruses from lineage II, found in Doko, might have been derived from strain 2015-114 (or a closely related sibling), which was the only strain from Kintinian classified in lineage II. Given the low nationwide rate of routine vaccination coverage, it is surprising that the outbreak was geographically confined to the region of Kankan. This localization could be the result of limited movement of populations. Without a developed long-range transportation network, individual geographic regions tend to be isolated, limiting the movement of human populations and thus restricting widespread virus transmission (*28*). Alternatively, the absence of widespread circulation may also indicate that VDPV strains are less transmissible than WPV.

In conclusion, epidemiologic and molecular data show that the outbreak VDPV strain circulated since 2013 in Guinea in an area of low vaccination coverage and substantial gaps in population immunity at a critical time in the polio endgame. This finding reiterates the need to enhance vaccination activities focused on risk groups to maintain high OPV coverage and strengthen routine vaccination and sensitive AFP surveillance systems to ensure the quick detection of cVDPV emergence. Our study shows how diversion of public health resources to the EVD outbreak in Guinea may have contributed to the spread of neurovirulent VDPVs. This finding argues strongly for enhancement of polio surveillance and vaccination efforts in other EVD-affected countries, such as neighboring Liberia and Sierra Leone, where vaccination systems have been weakened and where active AFP surveillance has only recently started (*29*). The fact that this outbreak occurred just before the withdrawal of type 2 from OPV adds significance because there was a particular urgency for stopping type 2 VDPV transmission before the withdrawal target date. In this context, as shown in our study, effective and sensitive surveillance for polio, as well as a good understanding of the extent and natural history of the outbreak, have proven critical for designing the best public health response.

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Characterization of a Feline Influenza A(H7N2) Virus

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During December 2016–February 2017, influenza A viruses of the H7N2 subtype infected ≈500 cats in animal shelters in New York, NY, USA, indicating virus transmission among cats. A veterinarian who treated the animals also became infected with feline influenza A(H7N2) virus and experienced respiratory symptoms. To understand the pathogenicity and transmissibility of these feline H7N2 viruses in mammals, we characterized them in vitro and in vivo. Feline H7N2 subtype viruses replicated in the respiratory organs of mice, ferrets, and cats without causing severe lesions. Direct contact transmission of feline H7N2 subtype viruses was detected in ferrets and cats; in cats, exposed animals were also infected via respiratory droplet transmission. These results suggest that the feline H7N2 subtype viruses could spread among cats and also infect humans. Outbreaks of the feline H7N2 viruses could, therefore, pose a risk to public health.

Influenza A viruses are endemic in humans and enzootic in other mammalian species including swine and horses; occasional infections of other mammalian species including whales, seals, sea lions, felidae in zoos, and other species have been reported (*1*). Reports of influenza A virus infections in dogs and cats were rare until 2004, when equine influenza viruses of the H3N8 subtype caused outbreaks in greyhounds in Florida (*2*). Since then, influenza viruses of the H3N8 and H3N2 subtypes have caused several outbreaks in dogs in the United States and South Korea (*3*–*5*).

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Until recently, only 1 major influenza A virus outbreak had been reported in cats (*6*). This changed in December 2016 with the outbreak of low pathogenic avian influenza A viruses of the H7N2 subtype in animal shelters in New York. Approximately 500 cats were infected in December 2016–February 2017; most of which experienced a mild illness with coughing, sneezing, and runny nose from which they recovered fully. Severe pneumonia developed in 1 elderly animal with underlying health issues, which was euthanized. A veterinarian who had treated an infected animal also became infected with the feline influenza A(H7N2) virus and experienced a mild, transient illness, suggesting the potential for these viruses to infect humans. While this manuscript was being prepared, Belser et al. reported that the H7N2 subtype virus isolated from the human case caused a mild disease in mice and ferrets, but was not transmitted among ferrets (*7*). We assessed feline H7N2 subtype viruses isolated from infected cats during the outbreak for their replicative ability, pathogenicity, and transmissibility in mammals; in contrast to the findings recently published by Belser et al. (*7*), we detected productive infection of co-housed ferrets, although with low efficiency. We also conducted extensive pathology and transmission studies in cats, and detected feline virus transmission via respiratory droplets to exposed cats. Our study provides additional data on the risk that the feline H7N2 subtype viruses pose to public health.

Methods

Cells and Viruses

The origins and growth conditions of all cell lines used in this study are described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/1/17-1240-Techapp1.pdf). The feline H7N2 subtype viruses used in this study were isolated from swabs collected from cats with influenza-like symptoms during the outbreak in an animal shelter in New York in December 2016. We obtained A/chicken/New York/22409–4/1999 (H7N2, A/chicken/

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NY/99) virus from the Agricultural Research Service, US Department of Agriculture (*8*). We deposited the viral gene sequences obtained in this study to GenBank. We amplified the feline virus in Madin-Darby canine kidney (MDCK) cells and the A/chicken/NY/99 virus in 10-day-old embryonated chicken eggs.

Growth Kinetics of Viruses in Cell Culture

We infected cells with viruses at a 0.005 multiplicity of infection, incubated them for 1 hour at 37°C, washed twice, and cultured with 1× minimal essential medium containing 0.3% bovine serum albumin and trypsin treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone at 33°C and 37°C (37°C and 39°C for chicken embryo fibroblast cells) for various periods. We determined virus titers at the indicated time points by use of plaque assays in MDCK cells. The statistical analyses are described in the online Technical Appendix.

Infection of Animals

To determine the pathogenicity of the viruses in infected mice, we anesthetized three 6-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) for each virus with isoflurane and inoculated intranasally with 10-fold serially diluted virus in a 50-μL volume. The mice were monitored daily for 14 days and checked for changes in body weight and morbidity and mortality. We euthanized animals if they lost more than 25% of their initial bodyweight.

To determine the pathogenicity of the viruses in infected ferrets and cats, we inoculated 6-month-old female ferrets (Triple F Farms, Sayre, PA, USA; 3 per group; serologically negative by hemagglutination inhibition assay for currently circulating human influenza viruses), and unvaccinated 4- to 5-month-old female specific-pathogen-free cats (Liberty Research, Waverly, NY, USA; 3 per group) intranasally with 10^6 PFU of viruses in 0.5 ml of phosphate-buffered saline. We monitored the animals daily for changes in bodyweight, body temperature, and clinical signs for 14 days.

For virus replication in organs and pathology analyses, we worked with groups of mice (12 per group), ferrets (6 per group), and cats (6 per group).We inoculated the animals intranasally with 10^5 PFU (mice) in 0.05 ml of phosphate-buffered saline or 10^6 PFU (ferrets and cats) of viruses in 0.5 ml of phosphate-buffered saline. On days 3 and 6 postinfection, we euthanized 6 mice, 3 ferrets, and 3 cats in each group for pathological analysis and virus titration in organs (by use of plaque assays in MDCK cells).

Virus Transmission Studies in Ferrets and Cats

For direct contact transmission experiments, we housed 3 ferrets per group in regular ferret cages and 3 cats per group in large dog transporter cages (online Technical Appendix Figure 1), and infected them intranasally with 10^6 PFU (500 μL) of viruses. One day later, we housed 1 virus-naive animal with each infected animal. We collected nasal washes from the infected ferrets and nasal swabs from the infected cats on day 1 after infection, and from the exposed animals on day 1 after exposure and then every other day (for up to 11 days). We determined virus titers in the nasal washes and swabs by performing plaque assays in MDCK cells. We monitored all animals daily for disease symptoms and changes in bodyweight and temperature for 14 days.

We performed airborne transmission experiments by using ferret isolators (Showa Science, Tokyo, Japan) (*9*–*11*) or regular cat cages. In these settings, there was no directional airflow from the infected to the exposed animals. We inoculated 3 animals per group intranasally with 10^6 PFU (500 μL) of viruses. One day after infection, we placed 3 immunologically naive animals (exposed animals) each in a cage adjacent to an infected animal. This setting prevented direct and indirect contact between animals but allowed spread of influenza virus by respiratory droplet. We spaced the ferret cages 5 cm apart and the cat cages 35 cm apart. We monitored the animals and assessed virus titers as described above.

Results

Genetic and Phylogenetic Analysis of Feline Influenza(H7N2) Viruses Isolated in Animal Shelters in New York, December 2016

We obtained swabs (collected on the same day) from 5 cats that experienced influenza-like symptoms during the outbreak at an animal shelter in New York, NY, in December 2016. After inoculation of these samples into MDCK cells, we isolated 5 pleomorphic influenza A viruses of the H7N2 subtype (Table 1; online Technical Appendix Figure 2). The HA consensus sequences of the 5 isolates (established by Sanger sequence analysis) displayed >99.9% similarity at the nucleotide level (Table 1). Phylogenetic analyses demonstrated that the 8 viral RNA segments of the 5 feline H7N2 viruses are most closely related to poultry influenza A(H7N2) viruses detected in the New York area in the late 1990s through early 2000s (Figure 1; online Technical Appendix Figures 3–9), suggesting that the 2016 feline H7N2 virus isolates descended from viruses that circulated more than a decade ago in the northeastern United States.

The HA protein of the 2016 feline H7N2 subtype virus encodes a single arginine residue at the hemagglutinin cleavage site (PEKPKPR↓G; the arrow indicates the cleavage site that creates the HA1 and HA2 subunits), indicative of low pathogenicity in chickens. Antigenically, A/feline/New York/WVDL-14/2016 (A/feline/NY/16) differs from other, closely related H7 viruses (online Technical Appendix Table 1); for example, its HA deviates by 27 aa from the closely related A/chicken/NY/22409–4/1999 HA. The

Table 1. Amino acid differences among feline influenza A(H7N2) virus isolates, New York, NY, USA*

	Amino acid positions in the viral proteins					
	PB2	PB1-F2	PA	NA		NS2
Virus	448	42	57	40	62	74
A/feline/New York/WVDL-3/2016	**S**	**C**	**Q**	**Y**	**C**	D
A/feline/New York/WVDL-9/2016	N	Y	R	H	**C**	**E**
A/feline/New York/WVDL-14/2016	**S**	**C**	**Q**	**Y**	**C**	**E**
A/feline/New York/WVDL-16/2016	**S**	**C**	**Q**	**Y**	F	**E**
A/feline/New York/WVDL-20/2016	**S**	**C**	**Q**	**Y**	**C**	D

*Consensus sequences among the 5 H7N2 subtype viruses are shown in bold. Amino acids: C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; H, histidine; L, leucine; N, asparagine; Q, glutamine; R, arginine; S, serine; Y, tyrosine. Viral proteins: NA, neuraminidase; NS, nonstructural protein; PA, polymerase acidic; PB, polymerase basic.

neuraminidase (NA) and ion channel (M2) proteins of the H7N2 viruses do not encode amino acids that confer resistance to neuraminidase or ion channel inhibitors. Inspection of the remaining feline H7N2 viral proteins revealed an absence of the most prominent amino acid changes known to facilitate adaptation to mammals, such as PB2–627K (*16*). These data thus suggest the 2016 feline H7N2 subtype viruses are avian-derived influenza viruses of low pathogenicity in avian and mammalian species.

Replication of Feline and Avian H7N2 Subtype Viruses in Cultured Cells

To characterize the replicative ability of the 2016 feline H7N2 viruses in cultured cells, we compared A/feline/NY/16 (which encodes the consensus amino acid sequence of the 5 isolates) with a closely related 1999 avian influenza virus, A/chicken/NY/22409–4/1999 (H7N2, A/chicken/NY/99) (Figure 1; online Technical Appendix Figures 3–9), which was isolated from a chicken in a live-bird market in New York state in 1999 (*8*). There are a total of 97 aa differences between A/feline/NY/16 and A/chicken/NY/99 viruses (12 aa differences in polymerase basic 2 (PB2), 7 in polymerase basic 1 (PB1), 12 in polymerase acidic (PA), 27 in hemagglutinin (HA), 8 in nucleoprotein (NP), 11 in neuraminidase (NA), 7 in matrix protein 1 (M1), 4 in matrix protein 2 (M2), and 9 in nonstructural protein 1 (NS1). Canine, human, feline, and chicken cells were infected at a multiplicity of infection of 0.005 at temperatures mimicking those of the upper and lower respiratory tract of the respective species (i.e., 37°C and 39°C for chicken cells; 33°C and 37°C for the remaining cells) (Figure 2). In canine MDCK, feline Clone81, and human Calu-3 cells, A/feline/NY/16 replicated at least as efficiently as A/chicken/NY/99 virus, while both viruses replicated to low titers in human A549 cells. Of note, A/feline/NY/16 virus replicated less efficiently than A/chicken/NY/99 virus in feline lung Fc2Lu cells. In chicken embryo fibroblast cells, A/feline/NY/16 virus replicated more slowly than A/chicken/NY/99 virus at early time points and reached its highest titers at later time points. When we compared virus growth at the 2 temperatures tested (i.e., 37°C and 39°C for chicken cells; 33°C and 37°C for the remaining cells), we observed similar trends (for example, in MDCK cells, A/feline/NY/16 replicated more efficiently than A/chicken/NY/99 at both temperatures tested).

Replication and Pathogenicity of Feline and Avian H7N2 Subtype Viruses in Mice

To assess the replication of A/feline/NY/16 and A/chicken/NY/99 viruses in mice, 3 mice per group were inoculated intranasally with 10-fold dilutions of viruses, and their bodyweight and morbidity and mortality were monitored daily for 14 days. Mice infected with A/feline/NY/16 virus did not experience weight loss or signs of disease, whereas infection with 10^6 PFU of A/chicken/NY/99 virus caused severe weight loss and required euthanasia (online Technical Appendix Figure 10).

A/feline/NY/16 replicated efficiently in the nasal turbinates and less efficiently in the lungs of infected animals (online Technical Appendix Figure 11); no virus was isolated from the other organs tested (i.e., brains, kidneys, livers, and spleens; data not shown). A/chicken/NY/99 replicated more efficiently in the lungs than in the nasal turbinates, consistent with immunohistochemistry analyses that detected A/feline/NY/16 virus antigens mainly in the upper respiratory organs of infected mice, whereas A/chicken/NY/99 virus antigens were detected more frequently in the lower respiratory organs (online Technical Appendix Figure 12).

Replication and Pathogenicity of Feline and Avian H7N2 Subtype Viruses in Ferrets

Ferrets intranasally infected with 10^6 PFU of A/feline/NY/16 or A/chicken/NY/99 virus did not lose bodyweight (online Technical Appendix Figure 13) but 2 of the ferrets infected with A/chicken/NY/99 virus had high fevers on day 1 postinfection. Both viruses replicated efficiently in the nasal turbinates and were also isolated from the trachea and lungs of some animals (Table 2), consistent with similar antigen distributions for both viruses (online Technical Appendix Figure 14). No viruses were isolated from any of the other organs tested.

Replication and Pathogenicity of Feline and Avian H7N2 Subtype Viruses in Cats

The infection of ≈500 cats with H7N2 subtype viruses in animal shelters in New York in December 2016 suggested

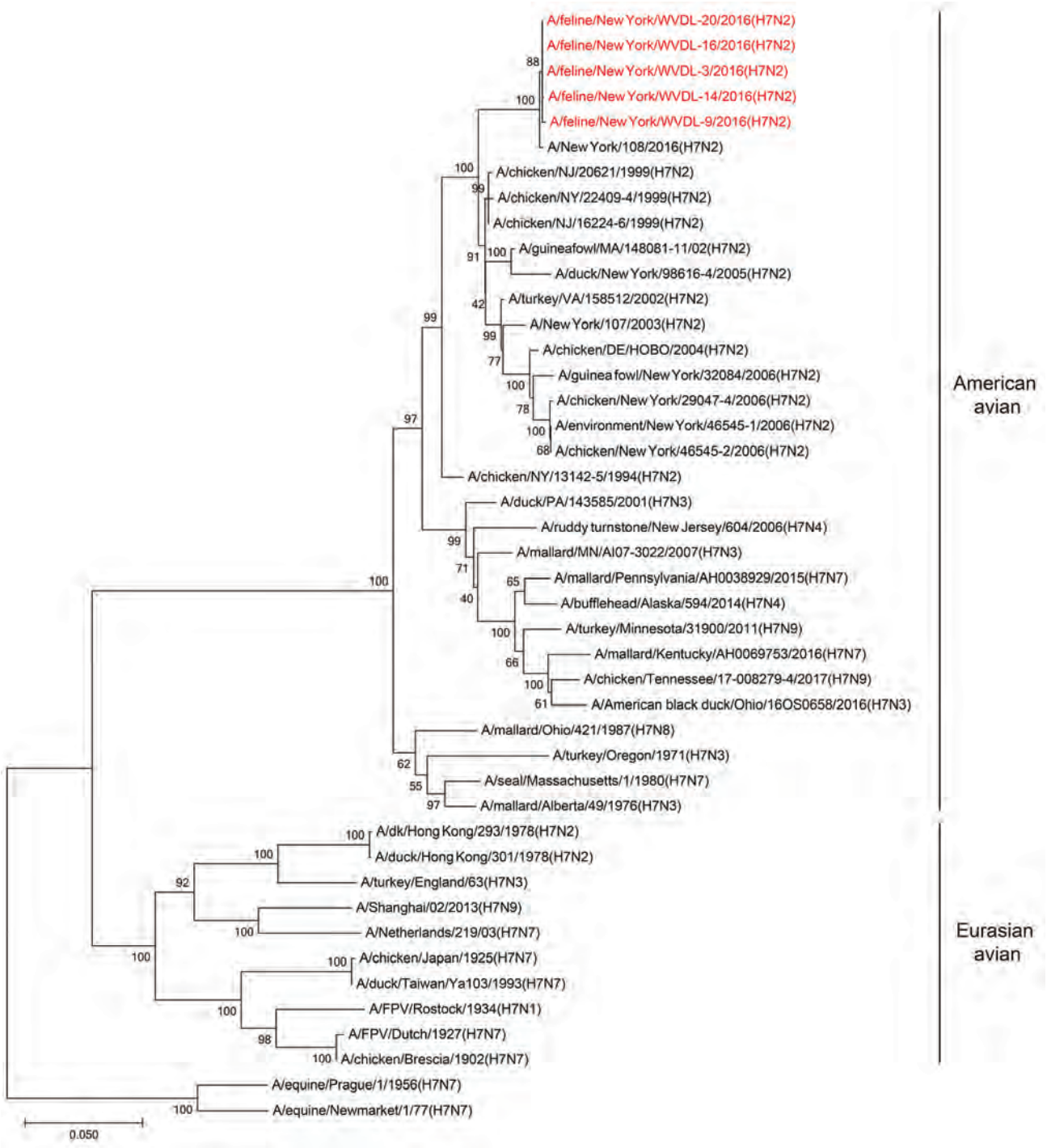

Figure 1. Phylogenetic tree of influenza A viral hemagglutin segments from New York, NY, USA, compared with reference viruses. Phylogenetic analysis was performed for selected influenza A viruses representing major lineages. The evolutionary history was inferred using the neighbor-joining method (*12*). The optimal tree with the branch length sum of 1.22521320 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (*13*). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method (*14*) and are in the units of the number of base substitutions per site. The analysis involved 44 nt sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. The final dataset contained a total of 1,612 positions. Evolutionary analyses were conducted in MEGA7 (*15*).

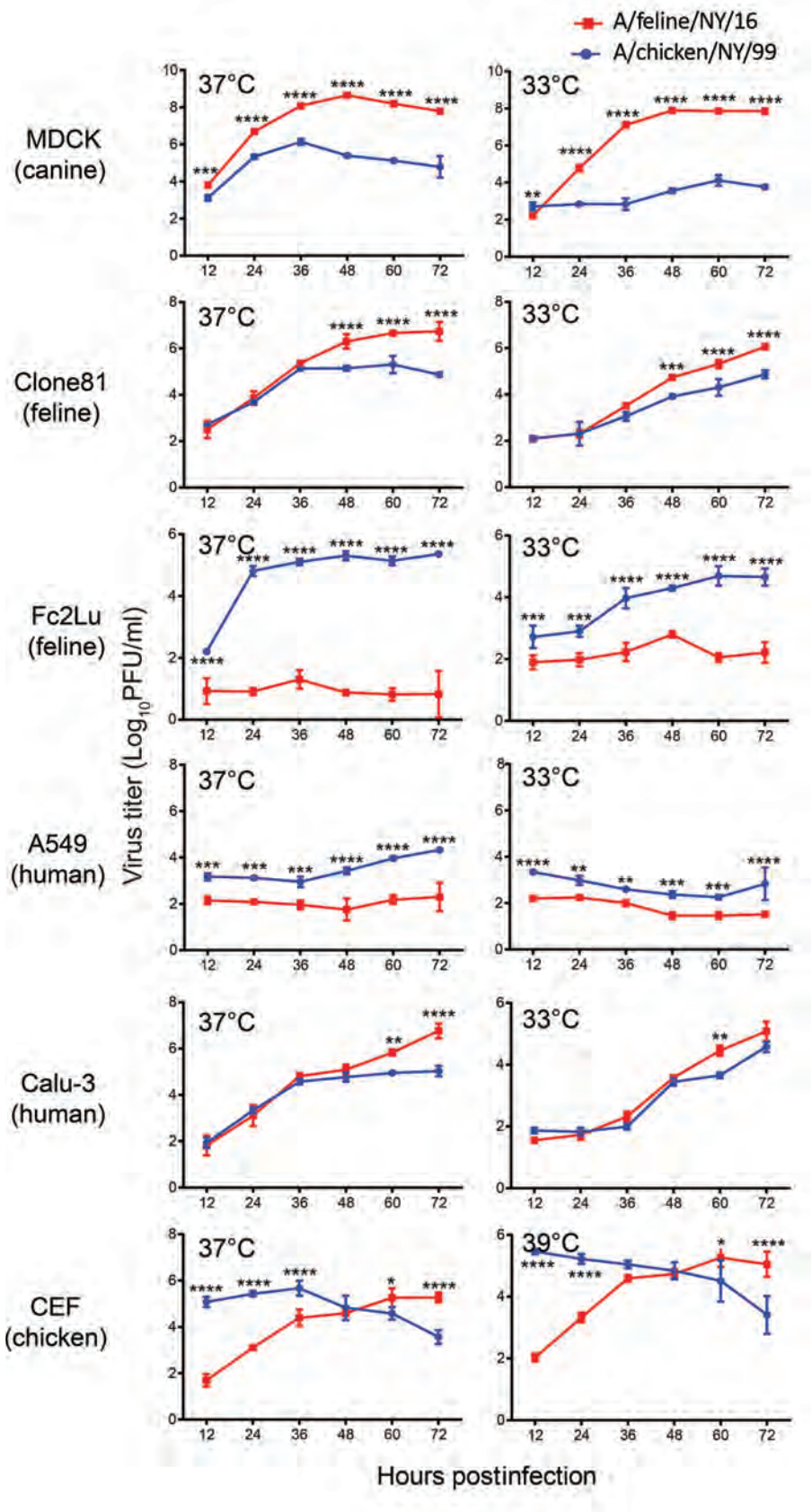

Figure 2. Growth properties of A/feline/NY/16 and A/chicken/NY/99 influenza A(H7N2) viruses in mammalian and avian cells at different temperatures, New York, NY, USA. Cells were infected with viruses at a multiplicity of infection of 0.005 and incubated at 33°C and 37°C (or at 37°C and 39°C for avian CEF cells). Supernatants were harvested at the indicated time points. Virus titers were determined by use of plaque assays in Madin-Darby canine kidney (MDCK) cells. The species from which the cell lines are derived are shown. The values presented are the averages of 3 independent experiments ±SD. Statistical significance was determined as described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/1/17-1240-Techapp1.pdf). *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. A549, human lung carcinoma epithelial cells; Clone81, cat kidney fibroblast cells; Fc2Lu, cat lung cells; CEF, chicken embryo fibroblast cells.

Table 2. Virus titers in organs of ferrets and cats infected with A/feline/NY/16 or A/chicken/NY/99 influenza A(H7N2) viruses, New York, NY, USA*

Species and virus	Days postinfection	Animal ID no.	Virus titers in organs of infected animals, log_{10} PFU/g					
			Nasal turbinates	Trachea	Lung	Small intestine	Colon	Other organs†
Ferret								
A/feline/NY/16	3	1	4.4	3.3	4.7	–	–	–
		2	5.2	–	2.4	–	–	–
		3	5.4	–	–	–	–	–
	6	4	3.1	–	–	–	–	–
		5	5.8	–	–	–	–	–
		6	6.0	–	–	–	–	–
A/chicken/NY/99	3	7	5.9	3.3	–	–	–	–
		8	6.0	–	–	–	–	–
		9	6.6	3.4	–	–	–	–
	6	10	4.2	–	–	–	–	–
		11	4.5	–	5.7	–	–	–
		12	4.4	–	–	–	–	–
Cat								
A/feline/NY/16	3	1	3.9	4.8	–	–	–	–
		2	4.1	6.6	5.8	–	–	–
		3	6.9	7.0	5.8	–	–	–
	6	4	6.3	6.2	6.1	–	2.3	–
		5	7.7	7.8	4.7	–	–	–
		6	5.9	6.2	6.7	3.8	–	–
A/chicken/NY/99	3	7	6.4	5.8	3.9	–	–	–
		8	2.0	–	–	–	–	–
		9	6.1	–	–	4.9	–	–
	6	10	6.4	–	5.0	–	–	–
		11	4.6	–	–	–	–	–
		12	6.7	4.0	–	–	–	–

*–, no virus detected.
†Brain, spleen, kidneys, liver, and pancreas.

efficient replication of these viruses in felines. However, it was unclear whether these viruses were restricted to the respiratory organs or caused systemic infection. Cats intranasally infected with 10^6 PFU of A/feline/NY/16 or A/chicken/NY/99 did not lose bodyweight (Figure 3); however, fever was detected in 1 animal infected with A/feline/NY/16, and 1 infected with A/chicken/NY/99 virus; and a different animal infected with A/feline/NY/16 sneezed intensely on day 3 postinfection, but recovered fully.

A/feline/NY/16 virus replicated efficiently in the nasal turbinates, trachea, and lungs of infected cats (with the exception of 1 cat with a virus-negative lung sample on day 3 postinfection; Table 2). We isolated A/chicken/NY/99 virus mostly from nasal turbinates, with limited replication in the trachea and lung. These findings are consistent with the detection of A/feline/NY/16 antigen in both the upper and lower respiratory organs of infected cats, whereas A/chicken/NY/99 antigen was detected mainly in the nasal turbinates (Figure 4). A/feline/NY/16 and A/chicken/NY/99 viruses were also isolated from the jejunum or colon of some of the infected animals (Table 2), although viral antigen was not detected in the intestines of cats infected with A/chicken/NY/99 or A/feline/NY/16 virus. These results demonstrate that the feline H7N2 virus replicates efficiently in the upper and lower respiratory tract of cats, reflecting adaptation of the virus to its new host.

All cats infected with the A/feline/NY/16 virus exhibited histologic lesions in their nasal turbinates, tracheas, and lungs. Nasal turbinate pathology was moderate to severe in 5 of 6 cats with multifocal to diffuse distribution of lesions (Figure 5, panel A). The tracheas of these cats exhibited mild to moderate histopathology (Figure 5, panel B), whereas the lungs exhibited multifocal to coalescing histopathology centered mostly on the bronchioles, with 3 of 6 cats possessing moderately severe lesions (Figure 5, panel C). Similar histopathological changes were found in cats infected with A/chicken/NY/99 virus. Appreciable histopathology was also noted in the small intestine (duodenum) of cats infected with A/feline/NY/16 and A/chicken/NY/99 viruses (Figure 5, panel D; cat ID nos. 1, 2, 4, 8, 10, and 12 in Table 2), although virus was detected in the intestines of only 3 cats (cat ID nos. 4, 6, and 9 in Table 2). The correlation between virus replication and histologic lesions in cat intestines is currently unknown.

Transmission of Feline and Avian H7N2 Subtype Viruses in Ferrets and Cats

The fulminant spread of the feline H7N2 subtype viruses among cats, and the confirmed H7N2 virus infection of a veterinarian who treated the animals, indicate that these originally avian influenza viruses have the ability to transmit among mammals. To test the transmissibility of feline and

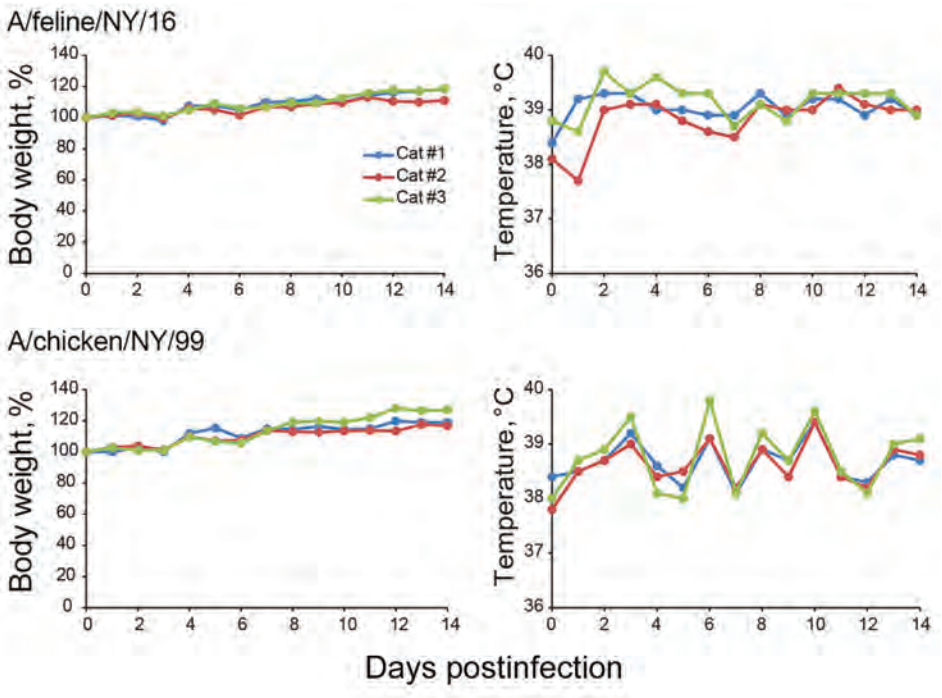

Figure 3. Body weight and temperature changes in cats infected with A/feline/NY/16 and A/chicken/NY/99 influenza A(H7N2) viruses, New York, NY, USA. Three cats per group were infected intranasally with 10^6 PFU of viruses and monitored for bodyweight and temperature changes.

avian H7N2 subtype viruses in ferrets, 3 animals per group (each placed in a separate cage) were infected intranasally with 10^6 PFU (500 μL) of A/feline/NY/16 or A/chicken/NY/99 virus. One day later, we housed 1 naive ferret with each of the infected ferrets (direct contact transmission experiment), or placed naive ferrets in wireframe cages (within

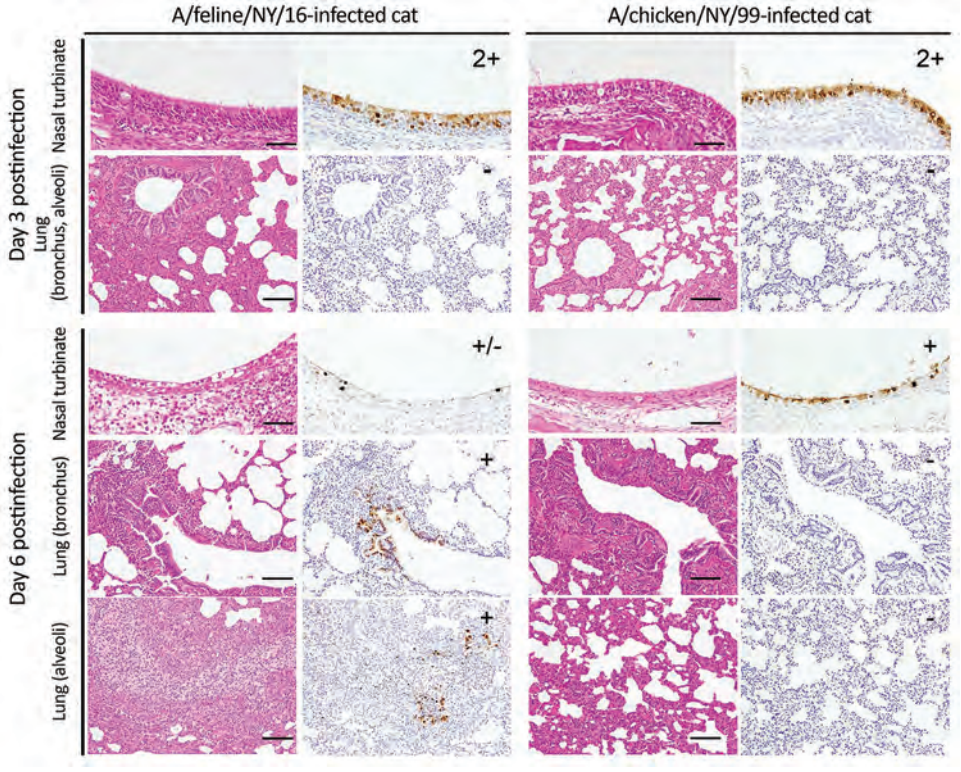

Figure 4. Immunohistochemistry findings in cats infected with influenza A(H7N2) virus, New York, NY, USA. Shown are representative sections of nasal turbinates and lungs of cats infected with the indicated viruses on days 3 and 6 postinfection. Three cats per group were infected intranasally with 10^6 PFU of virus, and tissues were collected on days 3 and 6 postinfection. Type A influenza virus nucleoprotein (NP) was detected by a mouse monoclonal antibody to this protein. For nasal turbinate sections: –, no NP-positive cells; +/–, NP-positive cells detected in 1–2 focal regions; +, NP-positive cells detected in >2 focal regions; 2+, NP-positive cells in large regions. For bronchus and alveolar sections: –, no NP-positive cells; +/–, ≥5 NP-positive cells; +, ≥6 NP-positive cells. NP-positive cells were detected in focal, but not in diffuse bronchial and alveolar sections. For all analyses, the entire sections were evaluated. Scale bars indicate 50 μm (nasal turbinates) or 100 μm (lung).

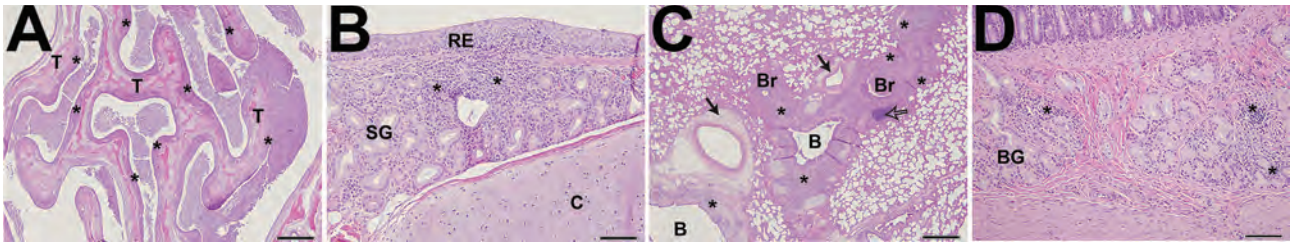

Figure 5. Pathology findings in cats infected with A/feline/NY/16 influenza A(H7N2) virus on day 6 postinfection, New York, NY, USA. A) In lungs, moderately severe histopathologic changes are present in the lower airways. The lamina propria of bronchi (B) and bronchioles (Br) and the surrounding interstitium are infiltrated by numerous histiocytes, lymphocytes, and plasma cells (*), which also extend into and expand neighboring alveolar septa. The infiltrates extend into and expand nearby alveolar septa. The lumina of bronchioles are filled with numerous foamy macrophages, viable and degenerating neutrophils, proteinaceous fluid, and sloughed respiratory epithelial cells. Hyperplasia of bronchiole-associated lymphoid tissue (open arrow) and perivascular edema (solid arrow) are present. Scale bar indicates 500 μm. B) In nasal cavities, copious amounts of exudate are present comprising numerous degenerating and necrotic neutrophils, cellular debris, proteinaceous fluid, and strands of mucin. The respiratory epithelium covering the nasal turbinates (T) is extensively eroded. The underlying lamina propria appears diffusely bluish-purple due to infiltration by moderate-to-large numbers of histiocytes, neutrophils, lymphocytes, and plasma cells (*). Scale bar indicates 500 μm. C) In the trachea, a locally extensive focus of inflammation is present in the tracheal wall. Moderate numbers of histiocytes, lymphocytes, and plasma cells, and a few neutrophils, infiltrate the respiratory epithelium (RE), lamina propria, and submucosa. Submucosal glands (SG) are surrounded by the inflammatory infiltrates and effaced in the areas of heaviest infiltration (*). Tracheal cartilage (C). Scale bar indicates 100 μm. D) In the duodenum, inflammatory cell infiltrates (*) in the submucosa of the duodenum are present between and around Brunner's glands (BG). Scale bar indicates 100 μm.

transmission isolators) ≈5 cm from the cages containing the infected ferrets as a respiratory droplet transmission experiment. We collected nasal wash samples from infected, contact, and exposed animals on day 1 after infection, contact, or exposure, and then every other day; we determined virus titers in nasal wash samples by use of plaque assays in MDCK cells (Table 3). In respiratory droplet transmission experiments, ferrets infected with A/feline/NY/16 or A/chicken/NY/99 virus secreted virus, but exposed animals were virus negative and did not seroconvert (Table 3). Among the direct contact animals, we detected virus in 1 ferret from the A/feline/NY/16-inoculated group and 2 from the A/chicken/NY/99-inoculated group; these 3 animals seroconverted, although the HI titer of 1 of the animals was low.

Table 3. Influenza A(H7N2) virus titers in nasal wash samples from ferret transmission studies, New York, NY, USA*

Virus and transmission mode	Pair	Action	Virus titers in nasal wash samples by days after infection, exposure, or contact, log_{10} PFU/mL						Seroconversion, HI titer†
			1	3	5	7	9	11	
A/feline/NY/16									
Respiratory droplets	1	Infection	4.2	5.6	5.0	–	–	–	320
		Exposure	–	–	–	–	–	–	<10
	2	Infection	4.6	4.3	5.4	–	–	–	320
		Exposure	–	–	–	–	–	–	<10
	3	Infection	5.3	5.0	4.8	2.8	–	–	640
		Exposure	–	–	–	–	–	–	<10
Direct contact	1	Infection	3.6	4.1	5.0	2.0	–	–	640
		Contact	–	–	–	–	–	–	<10
	2	Infection	5.5	5.1	4.3	1.3	–	–	320
		Contact	–	–	–	–	–	–	<10
	3	Infection	5.0	5.2	5.2	2.9	–	–	640
		Contact	–	–	4.2	5.3	4.6	–	320
A/chicken/NY/99									
Respiratory droplets	1	Infection	5.8	4.0	4.3	–	–	–	160
		Exposure	–	–	–	–	–	–	<10
	2	Infection	5.6	4.2	3.5	–	–	–	160
		Exposure	–	–	–	–	–	–	<10
	3	Infection	5.1	3.7	3.5	–	–	–	320
		Exposure	–	–	–	–	–	–	<10
Direct contact	1	Infection	4.3	4.3	3.0	–	–	–	160
		Contact	–	–	3.8	4.3	3.4	–	160
	2	Infection	4.2	3.8	3.8	–	–	–	160
		Contact	–	–	2.1	–	–	–	10
	3	Infection	4.9	3.9	4.3	–	–	–	320
		Contact	–	–	–	–	–	–	10

*HI, hemagglutination inhibition; –, no virus detected.
†Serum specimens were collected on day 18 after infection, exposure, or contact, and examined using an HI assay. The HI titer is the inverse of the highest dilution of serum that completely inhibited hemagglutination.

Table 4. Influenza A(H7N2) virus titers in nasal swab samples from cat transmission studies, New York, NY, USA*

Virus and transmission mode	Pair	Action	Virus titers in nasal swab samples by days after infection, exposure, or contact, log_{10} PFU/mL							Seroconversion, HI titer†
			1	3	5	7	9	11	13	
A/feline/NY/16										
Respiratory droplets	1	Infection	5.6	4.7	4.3	3.0	–	–	–	320
		Exposure	–	–	–	–	5.2	–	–	160
	2	Infection	4.5	2.7	5.0	4.6	–	–	–	320
		Exposure	–	–	–	–	–	5.4	–	80
	3	Infection	4.8	3.2	5.3	3.6	–	–	–	320
		Exposure	–	–	–	–	–	–	–	<10
Direct contact	1	Infection	5.9	4.6	3.4	–	–	–	–	320
		Contact	–	–	2.0	5.4	–	–	–	320
	2	Infection	6.0	5.0	4.6	–	–	–	–	640
		Contact	–	–	–	–	–	–	–	80
	3	Infection	4.9	4.9	4.4	4.2	–	–	–	640
		Contact	–	5.2	5.4	5.2	–	–	–	160
A/chicken/NY/99										
Respiratory droplets	1	Infection	4.0	3.7	4.7	–	–	–	–	320
		Exposure	–	–	–	–	–	–	–	20
	2	Infection	–	–	–	–	–	–	–	320
		Exposure	–	–	–	–	–	–	–	20
	3	Infection	2.6	1.6	2.3	–	–	–	–	80
		Exposure	–	–	–	–	–	–	–	160
Direct contact	1	Infection	4.5	2.4	4.5	–	–	–	–	80
		Contact	–	–	–	–	–	–	–	160
	2	Infection	3.4	4.8	4.1	3.6	–	–	–	160
		Contact	–	–	–	–	–	–	–	40
	3	Infection	3.4	3.5	3.2	3.3	–	–	–	160
		Contact	–	–	–	–	–	–	–	20

*HI, hemagglutination inhibition; –, no virus detected.
†Serum samples were collected on day 18 after infection, exposure, or contact, and examined by use of an HI assay. The HI titer is the inverse of the highest dilution of serum that completely inhibited hemagglutination.

We conducted the transmission study in cats in the same way as the study in ferrets; we spaced cages 35 cm apart to prevent direct contact between the inoculated and exposed animals (online Technical Appendix Figure 1, panel A). All infected cats secreted viruses for 5–7 days after infection and seroconverted, except for 1 cat infected with A/chicken/NY/99 virus, which seroconverted but did not shed virus (Table 4). We did not isolate A/chicken/NY/99 virus from contact or exposed animals, although these animals seroconverted (Table 4). Direct contact transmission of A/feline/NY/16 virus was detected in all 3 pairs of cats, with both seroconversion and virus isolation in 2 pairs (Table 4). Respiratory droplet transmission of A/feline/NY/16 occurred in 2 pairs of animals, with high virus titers detected in the nasal secretions of the exposed animals on days 9 and 11 postexposure, respectively; both of the exposed animals also seroconverted (Table 4). In the third transmission pair, the exposed animal did not shed virus or seroconvert. Taken together, we demonstrated that A/feline/NY/16 virus has the ability to transmit among cats via contact and respiratory droplets; the relative contribution of these modes of transmission to the H7N2 subtype virus outbreaks in cat shelters in New York is unknown.

Receptor-Binding Specificity of Feline and Avian H7N2 Subtype Viruses

Avian influenza viruses isolated from their natural reservoir (i.e., wild aquatic birds) are often restricted in their ability to infect mammalian cells because of their preferential binding to α2,3-linked sialic acids, whereas most human influenza viruses preferentially bind to α2,6-linked sialic acids (*17–19*). We performed glycan array analysis with A/feline/NY/16, A/chicken/NY/99, and Kawasaki/173-PR8, a control virus possessing the HA and NA genes of the seasonal human A/Kawasaki/173/2001 (H1N1) virus and the remaining genes from A/PR/8/34 (H1N1) virus. As expected, Kawasaki/173-PR8 virus bound to α2,6-linked sialosides (Figure 6; online Technical Appendix Table 2). A/chicken/NY/99 virus bound to both α2,6- and α2,3-linked sialosides, consistent with the dual avian/human receptor-binding specificity of influenza viruses isolated from land-based poultry (*1*). Of note, A/feline/NY/16 virus bound strongly to α2,3-linked sialosides (i.e., avian-type receptors) with negligible binding to human-type receptors.

Next, we examined the prevalence of α2,3- and α2,6-linked sialosides in the feline airway and intestines of an immunologically naive cat by using lectins that detect α2,3-linked (i.e., MAA I and MAA II) and α2,6-linked sialosides (i.e., SNAI). MAA I and MAIA II bound to epithelial cells throughout the feline airway, whereas SNA binding was detected only in the trachea and bronchus (Figure 7), consistent with the findings of other research groups (*20–22*). We did not detect sialosides in the cat intestine. The predominance of avian-type receptors in

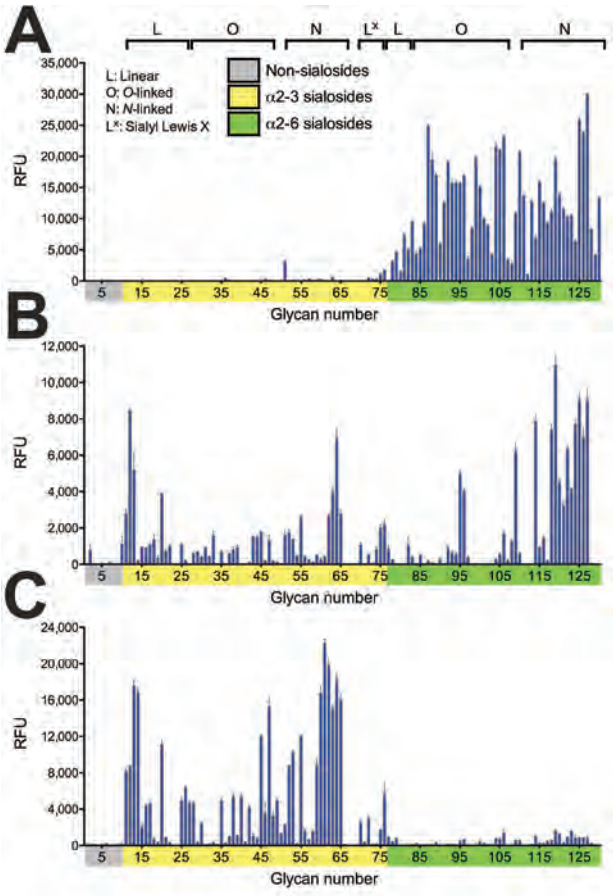

Figure 6. Receptor-binding specificities of influenza A viruses, New York, NY, USA. A) A representative human virus, A/Kawasaki/173-PR8(H1N1) is shown for comparison with B) the avian influenza A(H7N2) virus A/chicken/NY/99 and C) the feline influenza A(H7N2) virus A/feline/NY/16. Receptor-binding specificities of the avian and feline viruses were compared with those of the human virus in a glycan microarray containing α2,3- and α2,6-linked sialosides. Error bars represent SDs calculated from 4 replicate spots of each glycan. RFU, relative fluorescence units. A complete list of the glycans used is shown in online Technical Appendix Table 2 (https://wwwnc.cdc.gov/EID/article/24/1/17-1240-Techapp1.pdf).

the upper respiratory tract of felines may have led to the selection of feline H7N2 virus HA proteins with preferential binding to α2,3-linked sialosides.

Sensitivity to Neuraminidase Inhibitors

To test whether infections with the feline H7N2 viruses could be treated with neuraminidase (NA) inhibitors, we assessed the sensitivity of A/feline/NY/16 and A/chicken/NY/99 to several NA inhibitors (i.e., oseltamivir, zanamivir, and laninamivir) by determining the 50% inhibitory concentration (IC_{50}) of the NA enzymatic activity. We used A/Anhui/1/2013 (H7N9) virus as an NA inhibitor–sensitive control and its NA inhibitor–resistant variant, A/Anhui/1/2013-NA-R294K, as an NA inhibitor–resistant control (online Technical Appendix Table 3). A/feline/NY/16 and A/chicken/NY/99 were sensitive to all of the NA inhibitors tested (Technical Appendix Table 3), consistent with the absence of amino acid residues in the NA protein that are known to confer resistance to NA inhibitors. Hence, NA inhibitors could be used to treat persons infected with feline H7N2 subtype viruses.

Discussion

In our study, we demonstrated that a feline H7N2 subtype virus isolated during an outbreak in an animal shelter in New York in December 2016 replicated well in the respiratory organs of mice and ferrets but did not cause severe symptoms. The efficient replication of the feline H7N2 subtype viruses in the respiratory organs of several mammals, combined with the ability of these viruses to transmit among cats (albeit inefficiently) and to infect 1 person, suggest that these viruses could pose a risk to human health. Close contacts between humans and their pets could lead to the transmission of the feline viruses to humans. To protect public health, shelter animals (where stress and limited space may facilitate virus spread) should be monitored closely for potential outbreaks of influenza viruses.

Our findings of mild disease in mice and ferrets are consistent with the recent report by Belser et al. (*7*) who studied the H7N2 subtype virus isolated from an infected veterinarian. We also assessed feline H7N2 virulence in cats and detected efficient virus replication in both the upper and lower respiratory organs of infected animals, whereas an avian H7N2 subtype virus was detected mainly in the nasal turbinates.

Belser et al. (*7*) reported that intranasal or aerosol infection of ferrets with the H7N2 virus isolated from the infected veterinarian did not result in the seroconversion of co-housed or exposed animals, although nasal wash samples from some of the co-housed ferrets contained low titers of virus; these findings may suggest limited virus transmission that was insufficient to establish a productive infection. In contrast, we detected feline H7N2 virus transmission to co-housed ferrets in 1 of 3 pairs tested; this difference may be explained by the amino acid differences in the PA, HA, and NA proteins of the feline and human H7N2 isolates (online Technical Appendix Table 4) or by the small number of animals used in these studies. We also performed transmission studies in cats and detected feline H7N2 subtype virus transmission via direct contact and respiratory droplets. However, the group size used is a potential limitation of our study.

Cats are not a major reservoir of influenza A viruses, but can be infected naturally or experimentally with influenza viruses of different subtypes (*23*). Serologic surveys suggest high and low rates of seroconversion to seasonal

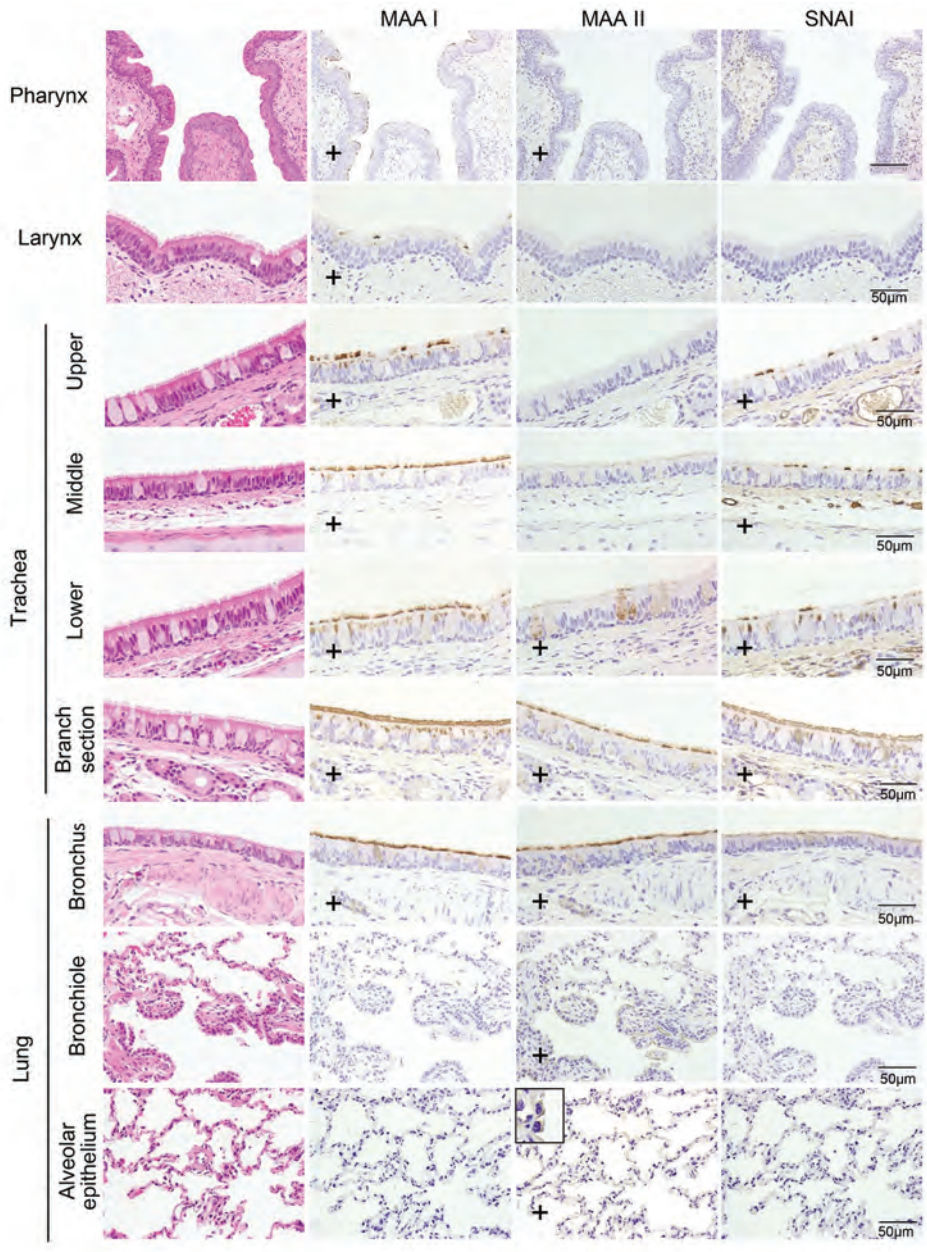

Figure 7. Distribution of α2,3- and α2,6-linked sialosides in the respiratory organs of a cat, New York, NY, USA. The α2,3- and α2,6-linked sialosides in the respiratory organs of a naïve cat were detected with biotinylated Maackia amurensis lectin I or II (MAA I, MAA II) or Sambucus nigra lectin (SNA I), respectively. Inset shows closer view of MAA III binding with alveolar epithelium in the lung. Plus signs (+) indicate that sialosides were detected. Scale bars indicate 50 μm.

human and highly pathogenic avian influenza viruses, respectively. Natural infections most likely result from close contact with infected humans or animals, and most of these infections appear to be self-limiting.

Few cases of human infections with influenza viruses of the H7 subtype were reported until 2013, and they typically caused mild illness; however, infection of a veterinarian with a highly pathogenic avian H7N7 virus had fatal consequences (*24*,*25*). Since 2013, influenza viruses of the H7N9 subtype have caused more than 1,300 laboratory-confirmed infections in humans, with a case-fatality rate of ≈30%. Although the current H7N9 and feline H7N2 subtype viruses do not exclusively bind to human-type receptors and do not transmit efficiently among humans, the spread and biologic properties of these viruses should be monitored carefully.

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Changing Geographic Patterns and Risk Factors for Avian Influenza A(H7N9) Infections in Humans, China

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The fifth epidemic wave of avian influenza A(H7N9) virus in China during 2016–2017 demonstrated a geographic range expansion and caused more human cases than any previous wave. The factors that may explain the recent range expansion and surge in incidence remain unknown. We investigated the effect of anthropogenic, poultry, and wetland variables on all epidemic waves. Poultry predictor variables became much more important in the last 2 epidemic waves than they were previously, supporting the assumption of much wider H7N9 transmission in the chicken reservoir. We show that the future range expansion of H7N9 to northern China may increase the risk of H7N9 epidemic peaks coinciding in time and space with those of seasonal influenza, leading to a higher risk of reassortments than before, although the risk is still low so far.

The third and fourth epidemic waves of avian influenza A(H7N9) human infections in China showed an apparent reduction in incidence compared to the spring 2013 and winter 2013–14 epidemic waves. However, during the winter of 2016–17, the incidence rose, growing to levels never observed before and reaffirming concerns of a pandemic threat posed by the H7N9 virus (*1*–*3*). Since 2013, more than 1,520 human cases of H7N9 virus infection have been reported, mostly located in eastern China, with a case-fatality rate ranging from 30% to 40% (*4*–*6*).

The H7N9 virus that caused the first epidemic wave in March 2013 originated from multiple reassortment events of avian influenza viruses from domestic poultry and wild birds (*7*). Mainly restricted to the Yangtze River Delta in eastern China, including urban areas of Shanghai, Jiangsu, and Zhejiang Provinces, in the first wave, the spatial range of H7N9 human cases increased during the second wave along the coast into Guangdong Province in southern China (*8*). Over time, phylogeographic inference suggested that H7N9 had become established in separate parts of China during the second and third waves, reassorting with local avian influenza viruses (*9*,*10*).

Humans are not a natural reservoir, but occasional spillover hosts of H7N9 human cases act as indicators, presumably reflecting the circulation of H7N9 in poultry (*10*), and are an effective way of studying the spatial distribution of H7N9 virus. Surveillance in poultry is difficult, as the virus has so far had a low pathogenicity in chickens (*11*,*12*), and the absence of clinical signs means that active and targeted sampling is needed. This difficulty has made the characterization of the spatial distribution of the virus reservoir inconclusive, although that may change in the future because of the recent evolution of a highly pathogenic strain of H7N9 (*13*–*15*).

In this study, we considered 3 sets of factors that may influence the spatial variation in H7N9 incidence. The first set of spatial risk variables, termed anthropogenic variables, included the distribution of live-poultry markets (LPMs)

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and human population density. Visits to LPMs are the main known risk factor for H7N9 human infection (*16*–*18*), and LPMs represent a key interface between humans and poultry. At a higher level, LPM networks may also support the spread and persistence of H7N9 virus through the network of LPMs and poultry farms linked by trade (*19*). In previous studies, we showed that a high density of LPMs in some specific areas could regionally increase the risk for H7N9 infection at the market level (*20*), which translates into higher risk at the county level, as observed in several studies (*21*–*23*). Human population density was included as a surrogate for surveillance bias and to account for any anthropogenic transmission mechanisms.

During the fifth wave, outbreaks in poultry farms started to be reported in higher numbers, so we included a second set of predictor variables, termed poultry, including the density of chickens and ducks, as these may regionally influence the risk of H7N9 virus transmission to humans. From 69% to 80% of H7N9 human patients in the 5 epidemic waves reported exposure to live poultry before infection, including LPM (52%–60%) and backyard poultry (13%–40%); these figures remained fairly stable with time (*1*). Although most of those exposures may correspond to LPM visits, other opportunities for contact with poultry along the production and value chain also exist. For example, poultry workers in Beijing were shown to be at a higher risk for H7N9 infection than the remaining population of the city (*24*). Poultry may become a reservoir when the circulation of avian influenza viruses through the production and value chains cannot be prevented; poultry-related variables were found to be key predictors of H7N9 risk in several previously published studies (*20*,*23*,*25*,*26*).

In addition, to account for the distribution and abundance of wild birds, we included 2 indicator variables of proximity to and abundance of water and wetlands. Although the most conservative hypothesis remains that human infections are linked to the circulation of H7N9 in domestic poultry with exposure in LPMs, it cannot be assumed that wild birds do not play any role in transmission. The virus precursors of the H7N9 virus in China were found in a wide variety of bird species, both wild and domestic (*7*); avian influenza viruses circulating in wild birds represent a gene pool that may recombine with H7N9 viruses and allow better adaptation and persistence. There is little information on the wild host specificity of H7N9, and data on the distribution of wild bird species are generally coarse, with populations varying strongly according to the season.

We studied the spatial variation of H7N9 incidence in the human population during the 5 epidemic waves in relation to these 3 sets of spatial risk factors. More specifically, we compared the association between these spatial factors and H7N9 infections across the 5 epidemic waves, to investigate the spatial distribution of repeated recurrences and the year-to-year variation in predictability of H7N9 infections.

Materials and Methods

Data

H7N9 Human Cases and Seasonal Influenza

We analyzed all confirmed H7N9 human cases during February 19, 2013–August 9, 2017. We collated information on laboratory-confirmed H7N9 human cases by collecting data from the World Health Organization (WHO) Monthly Risk Assessment Summary report, websites of the national and provincial Health and Family Planning Commission of China, FluTrackers (http://flutrackers.com), HealthMap (http://www.healthmap.org/en/), and avian influenza reports from the Centre of Health Protection of Hong Kong. When information was inconsistent, we used the WHO report as the primary source. A detailed description of case definitions, surveillance for identification of cases, and laboratory testing for H7N9 virus have been provided elsewhere (*4*,*27*,*28*). For each case, the information about place of residence and date of onset of symptoms was used and 6.5 days were subtracted from the date of onset of symptoms to estimate the dates of first contact with the virus, as estimated elsewhere (*29*). To compare the seasonality of H7N9 human cases with that of human seasonal influenza A in space and time, we extracted influenza sentinel surveillance data for January 2013–March 2017 from Influenza Weekly Reports, managed by the Chinese National Influenza Centre (http://www.chinaivdc.cn/cnic/zyzx/lgzb/). More information on the sentinel network supporting these data can be found in Yu et al. (*30*).

Live Poultry Markets and Permanent Closure Measures

We assembled a database recording the locations of 8,943 retail and wholesale LPMs from multiple sources. In addition, we compiled a database recording the market closure measures implemented since the first wave, with the start and end date of each measure. Both databases are described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/1/17-1393-Techapp1.pdf).

Spatial Predictor Variables

The first set of predictor variables included the LPM density (LPM/km^2) and human population density (persons/km^2). Some counties do not have LPMs but their inhabitants may easily go to LPMs in neighboring counties. LPMs may also act at a higher level by providing a network of markets through which the disease could spread and persist. The LPM density was computed by means of a Gaussian smoothing kernel function with the optimal

bandwidth found by Gilbert et al. (*20*). To account for closure of LPMs, the data on permanent market closures were used to remove the permanently closed markets from the full LPM database before the Gaussian smoothing, resulting in a different LPM density distribution for each epidemic wave. Human population density was taken from the 2010 census (http://www.stats.gov.cn/tjsj/pcsj/rkpc/6rp/indexch.htm).

The second set of predictor variables included chicken and domestic duck densities from a new dataset we produced using the Gridded Livestock of the World methodology applied to an extensively improved dataset we compiled using the 2010 reference year (*31*,*32*). Because a high correlation was noted between duck and chicken densities at the county level, and to reduce colinearity and to facilitate the interpretation of the results, we combined these variables to give a poultry density layer (chickens + ducks/km^2) and the chicken-to-duck ratio (chicken density/duck density).

The last set of predictor variables was indicative of water bird habitat. This included the distance to the largest lakes and reservoirs (km), measuring the distance between the county centroids and the nearest lakes (area ≥50 km^2) or reservoirs (storage capacity ≥0.5 km^3) (*33*), and the proportion (%) of the county covered by wetlands, according to the hybrid wetland map for China (*34*).

Analyses

The analyses involved the development of Poisson boosted regression tree (BRT) models to predict the daily incidence rate of H7N9 virus in the human population as a function of 6 predictor variables. (A description of the BRT models and a list of model parameters is provided in the online Technical Appendix.) The models were developed using the number of human cases as the dependent variable, with an offset term corresponding to the product of human population by the duration of the epidemic. The duration of each epidemic was defined as the period separating the 5th from the 95th percentile of the days of onset of illness in each wave. One model per epidemic wave was built to compare the effect of predictor variables and to assess the predictive capacity from one wave to another. The contribution of each predictor variable to the model was quantified by its relative contribution (RC), a measure of its overall importance in the model (*35*), and by its partial dependence plots, or BRT profiles, which provide a graphical description of its effect on the daily incidence rate after accounting for the average effects of all other predictor variables in the model (*36*). We tested the presence of spatial autocorrelation in the model residuals using spline correlograms (*37*) and we used the approach of Crase et al. (*38*) when autocorrelation was present in the model residuals. To evaluate the models for their capacity to discriminate between the presence and the absence of human cases at the county level, we converted the predicted daily incidence rate into a probability of having ≥1 human case in the county using a binomial model. Finally, we replicated the analysis with generalized linear models because BRT models do not explicitly allow the formal testing of the significance of individual risk factors.

Results

Table 1 presents the RC of the predictor variable in the different epidemic waves. The RCs of anthropogenic predictor variables were high initially but decreased strongly after the third epidemic wave (w1 = 41.66%; w2 = 50.99%; w3 = 39.93%; w4 = 17.31%; w5 = 21.52%). In parallel, the RC of poultry predictor variables increased and was greatest in the last epidemic wave (w1 = 10.39%; w2 = 5.57%; w3 = 2.12%; w4 = 28.53%; w5 = 36.37%). In this last epidemic wave, the most noteworthy predictor variables were, in decreasing order of RC values, the chicken-to-duck ratio (20.49%), the LPM density (18.41%), the poultry density (15.88%), and the distance to open lakes and reservoirs (7.31%). Figure 1 presents the BRT profiles of these 4 predictor variables in the different epidemic waves (the other profiles are provided in online Technical Appendix Figure 1). The chicken-to-duck ratio had a notable RC only in waves 4 and 5, when it showed a positive association with incidence up to a ratio of ≈30. The LPM density profile

Table 1. Relative contribution of the different Poisson BRT models across 5 epidemic waves of influenza A(H7N9), China*

Model	Relative contribution ± SD, %				
	Wave 1	Wave 2	Wave 3	Wave 4	Wave 5
Anthropogenic†	41.66	50.99	39.93	17.31	21.52
LPM density	39.81 ± 0.24	50.43 ± 0.42	12.22 ± 0.78	13.24 ± 0.78	18.91 ± 0.12
Human population density	1.85 ± 0.14	0.56 ± 0.03	27.71 ± 0.49	4.07 ± 0.22	2.61 ± 0.04
Poultry†	10.39	5.57	2.12	28.53	36.37
Chicken-to-duck ratio	5.33 ± 0.18	4.18 ± 0.06	0.54 ± 0.06	20.23 ± 0.3	20.49 ± 0.18
Poultry density	5.06 ± 0.14	1.39 ± 0.04	1.58 ± 0.13	8.3 ± 0.36	15.88 ± 0.08
Water habitat†	2.18	3.6	9.29	5.68	8.48
Proportion of wetlands	0.49 ± 0.02	1.13 ± 0.06	1.51 ± 0.1	0.74 ± 0.07	1.17 ± 0.03
Distance to lakes	1.69 ± 0.05	2.47 ± 0.11	7.78 ± 0.23	4.94 ± 0.19	7.31 ± 0.1
Autoregressive term	45.77 ± 0.27	39.84 ± 0.32	48.65 ± 0.65	48.49 ± 1.33	33.62 ± 0.17

*BRT, boosted regression tree; LPM, live poultry market.
†Sum of relative contribution for both categories.

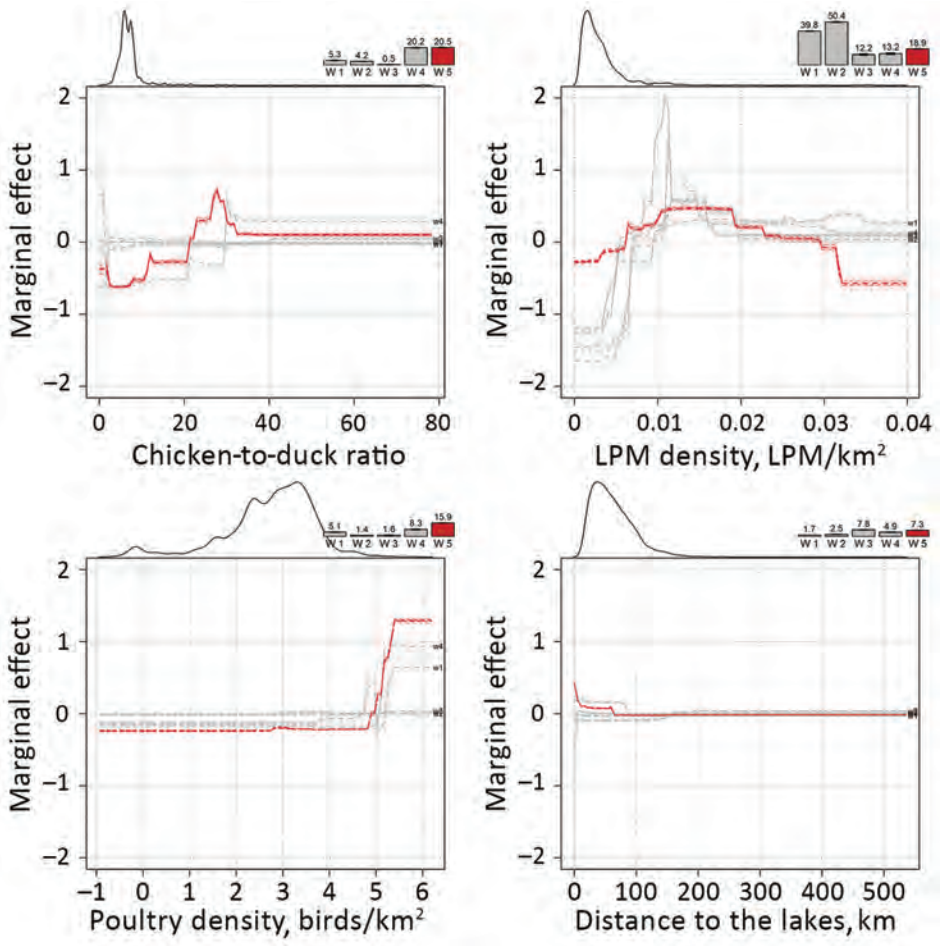

Figure 1. Marginal effect plots of the top 4 predictor variables on the predicted incidence rate of influenza A(H7N9) in China. Change in relative contribution over time is indicated by the bars on the top of each plot, showing the increasing relative contribution of the poultry predictor variables. The smoothed line on the top left part of each plot is indicative of the distribution of each variable.

of wave 5 also showed a positive association, leveling off at a density of 0.01, showing a profile that was relatively similar to those of the other epidemic waves. Wave 5, in contrast to previous epidemic waves, tended to associate lower incidence with the highest LPM densities (>0.03). The poultry density profile changed gradually over time, with an increasing RC, and the incidence rate in wave 5 is predicted to increase strongly in counties with a high density of poultry (>60,000 birds/km^2). Finally, the profile of the distance to lakes showed a decreasing association in the range 0–100 km.

The assessment of the BRT models' goodness of fit is presented in Table 2. With the exception of the fourth epidemic wave, the predictability of the models was moderate, with cross-validation correlation coefficients ranging from 0.42 to 0.55. For the presence/absence term, the models had a good discriminatory capacity, with areas under the curve (AUCs) ranging from 0.77 to 0.92, but this capacity decreased over the years (w1 = 0.92; w2 = 0.85; w3 = 0.83; w4 = 0.86; w5 = 0.77). This finding implies that it was easier to predict the presence or absence of a human case (good discrimination capacity and AUC values) than it was to predict the number of cases (moderate predictability and correlation coefficients). The discriminatory capacity was maintained from wave to wave, with a lower overall AUC in wave 5 (Table 3). The results obtained with the same risk factors and dependent variable from the generalized linear models (online Technical Appendix) show a similar pattern, with poultry variables becoming more apparent after the fourth epidemic wave.

Table 2. Goodness-of-fit metrics of the Poisson BRT models across 5 epidemic waves of influenza A(H7N9), China*

	Pearson correlation coefficient ± SD			AUC ± SD	
Wave	Training	Training, auto	Cross-validation	Training	Training, auto
1	0.793 ± 0.011	0.553 ± 0.002	0.487 ± 0.014	0.924 ± 0.001	0.907 ± 0.001
2	0.749 ± 0.004	0.345 ± 0.008	0.55 ± 0.014	0.849 ± 0.001	0.848 ± 0
3	0.588 ± 0.01	0.496 ± 0.003	0.424 ± 0.013	0.833 ± 0.002	0.811 ± 0.001
4	0.423 ± 0.005	0.292 ± 0.007	0.258 ± 0.009	0.855 ± 0.001	0.833 ± 0.001
5	0.586 ± 0.001	0.539 ± 0.001	0.446 ± 0.009	0.773 ± 0	0.75 ± 0

*AUC, area under the curve; BRT, boosted regression tree.

Table 3. Cross-predictability of the BRT models trained with the different epidemic waves of influenza A(H7N9), China, applied to the others, as measured by the area under the curve*

	Applied to				
Predictions	Wave 1	Wave 2	Wave 3	Wave 4	Wave 5
Wave 1	0.91	0.81	0.78	0.84	0.79
Wave 2	NA	0.85	0.78	0.83	0.76
Wave 3	NA	NA	0.82	0.82	0.74
Wave 4	NA	NA	NA	0.83	0.75
Wave 5	NA	NA	NA	NA	0.76

*BRT, boosted regression tree; NA, not applicable.

Figure 2 shows the distribution of the top 3 predictor variables (LPM density, poultry density, and chicken-to-duck ratio) in relation to the distribution of the human cases, distinguishing those from epidemic waves. The RGB (red/green/blue) composite plot (Figure 2, panel A) highlights areas in which all 3 predictor variables were high and where H7N9 persisted over time (Figure 2, panel B). A large area to the east of Taihu Lake on the urban areas of Wuxi, Suzhou, and Shanghai had high LPM densities and included several small hotspots of high poultry density. The RGB composite plot shows 3 additional areas with high LPM densities and high poultry densities: Guangdong Province, the Tianjin and Beijing urban areas; and the Chongqing urban area. These areas visually correspond to areas of high H7N9 recurrence in Figure 2, panel B, which contrasts counties with repeated recurrences from those with sporadic infections. Figure 2, panel C illustrates that the spatial pattern of wave 5 showed a marked geographic expansion from these previous hotspots of persistence, with 279 counties reporting H7N9 for the first time (66.11% of the total number of counties infected in wave 5). It is also apparent why LPM density was a less powerful predictor variable in wave 5 than in previous waves, as these newly infected counties no longer correspond to the green areas depicted in Figure 2, panel A. The heat maps shown in Figure 3 show that the majority of H7N9 human cases occurred around February and March (Figure 3, panel B), with a latitudinal gradient. The seasonality of common influenza A infection is different throughout China (Figure 3, panel C), with the provinces north of 34.1 degrees showing a much stronger annual winter seasonality of infection than do the more southerly provinces, where most cases occur during December–February. A comparison of Figure 3, panels B and C, shows that the peaks of H7N9 and seasonal influenza A have so far not coincided strongly in space and time. However, a geographic range expansion of H7N9 infections into the northern provinces, retaining the current seasonality, would bring the H7N9 and seasonal influenza A incidence peaks toward each other in both space and time.

Discussion

The results of our spatial models demonstrate a major shift over time from anthropogenic toward poultry predictor variables linked to H7N9 human cases, apparent in wave 4 and confirmed in wave 5. This shift was evident in both BRT and generalized linear models. More specifically, the predictive power of poultry variables increased over time and was greatest in the last epidemic, pointing to areas with high chicken densities and high

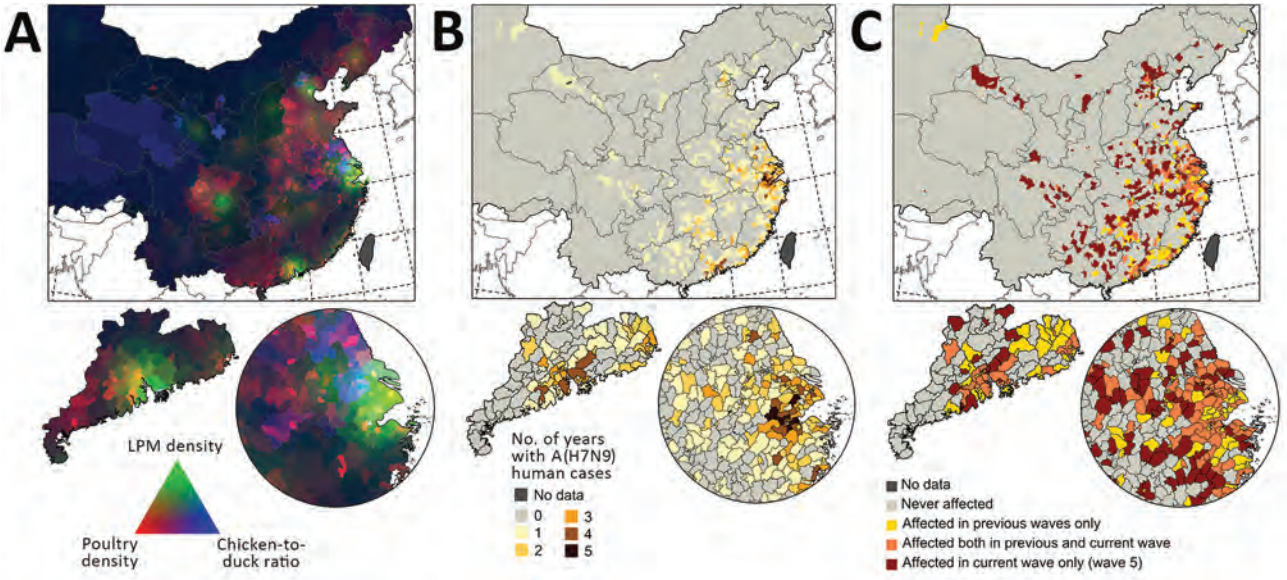

Figure 2. Distribution of predictor variables and influenza A(H7N9) infections in China, with 3 geographic extents: smallest extent around the location of human cases (top), Guangdong Province (bottom left), and Yangtze River Delta (bottom right). A) Visualization of poultry density (red), live-poultry market density (green), and chicken-to-duck ratio (blue). Dark areas correspond to low values and light areas to high values in all 3 predictors. B) Number of years with ≥1 human case per county. C) Distribution of the fifth wave of human infections compared with previous waves.

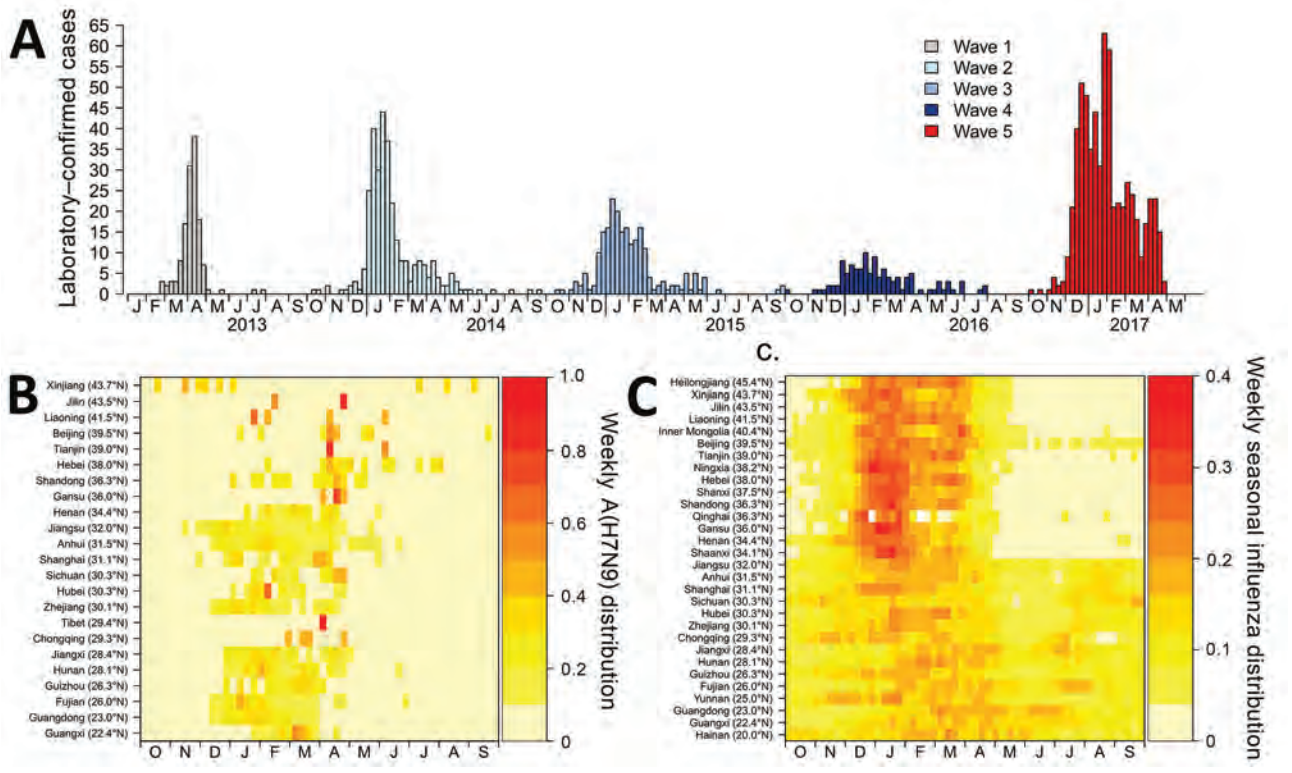

Figure 3. Seasonality of influenza A(H7N9) infections in comparison to seasonal influenza, by week, China, 2013–2017. A) Epidemic curve for H7N9. B) Seasonality for H7N9. C) Seasonality for seasonal influenza A.

chicken-to-duck ratios. A recent study on H7N9 human cases showed an increase in periurban and rural cases in the fifth wave and a comparatively higher number of cases among middle-aged persons (*1*). However, apart from the overall increase in cases, the study did not suggest any other major epidemiologic differences, and other authors have made similar observations when comparing waves 1–4 (*1*,*5*,*8*). Our results do not contradict the observation of a higher number of human cases in periurban and rural areas, because high poultry production regions are typically located in periurban and rural settings, but they strongly support the hypothesis that the H7N9 virus may have spread in the chicken reservoir much more extensively during the last 2 epidemic waves than was previously the case, with a particularly marked geographic range expansion in the last epidemic wave. This observation, based on human cases, can be linked to the emergence of HPAI H7N9 that was reported early in 2017 in southern China (*13*). Recently published results showed that human cases of HPAI H7N9 were already found beyond Guangdong, in Hunan and Guangxi Provinces in early 2017 (*15*). In parallel, a comparatively higher number of reports of H7N9-positive samples was found in chicken farms this year in comparison with previous epidemic waves, including reports of HPAI H7N9 in northern China, in Tianjin (*39*). The precise role of the gain in pathogenicity on the range expansion of H7N9 remains unclear, as do the main mechanisms of transmission along the poultry production and value chain networks. However, the fact that such a range expansion took place in parallel with the emergence of a highly pathogenic variant seems unlikely to be coincidental.

It should be noted that the measure of predictor weights in the model, the RC, is relative, so that the sum of RCs equals 1. If, therefore, the poultry variables become better predictors of H7N9 incidence in humans, the RC of other variables must decrease, even if their effect on the predicted incidence remains fairly constant. The contribution of LPMs may have remained high, but its combination with increasing transmission along the poultry production and value chains may be responsible for the geographic range expansion and higher incidence observed during the fifth wave.

Although some of the highest incidences of H7N9 were observed along Taihu Lake, the predictive capacity of variables associated with water birds had a much lower influence in the models than did the anthropogenic and poultry variables. Many interfaces combining wetlands, intensive poultry farming, and rice paddy fields are present in southeastern China and may have played a role in the initial

emergence of the H7N9 virus in the Shanghai area (*40*). As the virus spread in the following epidemic wave, however, the contribution of wild birds to overall disease circulation may be fairly low, which is reflected by the low relative contribution of the water bird habitat proxy variables.

The predictive capacity of the incidence models was only moderate, as these spatial models did not account for the variability in incidence linked to market closure measures. In contrast, the predictions of presence/absence were generally better because presence cannot be influenced by market closure measures; such measures usually followed human cases rather than preceded them, and few counties implemented market closure measures in the absence of human cases.

This moderate predictive capacity may also relate to some limitations of the study. There may be an underreporting of milder symptomatic infections (*30*), and the effect and geographic distribution of this bias is unknown. Another aspect is that the poultry dataset underlying our analyses is of uneven quality, with better and more detailed data in the east than in western parts of the country, as shown by Artois et al. (*26*). Finally, although all efforts were made to compile the most comprehensive LPM dataset possible, many LPMs may have opened and closed, including illegal ones, further adding to model uncertainty. Finally, we were not able to integrate poultry movement and trade data (legal or illegal) into this analysis because of the lack of centralized data; this may be a line of investigation for the future.

The geographic range expansion and increase in incidence of human cases in the fifth wave of H7N9 brings serious human health concerns. First, repeated human infection by avian influenza viruses increases the chances of virus recombination, mutation, or both, leading to human-to-human transmission. Second, the provinces affected by earlier H7N9 epidemic waves do not have a strong seasonal influenza A peak in January and February (*30*) that matches the peak of H7N9 cases (Figure 3). However, if the H7N9 virus continues to expand its range northward, in areas with a strong influenza A peak in January and February, there will be a higher chance of local coincidence of peaks of incidence between human cases of H7N9 and seasonal influenza A virus. This change may enhance the chances of coinfections that could lead to the emergence of reassortants with the capacity to transmit easily between humans. Third, the extent of the geographic range of the expansion is not yet fully known; in the absence of new measures, it may spread further within China and internationally through poultry value chains.

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Pneumonic Plague in Johannesburg, South Africa, 1904

Charles M. Evans, Joseph R. Egan, Ian Hall

Plague is a potentially dangerous reemerging disease. Because modern outbreaks are relatively infrequent, data for epidemiologic study are best found in historical accounts. In 1905, the Rand Plague Committee published a report describing an explosive outbreak of 113 cases of pneumonic plague that occurred in Johannesburg, South Africa, in 1904. Using these data, we investigated social, spatial, and temporal dynamics and quantified transmissibility as measured by the time-varying reproduction number. Risk for transmission was highest when friends, family members, and caregivers approached the sick. Reproduction numbers were 2–4. Transmission rates rapidly diminished after implementation of control measures, including isolation and safer burial practices. A contemporaneous smaller bubonic plague outbreak associated with a low-key epizootic of rats also occurred. Clusters of cases of pneumonic plague were mostly limited to the Indian community; cases of bubonic plague were mostly associated with white communities and their black African servants.

Since the epidemics of primary pneumonic plague in Manchuria in the early 20th century, few opportunities for the study of substantial epidemics have occurred (*1*). Often small, self-limiting (*2*), and occurring in inaccessible places, epidemics may well have run their course before medical teams arrived (*3*). When authorities are vigilant, patients and contacts can be quickly identified, isolated, and, when possible, given effective antimicrobial drugs (*4*). Nevertheless, the pneumonic form of *Yersinia pestis* infection remains a potential threat to public health (*5*), and an aerosolized preparation might be used as a biological weapon (*6*). The death rate among patients who do not receive treatment approaches 100%, which makes pneumonic plague one of the most lethal diseases known to humanity, and lately, antimicrobial drug–resistant strains have been detected (*7*).

Historical records are therefore a useful source of data for epidemiologic studies. One such epidemic, which has received limited attention, occurred in 1904 in Johannesburg, South Africa, adjacent to the Witwatersrand gold fields. In 1905, the Rand Plague Committee published a report (the RPCR) that documented the principal findings together with the data on which their inferences were based (*8*). A copy of the RPCR can be found in the Wellcome Library, 183 Euston Road, London NW1 2BE, UK. It was compiled by Walter Pakes (acting for Charles Porter, Medical Officer of Health for Johannesburg) and comprises 103 pages of text accompanied by plates and folded maps. We reexamined these data in light of modern knowledge and recently developed analytical techniques.

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Materials and Methods

Social, Political, and Spatial Context

In 1886, the Witwatersrand gold fields opened, and Witwatersrand soon became the largest gold-producing region in the world. After the second Boer war, in May 1900 British forces occupied Johannesburg, achieving economic and political dominance. Up to and beyond 1904, the racial makeup of Johannesburg was determined by the need to attract highly skilled workers and cheap labor. From 1860 on, workers from India entered the Colony of Natal under a system of indenture organized by the British and white farmers, and from 1896, the Chamber of Mines coordinated the recruitment of black African labor through the Witwatersrand Native Labour Association (*9*).

After the completion of indenture, Indian workers were permitted to buy "preferent" rights to parcels of land in Johannesburg, known as "stands," within a small area widely known as the Coolie Location (Figure 1). This area subsequently became a multiracial slum, causing the municipal authorities to repurchase it for redevelopment. Mahatma Gandhi made a name for himself by securing fair compensation for the Indian property owners (*10*). A photograph (Figure 2) depicts a ramshackle collection of dwellings, described as follows in the RPCR: "… although the Coolie Location is theoretically laid out in stands, the tin shanties were put in all sorts of positions… the whole of Stands 43 and 48 must be considered as one mass of little huts, with common latrines, etc where the inhabitants… were in very constant and close contact with… their neighbors."

The central focus of Johannesburg was the market square, where the post office, stock exchange, banks, and shopping outlets were located. The market square received

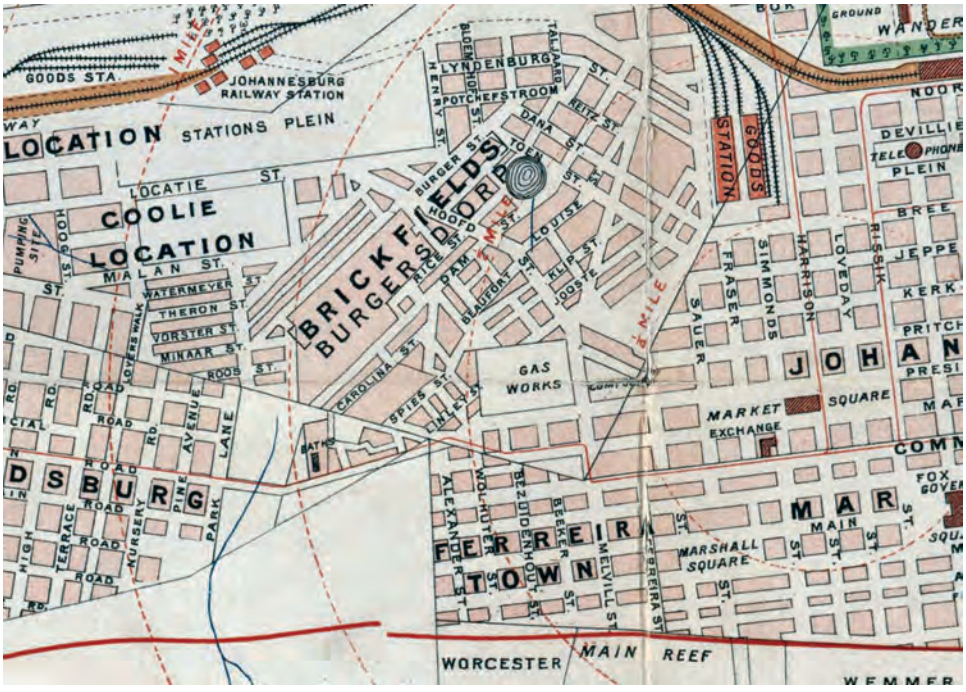

Figure 1. Central area of Johannesburg, South Africa, in 1904, showing the relative positions of the Coolie Location, Burgersdorp, and Market Square. Map held at the Witwatersrand Library, University of the Witwatersrand, Johannesburg, South Africa, and available at http://innopac.wits.ac.za/search/?searchtype=t&SORT=D&searcharg=plan+of+johannesburg+and+suburbs

goods, which were often auctioned on site, from the coast and elsewhere. Within the square, buildings housed additional shops, restaurants, and offices. Between the Coolie Location and market square was an area known as Brickfields or Burgersdorp. Originally established by poor Afrikaner families, who fabricated sun-baked bricks to supply the gold-driven property boom, this area also become a multiracial slum (*11*).

African workers recruited or coerced into supplying cheap labor for the mines were housed in compounds close to their place of work but some distance from Johannesburg center (*12*). Conditions there were poor, and pneumonia was commonplace (*13*). The Johannesburg census of April 17, 1904, recorded 118,917 male and 39,663 female inhabitants (*14*). The racial distribution was described as follows: "Europeans/whites," 52,042 male and 31,680 female; "all coloured races, including South African aboriginals and Asiatics," 66,875 male and 7,803 female.

The RPCR as Data Source

Outbreaks of primary pneumonic plague and bubonic plague together with an associated epizootic among rats were documented. Details were given of the procedures adopted by public health workers, methods used for the

Figure 2. The Coolie Location. Photograph kindly supplied by Museum Africa, 121 Lilian Ngoyi (formerly Bree) St., Newtown, Johannesburg, South Africa.

identification of *Y. pestis*, and postmortem examinations. Case types were defined as follows: "Pure pneumonic cases were those in which no buboes could be found, but in which there was definite broncho-pneumonia. The mixed cases were those in which there was definite broncho-pneumonia, as well as buboes, and the B. Pestis [sic] was recovered both from the foci in the lungs and from the bubo. The septicaemic cases were those without either signs of pneumonia or buboes."

A list of cases recorded from March 20 on indicated the date of death, type of infection, race, sex, occupation, and residential location for each patient. The RPCR also contained a retrospective study of 37 cases (originally thought to be pneumonia but which had occurred before March 20). Also noted were the cases of a Dr. Marais and 9 other patients with pneumonic plague (former inhabitants of the Coolie Location, who had fled to areas outside Johannesburg). To illustrate putative transmission pathways, all cases of pneumonic plague were logged on a fold-out chart; we used those dates of death for our analysis.

Estimation of the Time-Varying Reproduction Number for Cases of Primary Pneumonic Plague

The transmissibility of primary pneumonic plague can be quantified by estimating the time-varying reproduction number, R_t, defined as the average number of secondary infections resulting from an infectious person. We used a recently published method of estimating R_t (*15*), which requires a count of the observed number of patients who became symptomatic each day. However, the date of onset of many of the primary pneumonic plague cases could not be ascertained because the first indication was often the discovery of a deceased person and no witness to the date of disease onset. Thus, in our study, we back-calculated the incidence of symptom onset (*16*,*17*) from the death count by using the symptom onset to death distribution, based on 166 cases, previously estimated from the primary pneumonic plague outbreak in Manchuria (*1*). These data provide a mean (±SD) of 2.3 (±1.7) days. A second prerequisite for estimating R_t is the serial interval distribution (i.e., the time from symptom onset in a primary case-patient to symptom onset in a secondary case-patient). We used a distribution, based on 177 cases, previously estimated from 4 outbreaks that occurred during the 20th century (*18*), providing a mean of 5.4 (±3.0) days.

Results

Time Course of the Epidemic

The RPCR states, "On the evening of the 18th March, the District Surgeon, Dr. Mackenzie, was informed that a number of Indians were sick in the Coolie Location.… Dr. Alexander visited the Location and found a number of Indians suffering apparently from Pneumonia… during the evening of the 18th March, Mr. Gandhi, Dr. Godfrey and Mr. Madenjit… removed all the sick Indians they could find to Stand 36, Coolie Location…." Other reports suggest that Gandhi and his colleagues were the first to raise the alarm. William Godfrey, a doctor of Indian descent who graduated in 1903 from Edinburgh University (*19*), realized that plague had broken out and arranged for an empty house to be made available before a larger temporary hospital was later established. After bacteriologic confirmation of the presence of *Y. pestis*, a cordon was placed around the Coolie Location on March 20.

The first cases of pneumonic plague to be documented in the retrospective study occurred between early January and mid-March (Figure 3). Two cases of bubonic plague occurred in the weeks ending February 6 and March 5 and were mentioned as follows: "A very careful search through the death returns for several months before the 18th March, 1904, fails to reveal any probable case of bubonic plague (as distinguished from pneumonic plague) with the exception of Cases VII and XIV..." Both patients were attended by medical officers. Inguinal swellings were noted, although a diagnosis of bubonic plague was not made at that time. Death occurred within 2 days of the examinations, but the report maintains that no further cases seem to have arisen from them.

The pneumonic phase of the epidemic showed an "explosive" increase during the 12th and 13th weeks, whereas the bubonic cases peaked in week 14 and continued over the next 14 weeks. We note that pneumonic plague cases

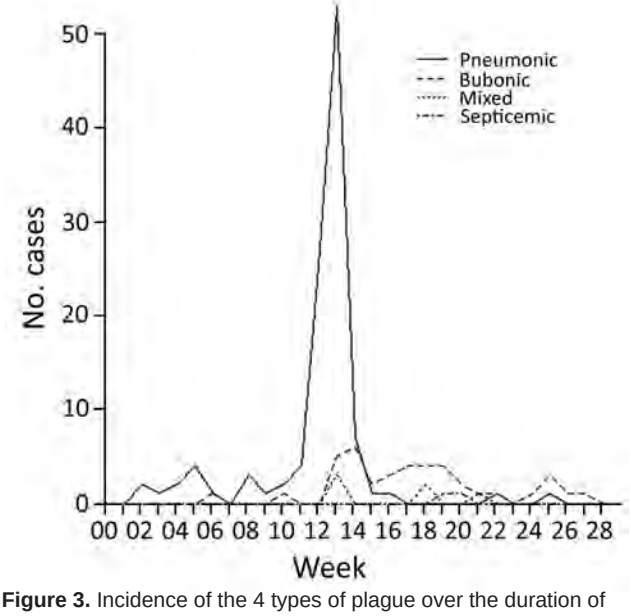

Figure 3. Incidence of the 4 types of plague over the duration of the epidemic in Johannesburg, South Africa, from week ending January 2 to week ending June 16, 1904.

were recorded on a date-of-death basis, whereas bubonic plague cases were recorded on an onset-of-symptom basis. The investigators were unable to trace the index case(s) and could only speculate that "Plague infected rice was imported from Bombay during December, 1903 and January 1904. From this rice a few Indians were infected with the pneumonic form of plague."

Case Incidence

The 113 cases of pneumonic plague far outnumbered the 40 bubonic, 6 mixed, and 2 septicemic plague cases (Table). The racial group most severely hit by primary pneumonic plague was Indians, even though they were a small minority within Johannesburg. Of the 40 bubonic cases recorded, 16 were in "whites," 14 in "natives," 4 in "coloureds," but only 6 were in "Indians." Relatively few cases in girls/women were reported, and parity between the sexes was approached by the white group only. Survival was reported for 31 patients with bubonic plague but only 2 with pneumonic plague.

Public Health Measures

In addition to the isolation of patients and the establishment of a cordon, inspectors were appointed to search the Coolie Location for additional sick persons. Within the Witwatersrand, every compound housing native mineworkers was eventually inspected and the movement of natives was controlled by the existing native pass system. Pneumonia was made a notifiable disease, and samples of sputum were routinely sent to government laboratories.

On April 8, the authorities razed the Coolie Location by burning it to the ground. The inhabitants, including "1,600 Asiatics, 142 coloured and 1,358 natives," were relocated to a camp 12 miles from the center of Johannesburg, the first step in the evolution of the township later to become known as Soweto (*20*).

We found no record of any special protective clothing or instructions being issued to those caring for primary pneumonic plague patients. A single nurse was allocated to the temporary hospital, but the Indian orderlies were left to their own devices. The RPCR also mentions that a nurse allocated to look after infected white children "…treated the pneumonic cases as bubonic cases. Certainly she kissed the children."

As the outbreak progressed, most deaths occurred in hospitals that allowed some control of burial practices. The RCPR states that "… in the case of Hindoos and the Mohammedans [sic]. The former were allowed to bury their dead: the latter, who have certain religious functions to perform were given a room in the mortuary to perform the rite. They were warned of the dangers of handling the cadavers, and it was suggested to them that the washing should be performed with a solution of corrosive sublimate."

Examinations for Bacteria and Pathologic Findings

Government health laboratories tested for *Y. pestis* in samples of sputum or tissues from organs including the lung, spleen, and liver. Bacteria were cultured, identified morphologically, and subsequently confirmed by inoculation into rabbits and guinea pigs.

The RPCR claims that postmortem examinations were conducted on every patient whose death was suspicious throughout the Rand. Before death, primary pneumonic plague patients exhibited typical symptoms of fever and some "scanty but blood-stained expectorations." Death occurred generally within 72 hours of symptom onset, and postmortem appearances were described as follows: "… signs of pleurisy were common and… extensive fibrinous exudation... B. Pestis [sic] was very abundantly present… but… not… sufficient pneumonic lung to account for death… in some… cases death is due to toxaemia and not to septicaemia."

Table. Plague cases by disease type and patient race and sex, Johannesburg, South Africa, 1904*

	White		Indian		Colored		Native		
Disease type	M	F	M	F	M	F	M	F	Total
Pneumonic									
Cases	4	4	88	4	0	0	12	1	113
Deaths	4	4	86	4	0	0	12	1	111
Bubonic									
Cases	10	6	6	0	3	1	14	0	40
Deaths	1	2	3	0	0	0	3	0	9
Mixed									
Cases	0	0	3	0	0	0	3	0	6
Deaths	0	0	3	0	0	0	3	0	6
Septicemic									
Cases	2	0	0	0	0	0	0	0	2
Deaths	2	0	0	0	0	0	0	0	2
Total									
Cases	16	10	97	4	3	1	29	1	161
Deaths	7	6	92	4	0	0	18	1	128

*The actual number of deaths from pneumonic plague recorded was 111 and not 67 as reported in Table 2, page 12, of the Rand Plague Committee report (*8*). Similarly, the number of deaths from bubonic infection was 9 and not 7. The text of the RPCR indicates that the category "coloured" refers to "coloured people other than Indians and Natives," but further clarification is not provided.

Time-Varying R_t for Cases of Primary Pneumonic Plague and Effectiveness of Public Health Measures

The number of deaths from pneumonic plague and back-calculated incidence for the explosive period in March are shown in Figure 4, panels A and B, respectively; Figure 4, panel C, shows the corresponding estimates of the time-varying R_t using a 7-day sliding window over which the transmission is estimated. The first estimate of R_t provided on March 14 covers all estimated dates of symptom onset from March 7 (i.e., the preceding week). The small numbers of estimated dates of onset before this time prevented our estimating transmission with any confidence; indeed, confidence intervals are much larger in the early stages of the outbreak. The fall and then rise in transmissibility that occurred March 14–19 reflect a similar pattern in the estimated incidence, giving R_t estimates of ≈2–4. As a consequence of the decreasing estimated incidence over the later part of March, the transmission estimates also decreased. By March 26, R_t decreased to <1, indicating that transmission levels were not sustainable and the outbreak was ending. The decrease in estimated transmissibility coincides with the start of the isolation process on March 18, suggesting that this strategy was probably effective.

Other factors that may also have contributed to the decrease in R_t include the local depletion of susceptible persons, because the primary pneumonic plague outbreak was manifestly confined to a limited number of dwellings within the Coolie Location and Brickfields, while others, prevented from fleeing, may well have sought to avoid contact with sick persons. The effectiveness of the cordon surrounding the Coolie Location is questionable because we have on record that 9 persons fled the area and died elsewhere. This action, and the later razing of the Coolie Location, is best thought of as part of a plan to allay the fears of white citizens.

Clustering of Primary Pneumonic Plague Cases

Of the 8 white persons with primary pneumonic plague, 7 formed a distinct cluster, including Dr. Marais, his wife, and 3 of his children living in the Fordsburg district. While nursing the Marais family, a female nurse and a male clerk acquired the disease. Within the Coolie Location, 45 of 64 cases were recorded in only 8 of 96 stands. Stand 93 housed 16 case-patients, stand 47 housed 7 case-patients, and stand 48 housed 5 case-patients. Similarly, of 20 cases recorded in the extensive Brickfields/Burgersdorp area, 15 were located at only 3

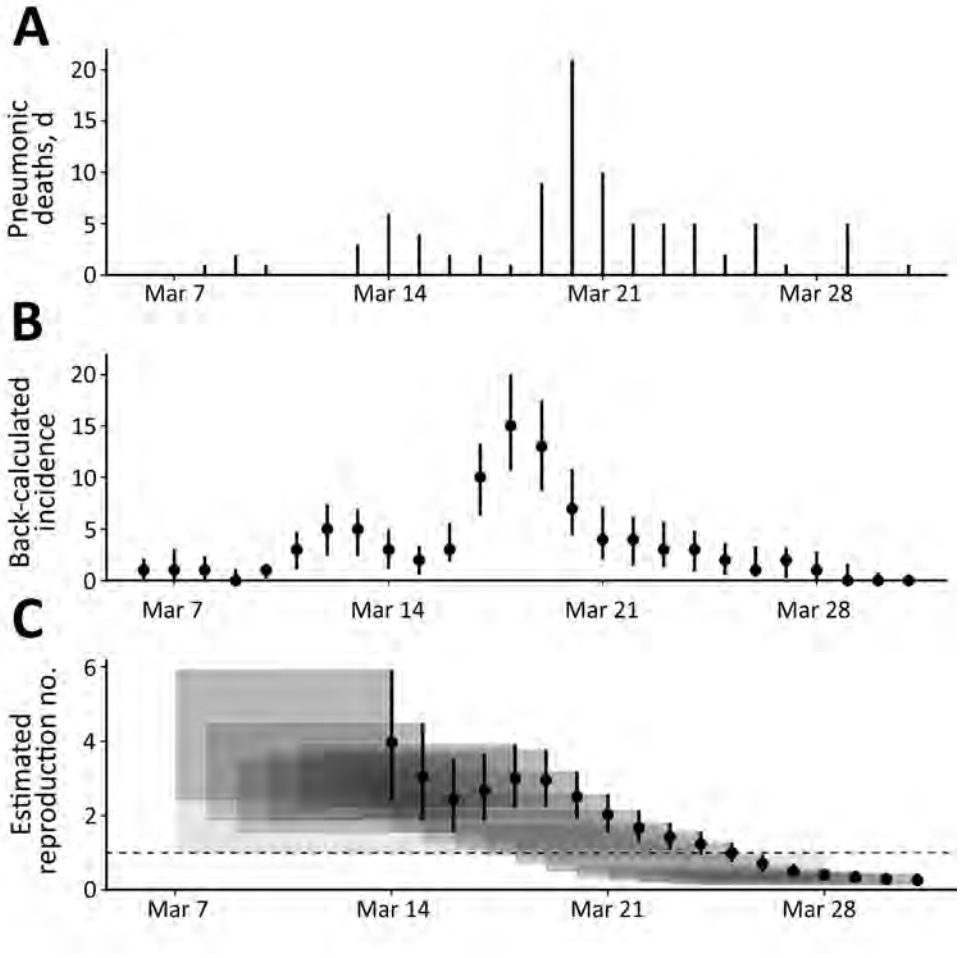

Figure 4. A) Deaths per day resulting from primary pneumonic plague in Johannesburg, South Africa, March 7–31, 1904. B) Back-calculated number of case-patients experiencing symptom onset. Circles represent most likely values; error bars represent 95% CIs. C) Transmissibility of primary pneumonic plague as measured by reproduction number, R_t. Circles represent the most likely values, error bars represent 95% CIs, and shaded polygons represent the period over which R_t was estimated. Uncertainty in the back-calculated incidence has not been accounted for in the transmission estimates, which means that the variations in the time-varying R_t are probably underestimated because the incidence curve is smoothed out somewhat by the back-calculation process (and also reduced slightly because of rounding to the nearest integer). However, because the 7-day sliding window has the effect of smoothing out the R_t estimates in any case, not accounting for the uncertainty in the back-calculation probably has a limited effect on panel C results.

addresses. Investigations of the Indian community identified 16 probable transmissions involving nursing, preparing bodies for funerals, attending funerals, or close family members. There was a considerable amount of social contact between residents of the Brickfields stands and stand 93 in the Coolie Location. The RPCR (p. 47) notes that "Among the inhabitants of Stands 47 and 93 were many fruit hawkers, and some of these were not only of the same caste, but actually came from the same village in India as the Indians employed at the S.A. Fruit Store… [inhabitants of Stand 618, Burgersdorp]… There is little doubt that they met one another at Stand 93."

In both communities, transmission seemed to follow relationship pathways involving intimate contact. However, there is no evidence that those who fled (some as far as Durban) transmitted the disease to others.

The Epizootic

The RPCR (p. 58) states that a municipal rat catcher was appointed on August 5, 1903, and from that date until March 17, 1904, a total of 8,972 rats were caught. During December 27, 1903–March 18, 1904, a total of 160 rats were sent to government laboratories, but only 13 (11.4%) of 114 examined were positive for *Y. pestis*. During March 18–July 31, 1904, a total of 1,657 rats were sent but only 95 (6.0%) of 1,583 examined were positive. Rats that were not examined had been mummified or were in an advanced state of putrefaction. There is no record of any investigation concerning rat fleas, and the species of rat was not identified, suggesting that the investigators did not yet appreciate the role of fleas in plague transmission.

The market buildings, post office, native pass office, and at least 4 restaurants and hotels near the market square were found to be infested with rats. The RPCR comments, "Upon pulling down the inside wooden lining, a terrible condition was found. Rat runs were seen throughout the [Market] buildings and a large number of dead and mummified rats were found…."

However, investigators consistently found little evidence of rats in the Coolie Location. For example, the RPCR states, "… during the course of the year 1903… the inhabitants of the Coolie Location stated most emphatically that there had been no rats in the location for some considerable time before the outbreak and the comparative absence of rats was abundantly proved… a well organized search was made, and as the result, not a dozen rats in all were found or caught."

Although rats were much more likely to be found near the market square than within the Coolie Location, a complete absence of rats in the location was unlikely. The RPCR states, "Tom Nesabi, a Basuto, was in the employ of a store-keeper at Stand 12… Tom did all the rough work in the store, carrying stacks of rice, cleaning up the shop and so on. His employer states that there were no rats in the store, but his mother states that Tom had a dog which used to kill the rats and the dog died shortly before Tom."

The Connection between the Epizootic and the Bubonic Plague Outbreak

In Johannesburg in 1904, a total of 48 cases of bubonic, mixed, and septicemic plague were reported. No information was available for 4 case-patients, but of the remainder, 15 (34%) lived or worked in buildings where rats were found and another 14 (32%) lived or worked in buildings where rats infected by *Y. pestis* were found. Furthermore, 26 (90%) case-patients associated with rats lived or worked within one half mile of the market buildings. Figure 5 illustrates the occupations of the white and native case-patients with bubonic, mixed, or septicemic plague. Most white case-patients were professional men and their family members, whereas more than half of the native case-patients worked as domestic employees.

Discussion

Arguably, the performance of the Johannesburg authorities in 1904 should be judged by the standards of the day, when antimicrobial agents were not available and the role of fleas

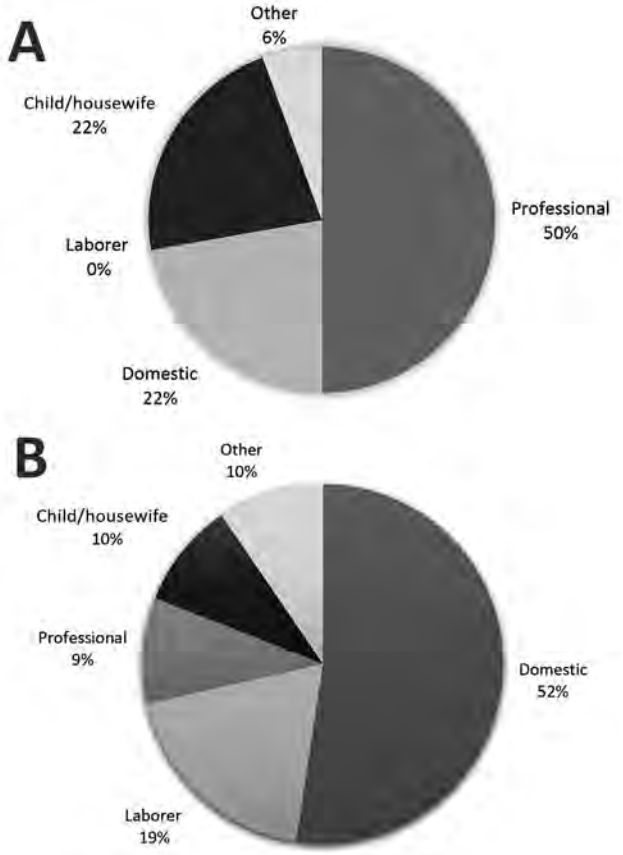

Figure 5. Occupations of white (A) and native (B) patients with bubonic, mixed, or septicemic plague, Johannesburg, South Africa, 1904.

as vectors had not yet been firmly established. Nevertheless, the standard of evidence presented in the RPCR was sufficient for us now to demonstrate that social, spatial, and environmental factors helped shape this epidemic.

A particularly noteworthy aspect of this outbreak of primary pneumonic plague was that none of the 9 escapees from the Coolie Location transmitted the disease to the wider population; the RPCR also lists lack of transmission by many other case-patients. Nevertheless, within social networks characterized by family connections, employment, caste, and so on, the disease spread rapidly. Recent outbreaks recorded in India (*21*) and China (*4*) followed this pattern in which the disease is transmitted to relatives, friends, or caregivers but not to more loosely associated contacts. The relative paucity of female patients is best explained by the preponderance of men attracted by the gold rush opportunity.

The fact that the explosive phase of the outbreak was surprisingly short-lived also deserves special attention. It seems improbable that *Y. pestis* could lose its virulence over 2 weeks, and we should look to the behavior of potential contacts and the implementation of public health measures. Here, the value of isolating infected persons was immediately appreciated by Dr. Godfrey and Mahatma Gandhi, and progress was made before municipal authorities first realized that the epidemic was fully under way.

The investigators were unable to identify the index case-patient(s) who initiated the outbreak of pneumonic plague, and there seems little chance of retrospectively doing so from the content of the RPCR. There is little evidence to confirm the conventional view that such cases originated through airborne transmission from patients with bubonic plague in whom secondary pneumonic plague had developed (mixed cases) and no evidence that a person from outside Johannesburg introduced pneumonic plague into the area.

The later outbreak of bubonic plague was the likely consequence of a modest epizootic that started in the previous year. Cases of bubonic plague were found in the environs of the market square and mainly involved white professionals and their families along with their black African servants. Surprisingly, the percentage of case-patients with pure bubonic plague who recovered was relatively high, which might suggest that this strain of *Y. pestis* was not especially virulent. However, against this argument, we have evidence that, when transmitted via the pneumonic route, the disease was almost 100% fatal.

It has been argued elsewhere that primary pneumonic plague is not as transmissible as some suppose (*22*). Indeed, it is well known that primary pneumonic plague rapidly incapacitates the patient, who is then incapable of reaching potential contacts within the most infectious period. Nevertheless, this study shows that relatively high rates of transmission were achieved in Johannesburg in 1904, as demonstrated by the peak values for the estimated time-varying R_t. These high rates are probably the consequence of crowding and spread through social networks, which facilitated transmission in a similar way to that observed in West Africa during the recent epidemic of Ebola virus disease (*23*), which is also not easily transmitted. In conclusion, we suggest that the pneumonic form of plague also remains a potentially serious threat in locations that are relatively inaccessible or that have limited capacity for a robust public health response.

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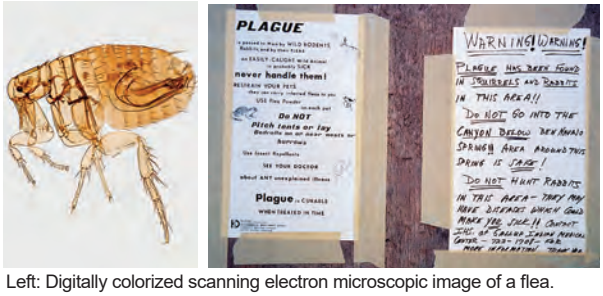
etymologia

Plague *[plāg]*

Ronnie Henry

Plague (from the Latin *plaga*, "stroke" or "wound") infections are believed to have been common since at least 3000 BCE. Plague is caused by the ancestor of current *Yersinia* (named for Swiss bacteriologist Alexandre Yersin, who first isolated the bacterium) *pestis* strains. However, this ancestral *Y. pestis* lacked the critical *Yersinia* murine toxin (*ymt*) gene that enables vectorborne transmission. After acquiring this gene (sometime during 1600–950 BCE), which encodes a phospholipase D that protects the bacterium inside the flea gut, *Y. pestis* evolved the ability to cause pandemics of bubonic plague. The first recoded of these, the Justinian Plague, began in 541 ACE and eventually killed more than 25 million persons.



Left: Digitally colorized scanning electron microscopic image of a flea. Fleas are known to carry a number of diseases that are transferable to humans through their bites, including plague, caused by the bacterium Yersinia pestis. Photo: Centers for Disease Control and Prevention (CDC), Janice Haney Carr. Right: Plague warning signs posted in regions where plague has been discovered. In remote areas with little human habitation, the most appropriate action may be to post signs on the roads entering the epizootic area to warn people, and provide information on personal protection and plague prevention. Photo: CDC, 1993.

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Dangers of Noncritical Use of Historical Plague Data

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Researchers have published several articles using historical data sets on plague epidemics using impressive digital databases that contain thousands of recorded outbreaks across Europe over the past several centuries. Through the digitization of preexisting data sets, scholars have unprecedented access to the historical record of plague occurrences. However, although these databases offer new research opportunities, noncritical use and reproduction of preexisting data sets can also limit our understanding of how infectious diseases evolved. Many scholars have performed investigations using Jean-Noël Biraben's data, which contains information on mentions of plague from various kinds of sources, many of which were not cited. When scholars fail to apply source criticism or do not reflect on the content of the data they use, the reliability of their results becomes highly questionable. Researchers using these databases going forward need to verify and restrict content spatially and temporally, and historians should be encouraged to compile the work.

In an article by Jones and Nevell (*1*), the authors argue that improved access to historical data through digitization projects has benefited research in different scientific fields. However, they also point out that digitization has some unintended consequences. A key issue they identified is the loosening of the rigorous standards of evidence and interpretation scientific researchers typically demand within their own disciplines (*1*). Although scholars regularly reprimand colleagues for misrepresenting evidence and misusing data to make arguments that their material cannot support, such issues are less frequently addressed when data sets transcend the border from one scientific discipline to the next. This discrepancy poses a problem in an age of greater interdisciplinary research.

Here we focus on the most frequently used record of historical plague outbreaks in Europe. This information was originally compiled >40 years ago by Jean-Noël Biraben as part of his 2-volume work, Les hommes et la peste en France et dans les pays méditerranéens, which documents plague outbreaks from the Black Death (1347–1352) to the 19th century (2,3). Using a digitized version of this data set (https://zenodo.org/record/14973), which includes a limited number of outbreaks in northern Africa, authors have boasted impressive collections of documented European plague outbreaks: 6,929 plague outbreaks across Europe during 1347–1900 (4), 7,711 outbreaks across Europe and Asia during 1347–1900 (5), 5,559 outbreaks across Europe and northern Africa during 1347–1760 (6), and 6,656 outbreaks across Europe during 1347–1760 (7). In one of these studies, the Biraben data set was supplemented with additional outbreaks from Russia and Turkey gleaned from secondary literature (5).

Biraben had the ambition of constructing a pan-European overview of recurring plague outbreaks, and although his work at the time was an extraordinary feat of scholarship, a complete documentation of the occurrence of plague throughout Europe could not be adequately concluded by any single researcher. From a historian's perspective, the most fundamental problem with Biraben's data is the lack of systematic justification for the sources used and only cursory referencing of the original documents. However, this article is not meant to be a criticism of Biraben's 1970s work but of the research published decades later by authors who interpreted Biraben's results at face value. Scholars who have used this data set have not applied adequate source critique expected within the field of history, failing to pose basic questions concerning how the data were collected and what they represent. The 4 aforementioned studies (*4–7*) are not the only instances in which the Biraben data set were not used critically; in fact, there are many examples (*8–13*). However, Büntgen et al., Schmid et al., and Yue et al. are the first to use a digitized version of the data set, which not only causes specific problems but also sets a dangerous precedent for future research (*4–7*).

Noncritical Use of Historical Plague Databases

In 2012, Büntgen et al. presented the digitized version of the Biraben data set in a short correspondence piece (*4*). This publication reflected little on the limitations of the data. The only concerns Büntgen et al. addressed were the imprecise geographic descriptions that impeded exact localization and the annual resolution of the data that

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precluded tracking of outbreaks within the same year. However, through digitization and subsequent publication in a top-ranked journal, the 4-decade-old data set was imbued with a false aura of trustworthiness and the impression of being new historical research. Subsequently, others used the resulting database noncritically, in some cases not referencing the original Biraben data at all (*6*). The perpetual reuse of these data without structural effort to add new archival evidence has given the impression that our knowledge of historical plague outbreaks is saturated and, moreover, has obscured the fact that large amounts of innovative research on the spatiotemporal spread of plague has been conducted by others since the mid-1970s.

These problems can be demonstrated through the maps that have been produced on the basis of Biraben's data. Büntgen et al. provided a map in the introduction to the database (*4*), and a copy (Figure 1) was later included in an article on the supposed link between plague spread and navigable rivers (*6*). However, looking at the map, 2 problems surface immediately. First, France is depicted as the major epicenter of plague activity across 4 centuries, something even accepted as a face-value truth by some scholars (*13*). However, more than likely, the concentration of plague activity reflected nothing more than the fact that Biraben was French and had exceptional knowledge of the archives in France (*14*). Second, there are vast areas where no plague was recorded across the whole of the late-medieval and early modern periods. For example, hardly any recorded plague outbreaks appear in a period of 4 centuries in much of the Low Countries in western Europe.

If we were to focus exclusively on the initial Black Death outbreak (1347–1352), this evidence would be in agreement with the literature of the mid-1970s. At that time, the consensus was that the Black Death somehow did not reach most parts of the Low Countries (*2*,*15*,*16*). Later this view was refuted, and proof that the Black Death was present in the Low Countries was established (*17*). In fact, a newly compiled data set of plague mentions shows that many regions of the Low Countries were hit by the Black Death (Figure 2) (*18*). When we add data of plague mentions across the entirety of the late Middle Ages (1349–1500), this map becomes filled to an even greater extent

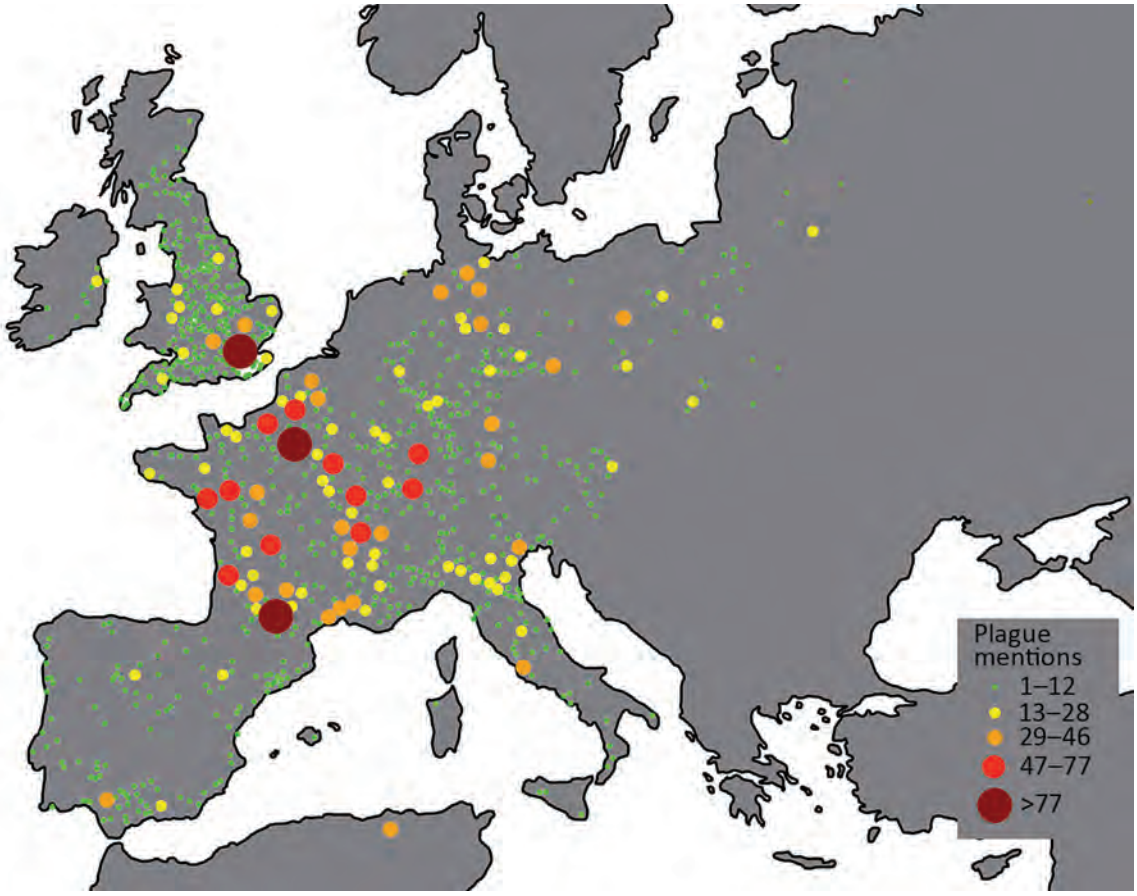

Figure 1. Plague outbreaks in Europe, 1347–1760. Map produced on the basis of data from Biraben (*2*,*3*). Map provided courtesy of Yue et al. Navigable rivers facilitated the spread and recurrence of plague in pre-industrial Europe. Sci Rep. 2016;6:34867 (*6*).

(Figure 3) (*18*), without even adding plague data from the 16th and 17th centuries, when many plagues, such as those in 1624–1625 and 1635–1636, hit almost every recordable locality of the Low Countries, both urban and rural (*19*). Biraben's data set, therefore, is not only hindered by being outdated but also by having crucial gaps in spatial coverage, leaving out large parts of the Low Countries, Denmark, Scotland, Ireland, and Central Europe (*20*). Even countries well known in the literature for having experienced numerous plagues of exceptional severity across the Middle Ages and during the early modern period, such as Italy (*14*,*21*), have very few plague markers on the maps produced with Biraben's data.

Biraben Data Set

In the examples we mention, 3 transgressions have been attributed to the scholars using the Biraben data set. First, reflection on the data collection process has been improper; second, what the data represent has not been recognized; and third, critique of the original sources has been inadequate. We argued that a critical consideration of any of these 3 elements would have led to the conclusion that the data set could not have been used at face value.

First, we address what the Biraben data represent. Three previously published graphs display the same data set (Figures 4–6) and yet, peculiarly, present the data differently. Büntgen et al. described the data as plague outbreaks,

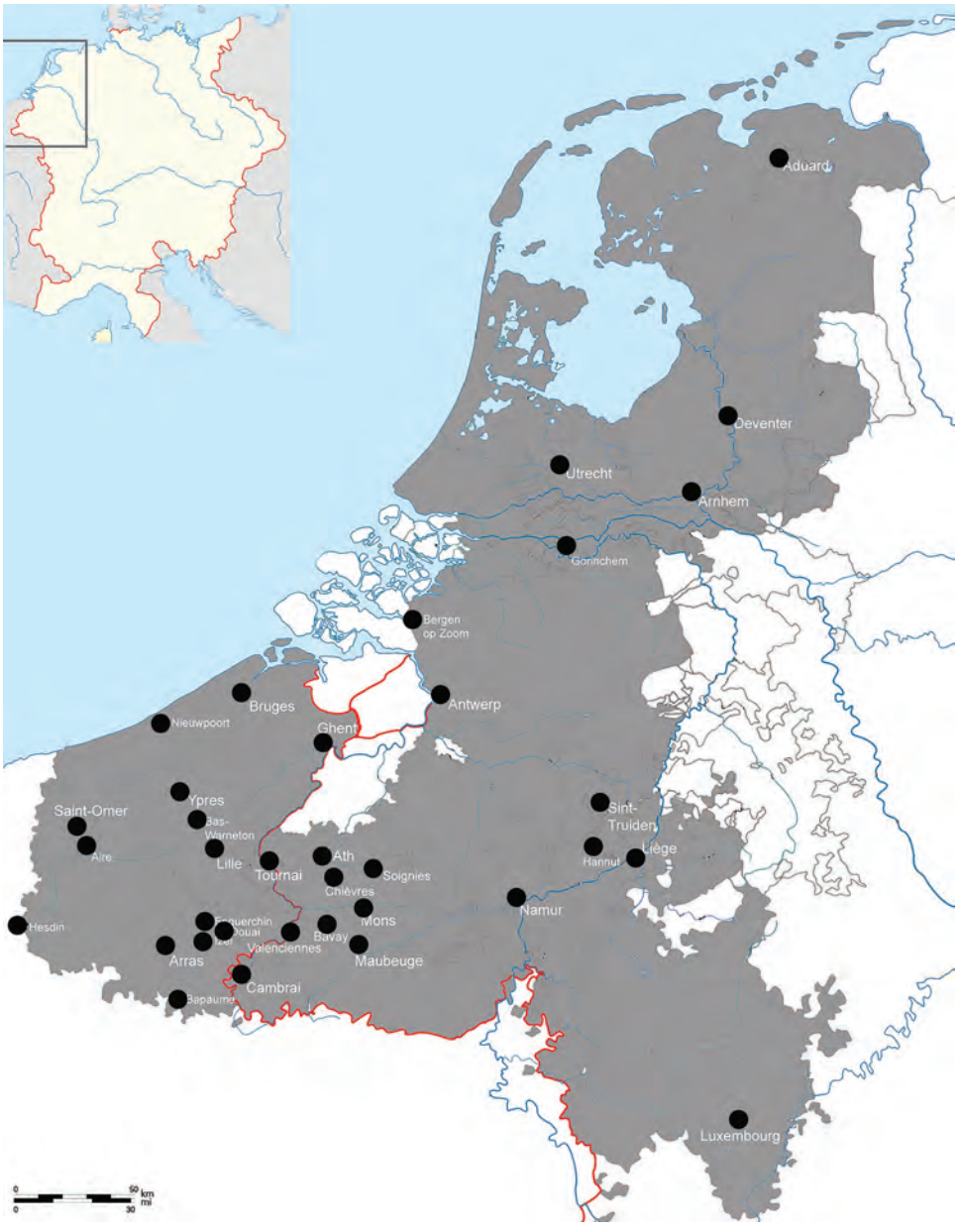

Figure 2. Plague mentions during the Black Death outbreak, Low Countries, 1348–1352 (*18*). Inset shows location of the Low Countries in western Europe.

Schmid et al. referred to the data as plague incidence, and Voigtländer and Voth regarded the Biraben data as plague epidemics. These 3 terms are not interchangeable. The lack of clarity on what the data set represents has led to the drawing of false conclusions.

Which of the 3 graphs uses the correct terminology? In fact, none of them do. The data collected by Biraben represent the availability of sources mentioning plague and not the severity or pervasiveness of the disease in any given year. More narrowly defined, the data set represents those sources Biraben was able to find in the timespan of researching his book while working in Paris. In no way does this data set represent the full coverage of all historical plague activity throughout the whole of Europe.

Furthermore, the Biraben data set has an urban bias. Most of the mentions of plague occurrences (particularly those outside of France) pertained to cities, perhaps because urban documents were more easily accessible. For example, in the database used to create Figure 1, the city of Paris was indicated as having 90 plague outbreaks, yet the middle-sized town of Soissons ≈100 km to the northeast only experienced 3 during the same period (1347–1760). We must view this result skeptically, given that this number would have meant that a new plague outbreak in Paris occurred on average every 3.5 years over a period of 320

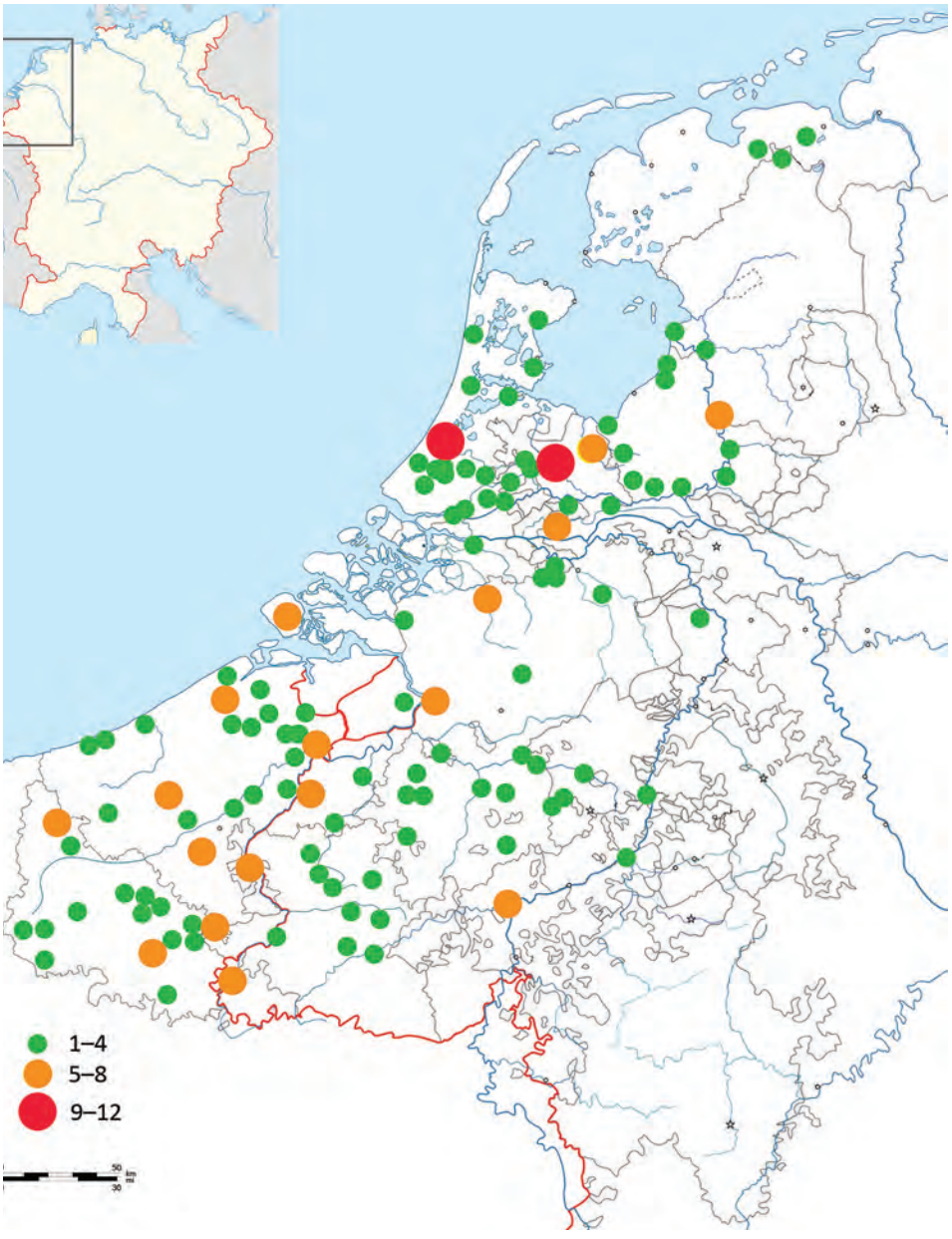

Figure 3. Plague mentions taken from archival sources, Low Countries, 1348–1500 (*18*). Inset shows location of the Low Countries in western Europe.

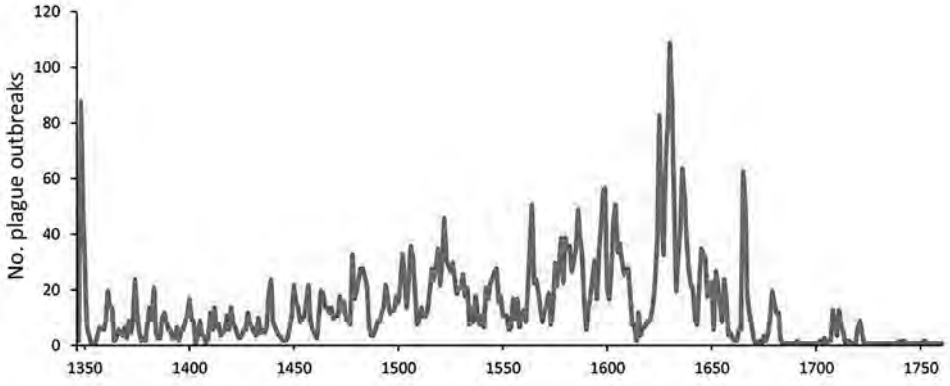

Figure 4. Plague outbreaks in Europe, 1347–1900. Graph produced on the basis of data from Biraben (*2,3*). Graph provided courtesy of Büntgen U et al. Digitizing historical plague. Clin Infect Dis. 2012;55(11):1586–8 (*4*). By permission of Oxford University Press.

years (the last plague in northern France was in the late 1660s). This average rate contradicts a wealth of scholarship that suggests that, after the Black Death, the average interval separating 2 plague occurrences in Northwest Europe was around 11–12 years in the 14th century, decreasing to 15–20 years by the late 15th century (*22*), and being anything from 10 to 20 years by the 17th century (*18*,*23*). Ultimately, by confusing mentions of plague in available sources as a representation of individual incidences or outbreaks of the disease, Biraben's data set has led to a gross overestimation of plague in big cities and a gross underestimation of plague in smaller towns and villages. This confusion is problematic, considering some scholars have linked plague spread to commerce (*9*), trade routes (*7*), or distance to navigable rivers (*6*), all factors highly conducive to the development of cities (*24*). Misinterpretation of Biraben's data set also feeds into a narrative describing plague as a fundamentally urban phenomenon when research is beginning to reveal this perception to be a fallacy (*14*,*19*).

Next, we address the question of how the data were collected. The collection process did not aim to attain a representative sample of all historical plague outbreaks across Europe, which would have been necessary for a data set attempting to offer a long-term pan-European overview. As previously mentioned, the data set has crucial gaps in geographic coverage; it does not provide an unbiased sample for every region in Europe and, within many regions, provides a clear urban bias. However, substantial gaps are evident in temporal coverage as well. For instance, the original data set gives the impression that the 16th and 17th centuries witnessed much higher plague activity than the 14th and 15th centuries. Yue et al. are especially not critical in this regard and suggest that more severe plague outbreaks occurred during the Thirty Years' War (1618–1648) than other periods (*6*), an association also suggested in other studies (*11*). Despite the fact that long and devastating wars occurred similarly throughout the 14th through 16th centuries in many parts of Western Europe, no consideration is given to why the Thirty Years' War would set off more severe plagues than, for example, the Hundred Years' War (1337–1453). Furthermore, absence of evidence cannot be interpreted as evidence of absence in the case of late-medieval plague outbreaks (*25*). The literature has explicitly pointed out the paucity of quantifiable evidence for the recurring epidemics of the late Middle Ages (1349–1500)

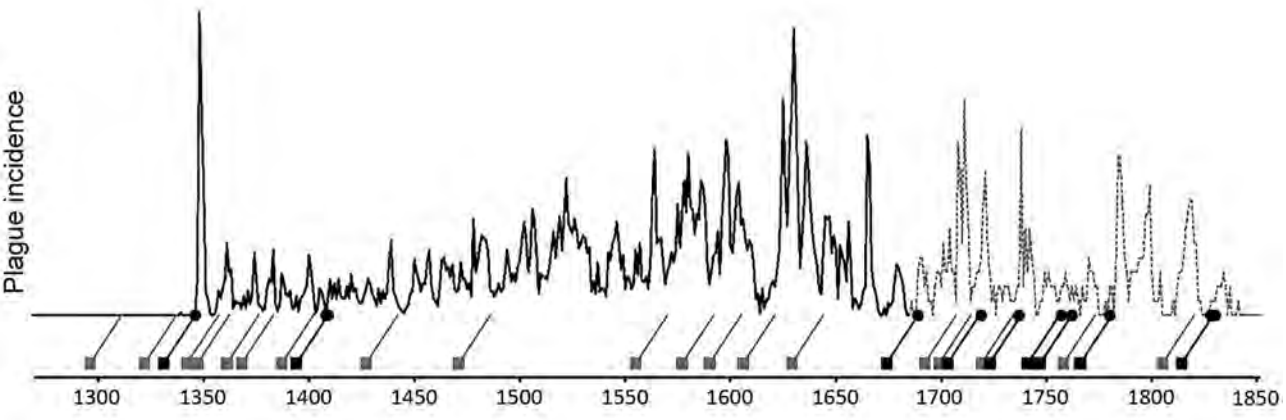

Figure 5. Plague incidences in Europe, 1347–1900. Graph produced on the basis of data from Biraben (*2,3*). Graph provided courtesy of Schmid BV et al. Climate-driven introduction of the Black Death and successive plague reintroductions into Europe. Proc Natl Acad Sci U S A. 2015;112:3020–5 (*5*).

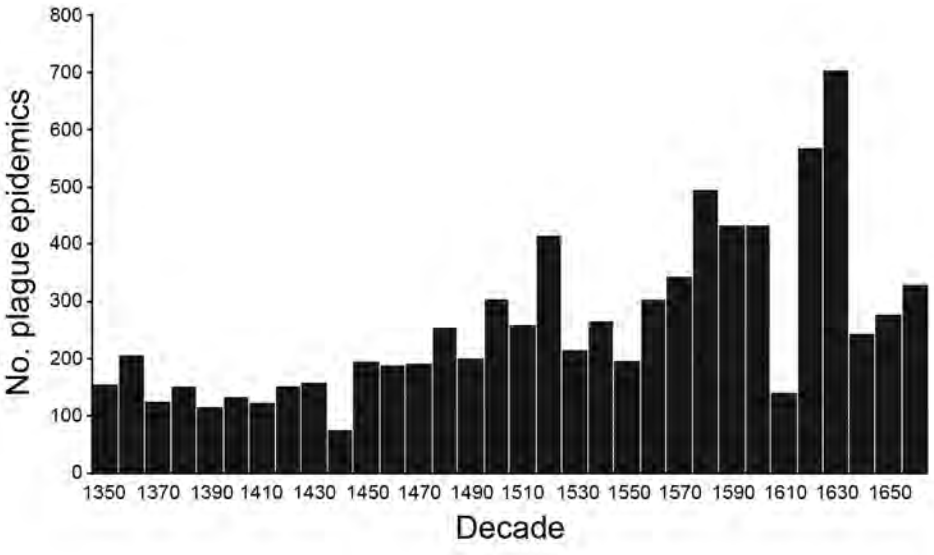

Figure 6. Plague epidemics in Europe, 1350s–1660s. Graph produced on the basis of data from Biraben (*2*,*3*). Modified graph provided courtesy of Voigtländer N, Voth H-J. Gifts of Mars: warfare and Europe's rise to riches. J Econ Perspect. 2013;27:165–86 (*12*).

(*26*); however, this paucity is also related to a polarization of the research focus between the initial Black Death outbreak and early modern outbreaks. To interpret both the incomplete recording of sources by Biraben and the less forthcoming nature of late-medieval plague documents as evidence of lower plague activity is unsatisfactory. This interpretation accepted by some researchers is yet another reason why the noncritical use of the data leads us to consternation over the results and interpretations produced.

Last, we address the third and final problem, the absence of source critique. Despite elucidating the basic symptoms one would expect to see with bubonic plague, such as fever, buboes, and vomiting, Biraben never justified how he came to identify certain localities in certain years as experiencing plagues in his own data set, and a structural overview of the original sources he used is missing. Because of his inadequate citation practices, we have little hope of checking the validity of Biraben's assertions, which undermines the reliability and accuracy of the data set that has been reused on a number of occasions by others. A further problem with not knowing the original sources is that equal weight in terms of accuracy and reliability cannot necessarily be attributed to different reference types (e.g., resources allowing for quantification of mortality rates, administrative sources with qualitative direct mentions of plague, and narrative sources with qualitative direct mentions of plague) (*17*). This problem is magnified further in light of increasing interest in germ theory–based nosology and the retrospective diagnosis of diseases (*1*). Medieval historians question the methods used for identifying diseases in the past (*27*,*28*). Laboratories have confirmed *Yersinia pestis* in burial sites connected to the initial Black Death outbreak of 1347–1352 (*29*), but few works have explicitly linked *Y. pestis* to burial sites of other specific, recurring, late-medieval plague outbreaks (*25*).

Accordingly for other late-medieval plagues, we are often reliant on anecdotal references by contemporaries in the absence of laboratory or even epidemiologic evidence. Using references by contemporaries is problematic, given the terms *peste* or *pestilentia* were often indiscriminate references to all sorts of afflictions (*30*). Only starting roughly around the second half of the 15th century do we find more explicit differentiation in the descriptions of diseases in the Low Countries and Italy (*31*,*32*), and even these descriptions still were by no means systematic. For many of the putative late-medieval outbreaks after the initial Black Death, most literary sources do not mention key signs or symptoms, such as the combination of buboes, fever, and a rapidity of death. When signs or symptoms are referenced, they are fragmentary and localized and, therefore, difficult to use as evidence for the occurrence of general epidemic outbreaks over large territories. Even in the early modern period, when disease differentiation became much more commonplace in sources (*19*,*33*), not every death spike could be accounted for, especially during periods such as the Thirty Years' War when a host of other diseases were present alongside plague (*18*). We must also bear in mind that even in modern times the diagnosis of plague on the basis of signs and symptoms is problematic for trained medical professionals (*34*). The only way to determine the etiologic agent responsible for a disease is by using molecular diagnostic tests. In other words, we are not sure that the mentions of plague identified originally by Biraben were in fact plague at all, especially for the medieval period, and as mentioned, we have no way of checking Biraben's data set without citations to the original manuscripts. This problem is further illuminated by some of the plagues Biraben identified, such as the plague of 1437–1440, which occurred during a period of extreme cold weather (*35*,*36*) and manifested as harvest failures and famine-related

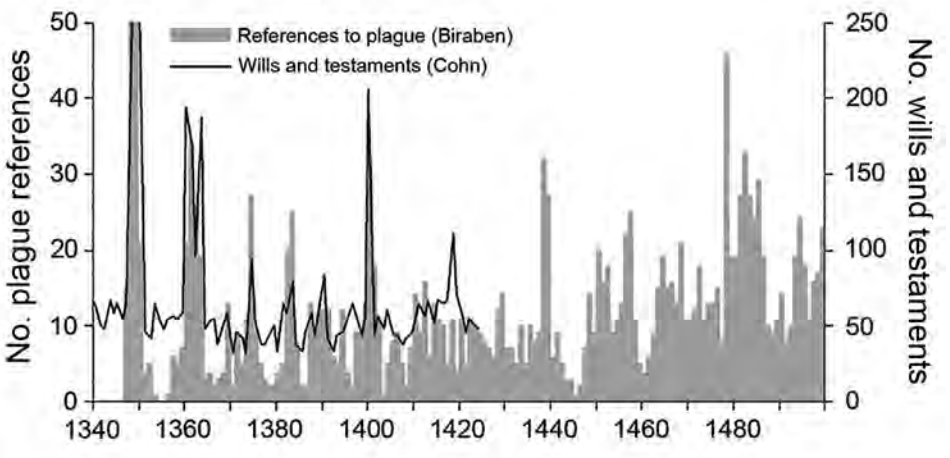

Figure 7. Comparison of Biraben's and Cohn's historical plague data sets. Biraben's data set included references to plague in various types of documents from Italy, Iberia, France, the Low Countries, and the British Isles that were written during 1345–1499 (*2*,*3*). Cohn's data set included information from a select set of documents (wills and testaments of 9 cities) that were drafted during 1340–1424 (*21*). Graph provided courtesy of Campbell B. The great transition: climate, disease and society in the late-medieval world. Cambridge: Cambridge University Press; 2016 (*39*).

diseases (*37*); research has suggested that waterborne infections were more likely the cause of this pestilence (*38*).

Moving Forward

We suggest 3 necessary steps to take to rectify some of the mistakes made with the use of digital databases of plague, which were often constructed by using Biraben's data. First, if we are going to pursue the Biraben database, we at least need to check his plague references with other forms of evidence rather than taking him at his word. Historians have done this in previous years with care by using only a select geographic sample of Biraben's evidence and comparing the data to other quantifiable indexes, such as the temporal distribution of will production (Figure 7) (*39*).

Second, scholars looking to test certain hypotheses, such as the effect of navigable rivers, commercialization, trade routes, or climatic fluctuations, should do so by using a historical plague data set of a much more restricted geographic or temporal scope to limit problems such as the inequalities in availability of source material or scholarly attention. We need to escape the confines of excessively localized and excessively macro scales and, instead, reap the benefits of a more workable historical laboratory at a regional level (*40*). This restriction method is similar to how epidemiologists try to control for confounders by limiting their data to a specific group of persons sharing a specific characteristic. A way of implementing this in practice is by moving away from using data sets that consolidate different kinds of references to plague through different kinds of evidence (often without justification) and moving toward using data sets that can show differences in plague characteristics by comparing the same type of source material, a method that offers greater control. For example, by using only data from church burial records from the 16th and 17th centuries over many parts of Europe, a systemic comparison can be performed between urban and rural localities over time and with regard to plague severity, seasonality, pervasiveness, and various kinds of selectivity (*14*,*18*). Epidemiologic information on plagues is better provided by using this approach than by using a random set of diverse manuscripts that may or may not refer to plague.

Third, it is clear that new databases of plague incidence have to be compiled by historians using data sets besides Biraben's. Given that this task is laborious and time-consuming, incentives are needed for historians to compile this information. One incentive could be the formal inclusion of trained medieval historians in large interdisciplinary scientific teams interested in charting and explaining the spread of plague.

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Recognition of Azole-Resistant Aspergillosis by Physicians Specializing in Infectious Diseases, United States

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Release date: December 14, 2017; Expiration date: December 14, 2018

Learning Objectives

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

- Assess clinician awareness of and experience with azole-resistant *Aspergillus fumigatus* infections based on an Emerging Infections Network survey of US infectious disease physicians
- Describe availability and use of *A. fumigatus* susceptibility testing in clinical settings
- Identify regional differences in availability and use of *A. fumigatus* susceptibility testing in clinical settings.

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Infections caused by pan–azole-resistant *Aspergillus fumigatus* strains have emerged in Europe and recently in the United States. Physicians specializing in infectious diseases reported observing pan–azole-resistant infections and low rates of susceptibility testing, suggesting the need for wider-scale testing.

Infections with strains of *Aspergillus fumigatus* that are resistant to all azole antifungal medications have become common in western Europe and have been documented in other regions since 1998 (*1*,*2*), but until recently, had

not been reported in the United States (*3*). The Infectious Diseases Society of America recommends voriconazole, an azole antifungal medication, as first-line therapy for invasive *A. fumigatus* infections (*4*). Pan–azole-resistant *A. fumigatus* strains that have TR_{34}/L98H and TR_{46}/Y121F/T289A mutations of the *Cyp51A* gene have been associated with higher rates of treatment failure and death (*2*). These mutations may be linked to agricultural and environmental use of azole fungicides (*5*–*7*) and not previous azole therapy, because patients with these infections frequently lack prior clinical exposure to azoles (i.e., were azole-naive) (*2*). These persons were likely exposed to *A. fumigatus* that developed resistance after exposure to environmental fungicides. Limited genetic diversity between strains isolated from noncontiguous countries suggests a common origin with capacity for extensive geographic spread (*1*).

Recent limited data suggest a low rate of illness caused by azole-resistant *A. fumigatus* exists in the United States (*1*,*3*,*8*). Isolates with mutations conferring pan-azole resistance have recently been documented (*9*–*11*); however, little is known about the broader epidemiology, because there is no national surveillance of *Aspergillus* spp. In addition, little is known about the degree to which *A. fumigatus* resistance testing is available to US clinicians. These data could inform future testing and clinical practice.

The Study

The Emerging Infections Network (EIN) surveyed US infectious disease physicians to better assess the availability of *A. fumigatus* susceptibility testing in clinical settings, the frequency with which clinicians request susceptibility testing, and the degree to which clinicians have observed azole resistance. The EIN is a provider-based emerging infections sentinel network supported by the Centers for Disease Control and Prevention and sponsored by the Infectious Disease Society of America (*12*). During May–June 2016, EIN distributed surveys to 1,584 members by email and fax; 709 (45%) responded.

Of the 709 respondents, nearly half (348, 49%) were familiar with the concept of azole-resistant *A. fumigatus*; 100 (14%) were aware of the possible link to agricultural or environmental antifungal products. During the previous year, 364 (51%) reported treating ≥1 patient who had been diagnosed with aspergillosis. Of those, 136 (38%) reported clinical failure of therapy for ≥1 patient, despite 290 (80%) physicians having used therapeutic drug monitoring to titrate azole therapy. Nine (2%) treating physicians reported observing azole resistance in an azole-naive patient.

Overall, 224 (62%) treating physicians who responded had access to susceptibility testing; 75 (21%) lacked access; and an additional 65 (18%) were unsure of availability. Of those with access, 182 (81%) reported that testing was physician-prompted; 162 (72%) reported that testing occurred off-site rather than in their hospital. For those reporting off-site testing, 8 (4%) physicians typically received results within 1 week and 42 (19%) reported receiving results ≥3 weeks after request, excluding the minority (n = 6, 4%), who were unsure. Of the 224 physicians who had access to susceptibility testing, 127 (57%) reported that ≥1 of their patients had an isolate tested, and 56 (25%) reported that >50% of patients had isolates tested. Forty-one (19%) reported a patient isolate with resistance to ≥1 azole, and 16 (7%) reported a patient with a pan–azole-resistant isolate.

Sixteen (8%) physicians practicing in the southern and 14 (9%) practicing in the western US census regions reported seeing >8 patients who had aspergillosis during the previous year, compared with 5 (3%) in the Northeast and 9 (5%) in the Midwest ($\chi^2 = 6.3$, $p = 0.18$). Other findings were generally similar across regions, including proportions reporting clinical failure, azole resistance in azole-naive patients, susceptibility testing availability, routine versus physician-prompted testing, and location of testing.

Of 224 physicians with access to susceptibility testing, 8 (16%) of 51 physicians in the South reported that >50% of their patients' isolates were tested, compared with 17 (27%) of 63 in the Midwest, 8 (21%) of 37 in the Northeast, and 22 (31%) of 70 from the West ($\chi^2 = 4.3$, $p = 0.37$). Of 51 physicians in the South, 13 (26%) reported observing isolates resistant to ≥1 azole, compared with 9 (14%) of 63 from the Midwest, 5 (14%) of 37 from the Northeast, and 13 (19%) of 70 from the West ($\chi^2 = 10.2$, $p = 0.04$). Pan–azole-resistant isolates were reported by 4 (8%) of 51 physicians in the South, 7 (11%) of 63 in the Midwest, 2 (5%) of 37 in the Northeast and 3 (4%) of 70 in the West ($\chi^2 = 4.4$, $p = 0.36$).

In summary, approximately 50% (348/709) of surveyed infectious disease physicians were familiar with azole-resistant *A. fumigatus* and 14% (100/709) were aware of a possible link to environmental fungicide use. Of physicians who had treated patients diagnosed with aspergillosis within the past year, 21% (75/364) lacked access to susceptibility testing and 57% (127/224) who had access tested an isolate in the previous year. A small proportion of 19% (41/224) reported observing any azole resistance and only 7% (16/224) reported pan-resistance. Of note, physicians in the southern states more commonly observed resistance to ≥1 azole, compared with physicians from other regions.

Because only a small fraction of patients with invasive aspergillosis have a positive culture (*13*), a survey of resistance in culture-positive aspergillosis is not necessarily representative of all cases; but this fact highlights the importance of monitoring available cultures to inform broader practice. Another gap in our understanding of azole-resistant *A. fumigatus* is that the Clinical and Laboratory Standards Institute has not established breakpoints for azole susceptibility for *A. fumigatus* because inadequate clinical data exist

to support breakpoints. The institute uses epidemiologic cutoff values, reflecting the minimal inhibitory concentration of 95% of wild-type isolates (*13*). However, there is some evidence that infection with resistant isolates by currently used thresholds is associated with worse outcomes in patients treated with azole monotherapy (*13*). Patients with hematologic or oncologic diseases are more likely to be infected with azole-resistant aspergillosis, and those with resistance have been shown to have higher case-fatality rates (*2*). However, it remains unclear to what degree these failures are attributable to underlying immunosuppression in these patients or to resistance-mediated treatment failure.

Conclusions

Our findings support that azole-resistant *A. fumigatus* infections, including those with pan-azole resistance, are occurring in the United States, and that broader susceptibility testing may be warranted to guide patient care. Systematic surveillance for aspergillosis, including collection of clinical data and isolates, could aid in detecting emergence of regional resistance patterns, assessing the role that resistance plays in treatment failure, and determining locally tailored treatment options. Awareness by physicians of azole-resistant aspergillosis and the possible link to environmental fungicide use are essential.

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Postmortem Findings in Patient with Guillain-Barré Syndrome and Zika Virus Infection

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Postmortem examination results of a patient with Guillain-Barré syndrome and confirmed Zika virus infection revealed demyelination of the sciatic and cranial IV nerves, providing evidence of the acute demyelinating inflammatory polyneuropathy Guillain-Barré syndrome variant. Lack of evidence of Zika virus in nervous tissue suggests that pathophysiology was antibody mediated without neurotropism.

Guillain-Barré syndrome (GBS) is an uncommon autoimmune disorder characterized by progressive, bilateral weakness and diminished deep tendon reflexes due to peripheral nerve damage. GBS is typically triggered by an acute infection and, less frequently, by vaccination (*1*). GBS has been associated with infection by Zika virus, a flavivirus transmitted primarily by *Aedes* species mosquitoes (*2*), and countries have reported increased GBS incidence during Zika virus outbreaks (*3*–*5*). Reports suggest Zika virus may result in a hyperacute immune response or have a direct viral neuropathic effect contributing to GBS (*6*). Further, case reports and series have noted higher rates of cranial neuropathy, such as facial palsy and paresthesia, among GBS patients with evidence of Zika virus infection, suggesting that the cranial nerves may be targeted by either virus or antibody (*3*,*6*–*9*).

Mortality rates among GBS patients in North America and Europe vary from 3% to 7% (*1*), and death often results from respiratory failure, autonomic dysfunction, or deep vein thrombosis (*10*). In Puerto Rico, the GBS in-hospital mortality rate before the introduction of Zika virus was estimated at 4% (*11*). Postmortem investigations of GBS are rare, but results may indicate underlying pathophysiologic mechanisms.

During a Zika virus epidemic in Puerto Rico in February 2016, an islandwide surveillance system was implemented to identify GBS cases and provide Zika virus diagnostic testing (*9*). Fatal GBS cases could be reported, and postmortem investigations were incorporated into an established fatal case surveillance system (*12*). Such investigations were implemented to clarify the pathophysiology of GBS patients with Zika virus infection.

The Study

In August 2016, a 78-year-old man living in the San Juan metropolitan area with a medical history of hypertension (for which he was taking amlodipine), diabetes, asthma, and prostate cancer visited a hospital emergency department with a 4-day history of worsening paresthesia of the lower and upper extremities and progressive bilateral lower and upper extremity weakness (Figure 1). Computed tomography without contrast of the head found no acute intracranial or other abnormalities. The patient was given albuterol nebulizer treatment, ipratropium bromide, and ceftriaxone and discharged home on the same day. Three days later, the patient returned to the emergency department with worsened weakness. He was admitted to the intensive care unit with respiratory distress and was intubated. Hospital staff suspected GBS due to monophasic illness progression, symmetric weakness, and loss of deep tendon reflexes. A 5-day course of intravenous immunoglobulin (30 g/d) was initiated, and serum and urine specimens were collected for Zika virus diagnostic testing. On day 7 after onset of neurologic illness, the patient had acute kidney injury and hyperuricemia. Electrodiagnostic studies performed 10 days post-onset identified demyelinating polyneuropathy consistent with GBS (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-1331-Techapp1.pdf). Given clinical features and electrophysiologic findings, the patient met level 2 of the Brighton Collaboration Criteria for GBS diagnostic certainty (*13*).

The patient was mildly responsive by day 15 and thereafter became unresponsive but hemodynamically stable. On day 16, pneumonia was diagnosed, and a

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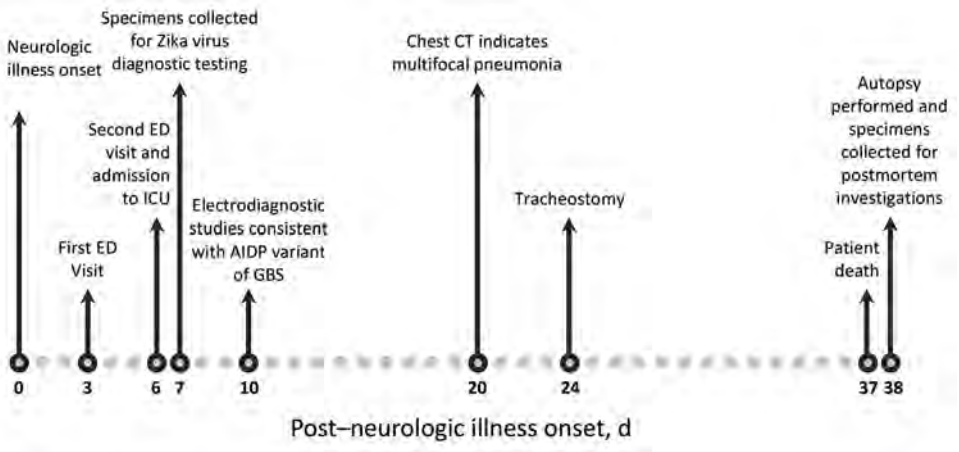

Figure 1. Timeline of key events surrounding the illness of a patient with GBS and confirmed Zika virus infection, Puerto Rico, 2016. AIDP, acute inflammatory demyelinating polyneuropathy; CT, computed tomography; ED, emergency department; GBS, Guillain-Barré syndrome; ICU, intensive care unit.

course of vancomycin was started. Multidrug-resistant forms of *Acinetobacter baumanii* were found in sputum (collected on day 14) and fecal samples (collected on day 18), and multidrug-resistant *Enterobacter aerogenes* and *Klebsiella pneumoniae* were isolated from sputum (collected on day 27). Computed tomography of the chest performed on day 20 indicated multifocal pneumonia with parapneumonic effusions involving the entire right lung and part of the left lung. Following an episode of cardiac arrest on day 23, tracheostomy for chronic respiratory failure was performed on day 24. The patient died on day 37 due to cardiac arrest and respiratory failure complicated by bilateral pneumonia, sepsis, and acute renal failure.

An autopsy was performed, and external evaluation noted edema of the left hand, scrotum, penis, legs, and feet, as well as decubitus ulcers on the back and in the gluteal area. Internal examination showed bilateral pleural effusions, hepatic congestion, ascites, and cardiomegaly. Microscopic examination showed focal pneumonia, acute tubular necrosis, and prostate adenocarcinoma. Brain examination showed subacute watershed infarcts of the left cerebral hemisphere; occasional perivascular lymphomononuclear cell infiltrates were also observed.

We detected Zika virus RNA by reverse transcription PCR (RT-PCR) in urine and Zika virus and dengue virus IgM antibodies in serum (both collected on day 7). Additional laboratory testing of premortem specimens was negative for *Shigella*, *Salmonella*, *Yersinia*, enteropathic isolates, *Campylobacter jejuni*, *Legionella*, *Mycoplasma pneumoniae*, HIV, influenza A and B, cytomegalovirus, and Epstein-Barr virus. We performed Zika virus RT-PCR and immunohistochemical (IHC) assays on formalin-fixed paraffin-embedded autopsy tissue specimens. We detected neither RNA nor antigen for Zika virus in any autopsy specimens tested (Zika virus RT-PCR, cranial nerve VII, sciatic nerve, and spinal cord including cauda equina; IHC, brain, spinal cord, sciatic nerve, and multiple solid organs).

We analyzed postmortem specimens of the peripheral and central nervous system and specimens from other organs by microscopic examination and special stains. A section of cranial nerve IV showed mononuclear lymphocytic infiltrate and mild myelin loss, while sections of the sciatic nerve showed inflammation-associated myelin loss. Myelin loss was highlighted by luxol fast blue staining (Figure 2). IHC staining for CD68 demonstrated an abundance of macrophages. Cranial nerves I, II, III, V, VI, and VII and spinal cord showed no substantial histopathologic findings. Pathology findings in lung, prostate, and spleen tissues were consistent with comorbid conditions. Findings for heart, testicle, adrenal gland, gastrointestinal system, and thyroid specimens were unremarkable.

Conclusions

We report postmortem findings of a fatal GBS case (online Technical Appendix). Confirmation of Zika virus infection during acute neurologic illness and negative diagnostic test results for other potential GBS triggers (e.g., *C. jejuni*) provide strong evidence for Zika virus as the GBS trigger. Consistent with the patient's electromyography results, histopathologic observation of demyelination of sciatic and cranial IV nerves provides phenotypic evidence of the acute demyelinating inflammatory polyneuropathy GBS variant. Although results from French Polynesia and Colombia found the acute motor axonal neuropathy variant (*3*,*9*), elsewhere, the acute demyelinating inflammatory polyneuropathy variant has predominated among GBS patients with evidence of Zika virus infection (*14*). Demyelination of both sciatic and cranial IV nerves is consistent with reported clinical signs of GBS patients with evidence of Zika virus infection. Despite reports of cranial neuropathy, including higher rates of facial palsy, ophthalmoplegia, and dysphagia, we observed no substantial pathologic findings in the patient's cranial nerves I, II, III, V, VI, or VII. We found no evidence of direct infection

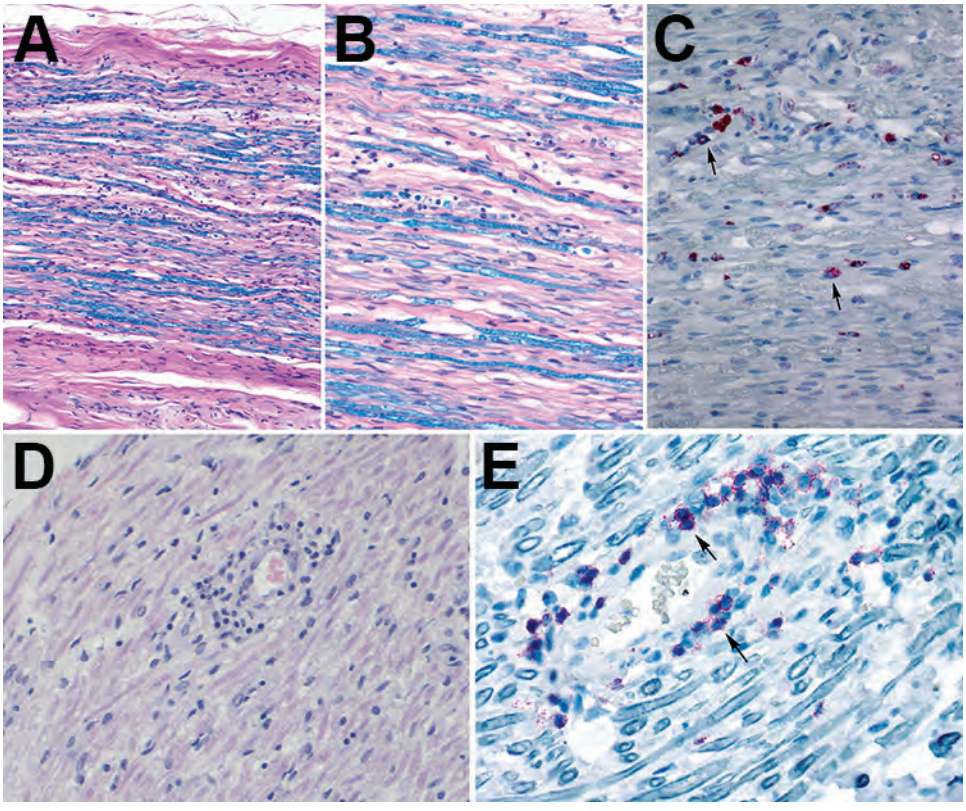

Figure 2. Histopathologic evaluation of tissue specimens collected postmortem from a patient with Guillain-Barré syndrome (acute demyelinating inflammatory polyneuropathy variant) and Zika virus infection, Puerto Rico, 2016. A, B) Luxol fast blue-periodic acid-Schiff myelin stain of sciatic nerves show patchy myelin loss and variable inflammation. Original magnification x10(A) and x20(B). C) Detection of CD68–positive cells (microphages) by immunohistochemistry (arrows) in sciatic nerve. Original magnification x20. D) Hematoxylin and eosin stain of cranial nerve IV shows perivascular lymphocytic infiltrate. Original magnification x20. E) Detection of T-lymphocytes by immunohistochemistry (arrows) in the same area where lymphocytic infiltrates were observed by hematoxylin and eosin stain. Original magnification x40.

of the peripheral or central nervous tissue, suggesting expected antibody-mediated pathophysiologic mechanisms for GBS were triggered by Zika virus and not neurotropism.

Our report has several limitations. First, findings from a single patient might not be representative. Second, because antecedent illness was denied, timing of Zika virus infection could not be defined; however, time-to-loss of detection of Zika virus RNA in the urine of patients with symptomatic Zika virus infection is a median of 8 days (*15*). Last, neurotropism concomitant to antibody-mediated damage cannot be ruled out because viral antigen and RNA may have been cleared before autopsy or false-negative laboratory results could have occurred. Further, Zika virus RT-PCR results were only available for cranial nerve VII, 3 sections of sciatic nerve, spinal cord, and cauda equina.

Despite these limitations, our findings provide crucial insights into GBS pathophysiology, specifically among GBS patients with evidence of Zika virus infection. Future investigations should collect postmortem specimens from GBS patients, particularly when death occurs soon after neurologic illness onset.

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High Seroprevalence of Jamestown Canyon Virus among Deer and Humans, Nova Scotia, Canada

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Using residual serum samples from Nova Scotia, Canada, we found that 87.8% of tested deer and an estimated 20.6% of the human population were infected with Jamestown Canyon virus. Human seropositivity reached 48.2% in 1 region. This virus may be an underrecognized cause of disease in Nova Scotia.

Jamestown Canyon virus (JCV), a mosquitoborne arbovirus, belongs to the California serogroup (CSG) in the *Bunyaviridae* family. The primary reservoir host for JCV is considered to be the white-tailed deer, although JCV antibodies have been observed in other mammals, including horses, sheep, and cattle (*1*). JCV-associated illness among humans is rarely documented; rates of ≤0.01 cases/100,000 population have been reported in the United States (*2*,*3*). Infections can be asymptomatic or associated with a variety of manifestations, including fever, headache, myalgia, weakness, and seizure (*4*–*6*).

Evidence of JCV in Atlantic Canada was reported in 1981 when 6 of 289 hunter-killed moose during the 1977–1978 hunting season in Nova Scotia (NS) were seropositive (*7*). There is no surveillance for JCV in NS, nor is it on the list of diseases notifiable to Provincial Public Health as defined in the NS Health Protection Act (*8*), but it could be reported as an unusual disease occurance or a disease occuring more frequently than expected. We undertook this study to determine the seroprevalence of JCV in humans and white-tailed deer in this province.

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The Study

During October 30–November 4, 2009, Public Health Agency of Canada (PHAC) staff or private contractors collected blood from the chest or organs of hunted deer in 2 check stations in NS: Italy Cross (community A) and Lunenburg (community B) (Figure). Hunters visiting a check station collected approximately 10% of all samples analyzed. Blood samples were collected from 82 deer (40 from community A, 42 from community B). All except 3 of the deer were harvested in zone 102 (https://novascotia.ca/natr/draws/deerdraw/ddZones.asp#zone102) in southern NS, which includes community A and community B.

To evaluate prevalence of JCV among humans, we used residual human serum specimens submitted for prenatal screening and testing for cholesterol, electrolytes, or HIV during May 1–August 30, 2012, that had been used in a previous serosurvey (*10*). We calculated sample size based on estimated seroprevalence of 20% among the NS population, with precision of ±5% and the ability to detect a statistically significant difference ($p = 0.05$) between District Health Authority (DHA) 1, DHA 9, and the remaining DHAs (Figure). Because of high seropositivity among hunter-killed deer in DHA 1, humans were oversampled in DHA 1 to facilitate comparisons with other DHAs. We based human population estimates for 2012 on census data from Statistics Canada. We used Stata version 13.0 (StataCorp, College Station, TX, USA) for statistical analysis and used the Stata *svy* prefix command to incorporate sampling weights to produce estimates representative of the NS population. We stratified serum samples by age, sex, and DHA, with sampling proportionate to the NS population in 10-year age groups for those 10–59 years of age and a 5-year age group for those 60–64 years of age. We used Pearson χ^2 and corrected χ^2 statistics to test for significant differences based on DHA and sex, respectively. We used logistic regression to test for a significant increasing trend in seroprevalence by age group.

The Research Ethics Board of each DHA approved the serosurvey; 1 board required an opt-out option by publicizing the study and asking patients to self-identify nonparticipation at the time of collection. No patient declined participation.

We shipped the samples to the National Microbiology Laboratory, where serum samples were stored at –80°C.

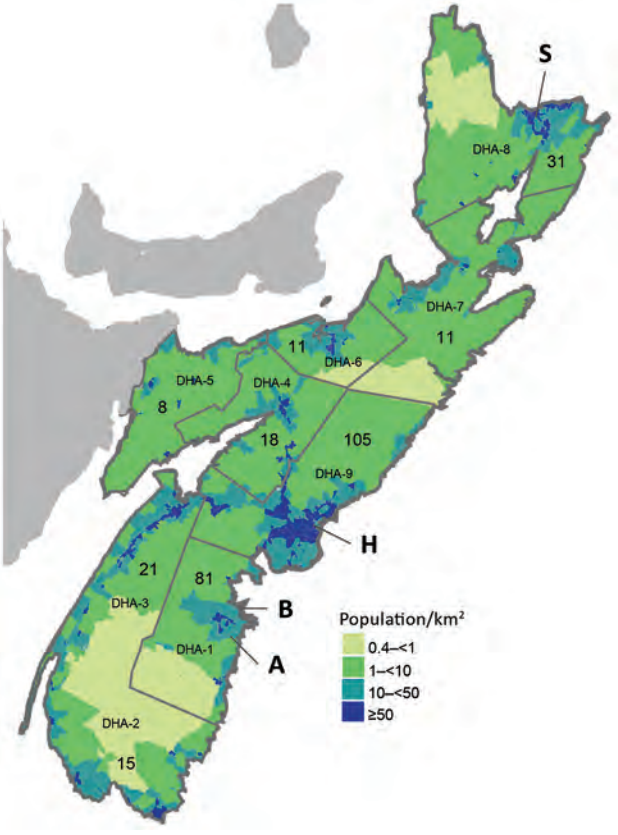

Figure. Population density and boundaries of DHAs in Nova Scotia, Canada, at the time of sample collection from white-tailed deer in 2009 and humans in 2012 for study of Jamestown Canyon virus seroprevalence. H, capital city of Halifax; S, Sydney; A, community A; B, community B. Numbers on map indicate number of human serum samples screened in each DHA. Population density map modified from its original format (*9*). DHA, District Health Authority.

JCV antibodies in human and deer specimens were determined by using an arbovirus plaque-reduction neutralization assay (PRNT); a titer of ≥1:20 was considered positive (*11*). Snowshoe hare virus (SSHV) or JCV and various dilutions of deer serum samples were incubated at 37°C for 1 h in tissue culture media, then added to Vero cell monolayers. After 1 hour of incubation at 37°C, an agar overlay was added; the plates were incubated in CO_2 for 3 days. An overlay containing neutral red was added to visualize plaque formation. Serum samples that inhibited >90% of plaque formation relative to virus controls, the highest positive serum dilution of the titration endpoint, were considered positive for viral antibodies. PRNT results were considered positive if the neutralizing antibody titer was ≥20; additional PRNT endpoint titrations were used to discriminate between related California serogroup viruses. Serum samples that demonstrated a 4-fold or greater difference in PRNT titers between SSHV and JCV were used to identify the virus to which the individual was previously exposed. JCV and SSHV strains used in PRNT assays were NY74-31 and SK75-93, respectively.

JCV antibody titers in samples from 72 of 82 deer ranged from 1:20 to >1:1,280; overall seroprevalence was 87.8% (95% CI 78.7%–94.0%; Table 1). Measured by using the Fisher exact test, seropositivity did not differ significantly based on sampling location, and JCV exposure did not differ by sex: 87.5% (35/40) of male deer and 91.9% (34/37) of female deer were seropositive (Table 1). Seropositivity increased with age (75.0% of immature deer and 93.1% of adult deer were JCV-positive), but the difference was not statistically significant (p = 0.0567).

Of 301 human serum specimens tested for JCV antibodies, 81 were positive (Table 2). After adjusting for survey design, the estimated overall seroprevalence for JCV in NS was 20.6% (95% CI 16.0%–25.9%). The DHA 1 seropositivity of 48.2% (95% CI 37.2%–59.1%) was significantly higher than those of DHAs 2–8 (combined seroprevalence: 22.6%, 95% CI 15.8%–31.4%) and that of DHA 9 (seroprevalence 15.2%, 95% CI 9.5%–23.5% [Table 2]). Estimated seroprevalence was higher for men and boys than for women and girls (26.8% vs. 14.4%, respectively; p = 0.013) and increased with age (p = 0.024): 10.8% were seropositive among those 10–19 years of age, and 33% were seropositive among those 60–64 years of age (Table 2).

Conclusions

In this study, we found that >20% of the tested residual serum samples of persons from Nova Scotia had antibody evidence of infection with JCV. The highest seroprevalence was 48% in specimens tested from 1 of the 9 DHAs. JCV antibodies were found in 88% of hunter-killed deer, higher than in studies in the United States (*12*,*13*).

A seroprevalence rate of 20.6% in persons living in NS is comparable to that seen in reports of other locations (*14*,*15*). The lowest rates of JCV in NS are in DHAs in which the province's 2 most populous cities, Halifax

Table 1. White-tailed deer seropositivity for Jamestown Canyon virus, Nova Scotia, Canada*

No. white-tailed deer	No. (%) seropositive	No. seronegative	p value
Total, n = 82	72 (87.8)	10	NA*
Community			1.000
A, n = 40	35 (87.5)	5	
B, n = 42	37 (88.1)	5	
Sex			0.713
Male, n = 40	35 (87.5)	5	
Female, n = 37	34 (91.9)	3	
Unknown, n = 5	3 (60.0)	2	
Age			0.0567
Adult, n = 58	54 (93.1)	4	
Immature, n = 24	18 (75.0)	6	

*NA, not applicable.

Table 2. Human Jamestown Canyon virus seropositivity by sex and age, Nova Scotia, Canada

Characteristic	No. tested	No. (%) positive	Adjusted proportion (95% CI)	p value*
Sex				0.013
M	151	51 (33.8)	26.8 (19.9–35.0)	
F	150	30 (20.0)	14.4 (9.3–21.5)	
Age group, y				0.024
10–19	43	6 (14.0)	10.8 (4.0–25.8)	
20–29	54	13 (24.1)	20.4 (11.0–34.5)	
30–39	52	12 (23.1)	14.1 (6.9–26.7)	
40–49	60	15 (25.0)	20.4 (1.6–33.3)	
50–59	61	21 (34.4)	27.8 (17.1–41.7)	
60–64	31	14 (45.2)	33.0 (17.7–53.0)	
District Health Authority				0.004
1	81	39 (48.2)	48.2† (37.4–59.1)	
2–8	115	26 (22.6)	22.6† (15.8–31.4)	
9	105	16 (15.2)	15.2† (9.5–23.5)	
Total no.	301	81 (26.9)	20.6 (16.0–25.9)	

*Pearson χ^2 for test by DHA; corrected Pearson χ^2 for test by sex; logistic regression test for trend by age group.
†Adjustment was unnecessary for estimates made by the DHA because weighting was done by this authority.

(DHA 9) and Sydney (DHA 8), are located. In addition to correlating JCV seropositivity with geographic location, this study demonstrated associations of seropositivity with male sex and increasing age in both sexes. These findings support prior data that identified increasing age as predictive for JCV infection, reporting an approximate 10-fold increase in JCV antibodies among persons in Alaska when comparing those <4 years of age with those >65 years of age (*15*).

A limitation of this study is the relatively small number of serum samples, which may not represent the population at risk for this zoonotic disease. The use of anonymized residual serum samples did not enable us to collect data on outdoor activities, exposure history, or travel, so we cannot rule out the possibility that exposure to JCV occurred outside the respective DHA.

We provide serologic evidence of JCV infection in humans and deer in NS and suggest that central nervous system infections (e.g., viral encephalitis and viral meningitis) caused by JCV have been unrecognized. Until more is known of JCV's clinical effect in this province, evidence of its circulation should prompt consideration as a possible etiology of aseptic central nervous system disease.

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Serologic Evidence of Fruit Bat Exposure to Filoviruses, Singapore, 2011–2016

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To determine whether fruit bats in Singapore have been exposed to filoviruses, we screened 409 serum samples from bats of 3 species by using a multiplex assay that detects antibodies against filoviruses. Positive samples reacted with glycoproteins from Bundibugyo, Ebola, and Sudan viruses, indicating filovirus circulation among bats in Southeast Asia.

The genus *Ebolavirus* comprises 5 virus species: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Bundibugyo ebolavirus* (BDBV), *Taï Forest ebolavirus* (TAFV), and *Reston ebolavirus* (RESTV). The genus *Marburgvirus* comprises 1 species, *Marburg marburgvirus*, which includes 2 closely related virus strains: Marburg virus (MARV) and Ravn virus (RAVV). Viruses within the *Ebolavirus* and *Marburgvirus* genera are zoonotic; EBOV was the causative agent of the 2014–2016 Ebola virus disease epidemic in West Africa (*1*). *Rousettus* bats in Africa have been identified as *Marburgvirus* hosts (*2*), and viral nucleic acid and serologic evidence suggests that bats are also natural hosts of *Ebolavirus* spp. (*3*). Yet it remains unclear which species are the definitive reservoirs of filoviruses.

Ecologic models of *Ebolavirus* and *Marburgvirus* geographic distribution and habitat ranges of potential reservoir bat species suggest that both groups are distributed throughout Asia (*3*,*4*). Serologic evidence of filoviruses in frugivorous bats in Bangladesh, China, and the Philippines has been reported (*5*–*7*), and RESTV nucleic acid was detected in an insectivorous bat in the Philippines, where RESTV is considered endemic (*8*). We examined pteropodid bats of 3 species: *Cynopterus brachyotis*, *Eonycteris spelaea*, and *Penthetor lucasi*, which are widely distributed across Southeast Asia and share ecologic niches (*9*).

The Study

During 2011–2016, we collected serum from bats of the 3 aforementioned species in Singapore and screened samples for evidence of exposure to filoviruses. Samples were collected with permission from the National University of Singapore Institutional Animal Care and Use Committee (B01/12) and the National Parks Board (NP/RP11–011–3a). We diluted venous blood 1:10 in phosphate-buffered saline and then centrifuged, recovered, and heat-inactivated the serum at 56°C for 30 minutes and stored it at –80°C.

We developed a Bio-Plex (Bio-Rad, Hercules, CA, USA) bead-based multiplex assay that simultaneously probes serum for immunoglobulins specific to the viral envelope glycoproteins (GPs) from representative strains of all described *Ebolavirus* and *Marburgvirus* species (Table 1). A human FreeStyle 293-F stable cell-line expression system was used to produce the *Ebolavirus* and *Marburgvirus* spp. GPs as a soluble GP consisting of the entire ectodomain, $sGP_{(1,2)}$, which retains a native-like oligomeric conformation, as described previously with modifications (*10*). In brief, each $GP_{(1,2)}$ coding sequence was truncated at the C-terminus to remove the predicted transmembrane domain and cytoplasmic tail, then appended with the GCN trimerization peptide sequence (*10*) together with a factor Xa protease cleave site and a Twin-Strep-tag sequence (IBA Lifesciences, Göttingen, Germany). The $sGP_{(1,2)}$ proteins were produced in serum-free conditions and purified by Strep-Tactin XT technology (IBA Lifesciences). The Twin-Strep-tag was removed by factor Xa enzymatic cleavage; factor Xa was removed by Xarrest Agarose (Merck Millipore, Billerica, MA, USA); $sGP_{(1,2)}$ was purified further by S-200 size exclusion chromatography, concentrated, and stored frozen. These $sGP_{(1,2)}$s were coupled to carboxylated beads (Bio-Rad). Screening was performed on a Bio-Rad Bio-Plex 200.

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Table 1. *Ebolavirus* and *Marburgvirus* species soluble envelope glycoproteins conjugated Bio-Plex beads used in multiplex assay to detect antibodies against filoviruses*

Virus	Isolation host/location	Bio-Plex bead no.	NCBI accession no.
Ebola virus/H.sapiens/COD/1976/Yambuku-Mayinga	Human/DRC	33	NC_002549.1
Bundibugyo virus/H. sapiens/UGA/2007	Human/Uganda	64	FJ217161.1
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-CI	Human/Côte d'Ivoire	57	NC_014372
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	Human/Uganda	77	NC_006432.1
Reston virus/M. fascicularis/USA/1989/Pennsylvania	Macaque/USA	85	AF522874.1
Reston virus/S. domesticus/PHL/2008/Reston08-A	Swine/Philippines	72	FJ621583.1
Marburg virus/H. sapiens/KEN/1980/Musoke	Human/Kenya	37	Z12132 S55429
Marburg virus/H. sapiens/AGO/2005/Ang0126	Human/Angola	28	DQ447656.1
Ravn virus/H. sapiens/KEN/1987/Kitum cave-810040	Human/Kenya	49	NC_024781.1

*Bio-Plex manufactured by Bio-Rad (Hercules, CA, USA). DRC, Democratic Republic of the Congo; NCBI, National Center for Biotechnology Information.

In the absence of confirmed filovirus-negative bat serum, we used methods developed by Peel et al. to establish a median fluorescence intensity (MFI) cutoff value (*11*). We confirmed a cutoff value of 200 MFI (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-0401-Techapp1.pdf), as was previously used for *Eidolon helvum* bat serum in a Bio-Plex serologic assay (*12*). We screened 409 samples with our *Ebolavirus* and *Marburgvirus* spp. $sGP_{(1,2)}$ Bio-Plex assay modified from that described by Bossart et al. (*13*). Samples were diluted 1:100 and tested in duplicate; the $sGP_{(1,2)}$-coupled beads were mixed with individual samples; and a 1:1 combination of recombinant biotinylated-protein A/protein G (1:500) (Pierce, Rockford, IL, USA) was added to the wells, followed by addition of streptavidin-phycoerythrin (1:1,000) (Bio-Rad) and determination of MFI.

Samples were positive for 17 (9.1%) of 186 *E. spelaea*, 13 (8.5%) of 153 *C. brachyotis*, and 3 (4.3%) of 70 *P. lucasi* bats (Figure 1). Positive samples reacted with EBOV, BDBV, SUDV, or TAFV $sGP_{(1,2)}$. However, no samples were positive for RESTV, MARV, or RAVV $sGP_{(1,2)}$. We further examined positive samples to determine cross-reactivity between the *Ebolavirus* spp. $sGP_{(1,2)}$ (Table 2). Twelve (71%) samples from *E. spelaea* bats cross-reacted with ≥2 *Ebolavirus* spp. $sGP_{(1,2)}$ (BDBV, EBOV, SUDV, or TAFV). In contrast, 8 (62%) *C. brachyotis* and 2 (66%) *P. lucasi* samples were positive for only 1 $sGP_{(1,2)}$ (BDBV or SUDV).

To further determine the cross-reactivity of positive samples and to corroborate Bio-Plex assay results for a selected number of samples, we performed Western blot (WB) assays (Figure 2). The filovirus $GP_{(1,2)}$ is a trimer of heterodimeric GP_1 and GP_2 subunits. The trimeric-like $sGP_{(1,2)}$ is the antigen in the multiplex Bio-Plex assay, whereas linearized monomeric sGP_1 and sGP_2 subunits are the antigens in WBs. Reduced and denatured EBOV or BDBV unconjugated $sGP_{(1,2)}$ was loaded on 8% sodium dodecyl sulfate–polyacrylamide electrophoresis gels, transferred to a polyvinylidene difluoride membrane, and probed with 1:100 dilutions of positive and negative bat serum, as previously determined by the Bio-Plex assay. All 3 *E. spelaea* bat samples and 2 of 3 *C. brachyotis* bat samples that were Bio-Plex positive were also positive by WB and displayed reactivity with EBOV and BDBV GP_1 and GP_2 antigens; no *P. lucasi* bat samples positive by Bio-Plex were positive by WB.

Conclusions

We present evidence of antibodies specific to filoviruses antigenically related to *Ebolavirus* spp. in 3 species of fruit bats widely distributed throughout Southeast Asia. We detected seroreactivity with *Ebolavirus* spp. but not *Marburgvirus* spp. GP. Despite the close relatedness of the viruses, we detected samples reacting with only SUDV, not RESTV, GP. This finding contrasts

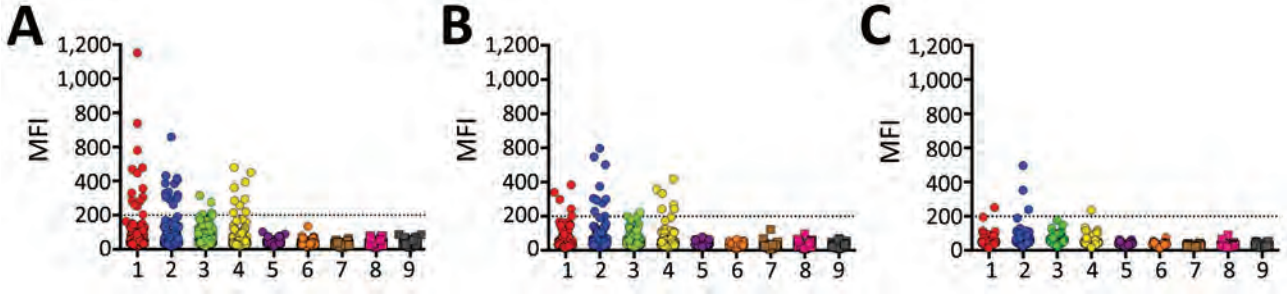

Figure 1. Mean fluorescence intensity (MFI) values obtained from Bio-Plex assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples from bats of 3 species with soluble filovirus glycoproteins. Dashed line indicates the cutoff value at 200 MFI. 1, *Zaire ebolavirus*; 2, *Bundibugyo ebolavirus*; 3, *Taï Forest ebolavirus*; 4, *Sudan ebolavirus*; 5, *Reston ebolavirus*–monkey; 6, *Reston ebolavirus*–pig; 7, Marburg virus–Musoke; 8, Marburg virus–Angola; 9, Ravn virus.

Table 2. Bio-Plex median fluorescence intensity values for bat serum samples positive for ≥1 filovirus antigen*

Bat species, ID	EBOV	BDBV	TAFV	SUDV	RESTVm	RESTVp	MARV(Mus)	MARV(Ang)	RAVV
Eonycteris spelaea, n = 186									
0805149†	**738**	124	68	40	44	22	23	21	24
080814	86	**318**	105	**258**	26	12	17	16	20
082154	143	161	113	**214**	35	41	21	31	39
052313	**284**	**408**	177	**285**	89	72	29	23	30
052335	**203**	191	124	**219**	42	21	38	38	24
052339	**357**	**306**	141	**293**	54	31	26	26	42
071839	**330**	**299**	164	**480**	65	44	28	33	45
071842	**446**	**327**	**202**	**362**	65	49	42	38	57
110733	126	**416**	166	95	58	42	34	42	58
011603†	**1151**	130	91	69	36	32	51	35	39
011616	**252**	294	168	175	32	49	47	29	50
011656	**306**	**386**	**204**	**394**	89	73	18	39	37
012309†	**579**	**659**	**315**	69	35	31	27	33	35
021303	**478**	**431**	188	**450**	52	37	24	30	47
111903	**469**	**384**	**276**	113	52	57	37	69	54
111907	**285**	**336**	**213**	158	39	36	29	50	30
042722	**260**	**262**	174	**1**67	75	31	54	24	42
Cynopterus brachyotis, n = 153									
051253	121	133	59	**242**	40	41	19	25	68
0516613	146	**293**	127	73	47	36	25	29	22
0516632	138	139	86	**356**	35	25	28	34	34
0726122†	119	**501**	100	60	40	46	25	19	29
1103241	84	141	128	**241**	50	47	66	38	34
100903	148	**201**	71	108	42	33	18	16	36
100914	74	**228**	70	55	39	38	30	27	26
100925	166	**304**	109	116	43	18	33	30	28
021357	**201**	**299**	179	**264**	65	44	25	55	47
050804	**242**	**276**	140	124	41	30	34	33	44
050818	**383**	**374**	198	**332**	60	55	29	26	68
040807†	**297**	**597**	194	192	40	38	122	95	32
042701†	**339**	**547**	**222**	**417**	60	78	54	25	62
Penthetor lucasi, n = 70									
062590†	34	**496**	93	39	36	18	23	17	23
070409†	95	**238**	129	89	62	27	34	36	37
112112†	**251**	**352**	148	**235**	51	29	23	23	29

*Bio-Plex manufactured by Bio-Rad (Hercules, CA, USA). Boldface indicates positive results. BDBV, Bundibugyo virus; EBOV, Ebola virus; ID, specimen identification number; MARV(Mus), Marburg virus–Musoke; MARV(Ang), Marburg virus–Angola; RESTVm, Reston virus–monkey; RESTVp, Reston virus–pig; SUDV, Sudan virus; RAVV, Ravn virus; TAFV, Taï Forest virus.
†Sample screened by Western blot and shown in Figure 2.

with previous reports of bat serum cross-reactivity with RESTV nucleoprotein (*5*,*7*,*14*). Possible explanations include 1) the fact that our customized Bio-Plex assay is based on conformational $sGP_{(1,2)}$, which can differentiate antibody specificity better than the more sequence conserved nucleoprotein, and 2) the lack of evidence of RESTV GP positivity with *Cynopterus* and *Eonycteris* bat serum samples, which is in line with previous findings (both species were negative while only *Rousettus amplexicaudatus* bats were positive) (*7*). *E. spelaea* bats were previously predicted to be filovirus hosts (*15*), and sequences of novel filoviruses have been discovered in *E. spelaea* bat populations in Yunnan, China (*14*). Our data provide additional empirical evidence that populations of *C. brachyotis*, *E. spelaea*, and *P. lucasi* bats in Southeast Asia are hosts of filoviruses, which seem antigenically more closely related to EBOV, BDBV, and SUDV than to RESTV.

Examination of cross-reactivity of positive samples from *E. spelaea*, *C. brachyotis*, and *P. lucasi* bats revealed no clear patterns of preferential reactivity with EBOV, BDBV, or SUDV GP. Factors that might contribute to the lack of *P. lucasi* positivity by WB include sensitivity differences between Bio-Plex and WB assays paired with the change in $sGP_{(1,2)}$ conformation. Two Bio-Plex EBOV-positive samples (*E. spelaea* samples 0805149 and 011603) reacted with EBOV sGP_2 and BDBV sGP_1 in the WB. Bio-Plex and WB data strongly suggest the presence of yet-undetected batborne filoviruses, which are antigenically related to but distinct from BDBV, EBOV, and SUDV circulating in local bat populations. Reasons why these filoviruses have remained undetected include their inability to cross the species barrier, the rarity of spillovers into humans or domestic animals, or the fact that spillover events cause mild or no disease. We suggest that a yet-undescribed diversity of filoviruses exists in Southeast Asia bat populations, a hypothesis supported by the recent identification of filovirus sequences in *E. spelaea* and *R. leschenaulti* bats in China (*14*,*16*). Comprehensive surveillance including serology and detection of viral nucleic acid, along with virus

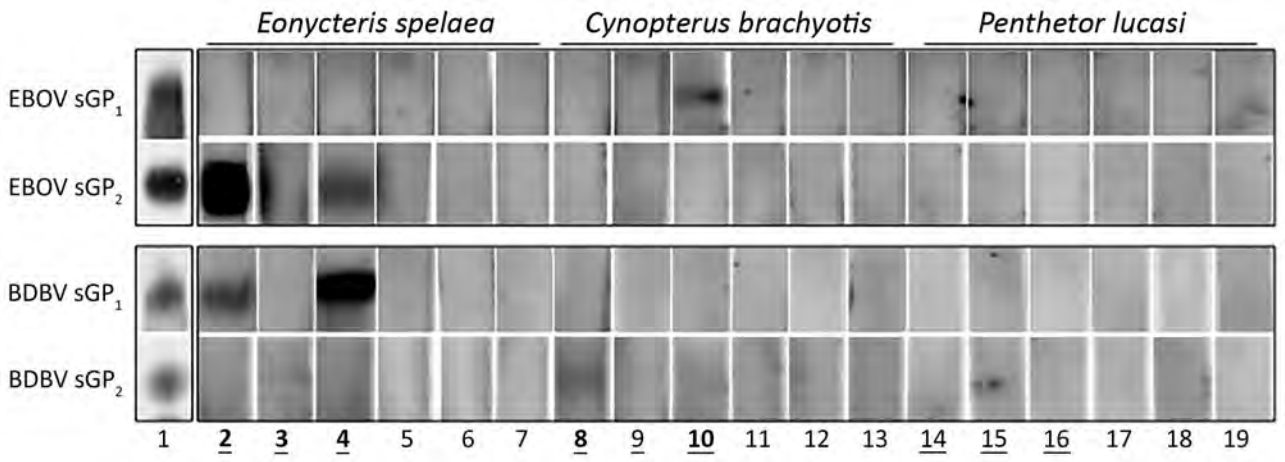

Figure 2. Western blot results of individual bat serum samples probed against *Zaire ebolavirus* and *Bundibugyo ebolavirus* glycoproteins 1 and 2 (GP_1, GP_2). Boldface indicates positivity by Western blot and underlining indicates positivity by Bio-Plex (Bio-Rad, Hercules, CA, USA). 1, soluble GP_1 and GP_2 blotted with control anti–Ebola virus nonhuman primate polyclonal serum that demonstrates cross-reactivity with *Bundibugyo ebolavirus* soluble GP. Other numbers along baseline correspond to the following sample identifiers, also used in Table 2: 2, 0805149; 3, 012309; 4, 011603; 5, 0116048; 6, 0719036; 7, 1128015; 8, 0726122; 9, 042701; 10, 040807; 11, 0512540; 12, 1009010; 13, 0408029; 14, 070409; 15, 112112; 16, 062590; 17, 0228004; 18, 0919025; 19, 0625095. BDBV, Bundibugyo virus; EBOV, Ebola virus.

isolation, will help elucidate the characteristics of filoviruses endemic to Asia and identify bat species that function as maintenance populations and reservoirs.

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Expected Duration of Adverse Pregnancy Outcomes after Zika Epidemic

Rosalind M. Eggo, Adam J. Kucharski

Evidence is increasing that Zika virus–related adverse outcomes can occur throughout pregnancy. Mathematical modeling analysis using reported outcome data suggests that surveillance for these outcomes should begin as soon as an outbreak is detected and should continue for 40 weeks after the outbreak ends.

Quantifying the risk for adverse pregnancy outcomes (APOs) after Zika virus infection is of critical public health importance. Recent studies have suggested that risk for microcephaly is concentrated in pregnancies in which infection occurs during the first trimester (*1*,*2*). However, microcephaly is at the severe end of the APO spectrum and might have a different risk profile from other outcomes: brain abnormality and malformation, eye anomalies, neural tube defects, arthrogryposis, congenital deafness, and others (*3*). In particular, estimates of APOs after symptomatic confirmed Zika virus infection suggest risk for fetal injury throughout pregnancy (*3*,*4*). Thus, a better understanding of the likely duration and risk for APOs after Zika virus outbreaks is urgently needed (*2*). We used surveillance and clinical data to estimate the timing and number of expected APO events after observed Zika outbreaks in 9 regions of Brazil during April 2015–July 2017.

The Study

To quantify APO risk, we used data from a study that recruited 345 pregnant women with rash in the previous 5 days, of whom 134 tested positive for Zika virus infection (*4*). Excluding 9 losses to follow-up, we followed a cohort of 125 women during pregnancy; surviving infants were examined for APOs. A total of 58 APOs occurred in this group; microcephaly was infrequent (4 [6.9%] of 58) but severe (*4*).

We fitted a logistic model to individual-level data for the 125 followed-up women to estimate the proportion of APOs after symptomatic Zika virus infection at each week of gestation (Figure 1, panel A). Although the fitted linear model suggested a decline in risk over time, the model did not perform significantly better than a model with constant risk for APO at any gestational age (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-0482-Techapp1.pdf). For comparison, we also considered a theoretical risk profile in which APO risk occurs only during the first trimester (Figure 1, panel A).

We used these risk profiles to estimate the period through which an elevated rate of APOs would be expected after the 2015–2016 Zika epidemic in 9 regions of Brazil (Figure 1, panels B–J). We superimposed the timing of confirmed microcephaly cases in each region to assess the relationship between observed microcephaly and expected duration of elevated APO risk but did not fit explicitly to microcephaly incidence data. If risk were assumed to occur only during the first trimester, the period of APOs would be shorter than the duration of observed microcephaly events. In contrast, the predicted durations of APOs based on risk throughout pregnancy were more consistent with the observed distribution of microcephaly in these regions.

To examine the potential duration and risk for Zika-associated APOs more generally, we also predicted the pattern of APOs under 3 hypothetical epidemic scenarios: single outbreak, multipeaked epidemic, and endemic transmission (Figure 2). For each epidemic scenario, the model suggested that the duration of elevated risk was much longer than the duration of cases if APOs could occur from infection in any gestational week. This observation means that in areas where seasonal outbreaks of Zika occur, the risk for APOs might not return to baseline levels between epidemics, and Zika-specific interventions based on timing of pregnancy might be less effective (*7*).

Our findings are subject to several limitations. First, we based the estimation of APO risk by gestation period on a cohort study of symptomatic infection with rash, which does not occur with all Zika virus infections (*8*). However, recent evidence suggests the risk for APOs is similar for symptomatic and asymptomatic infection (*9*). We included pregnancy loss during the first trimester (miscarriage) as an APO, but excluding these 5 cases did not alter the findings (online Technical Appendix, Sensitivity Analysis on Inclusion of Miscarriages section). Moreover, evidence suggests that APOs might not be detectable at birth but appear later, which would underestimate the frequency of APOs (*10*).

Second, the data were from patients recruited in Rio de Janeiro, whereas we considered potential risk across all regions of Brazil. Although the cohort was large and APO data detailed, numbers of exposed women in each gestational

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week were low, leading to large CIs on the risk profile (Figure 1, panel A). We therefore used a linear model to estimate the risk at each gestational week because data were insufficient to fit a more complex risk function. The range of data (6–39 weeks' gestation) also constrained our estimates.

Third, publicly available epidemiologic reports from Brazil recorded microcephaly cases, rather than all forms of APO. We qualitatively compared these microcephaly reports with our estimates for the duration of risk for APOs, but the risk for microcephaly by gestational week might differ from the overall risk for APOs. Different regions are likely to have differing baseline levels of APOs in the absence of Zika virus infection; we therefore focused our analysis on the risk for APOs associated with Zika virus

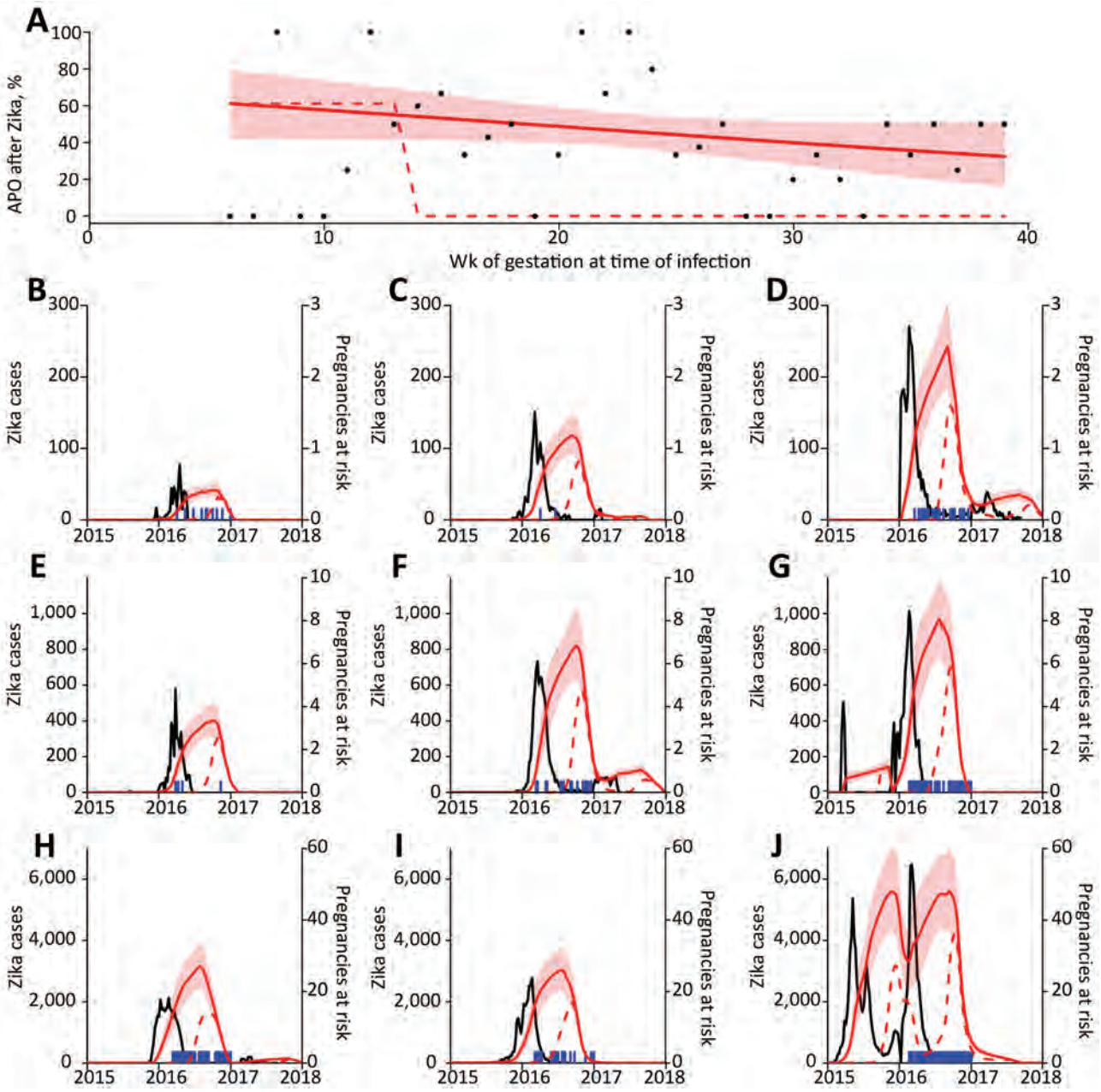

Figure 1. Relationship between Zika virus infection and expected related APOs per 1,000 pregnancies in Brazil during April 2015–July 2017. A) Percentage of APOs (fetal loss at any gestational age, stillbirth, neonatal abnormality) given symptomatic PCR-confirmed Zika virus infection. Points show weekly proportion with APO (*4*); red line indicates fit to data with a generalized linear model, and shading indicates 95% CIs; dashed line indicates fixed risk in first trimester only (*5*). B–J) Blue lines indicate suspected Zika cases in different regions; red lines indicate expected number of births with Zika-associated APO in subsequent weeks based on the 2 risk distributions in panel A. Shaded regions indicate 95% CIs. Model assumes 17% of Zika virus infections are reported (*5*,*6*). APO, adverse pregnancy outcome.

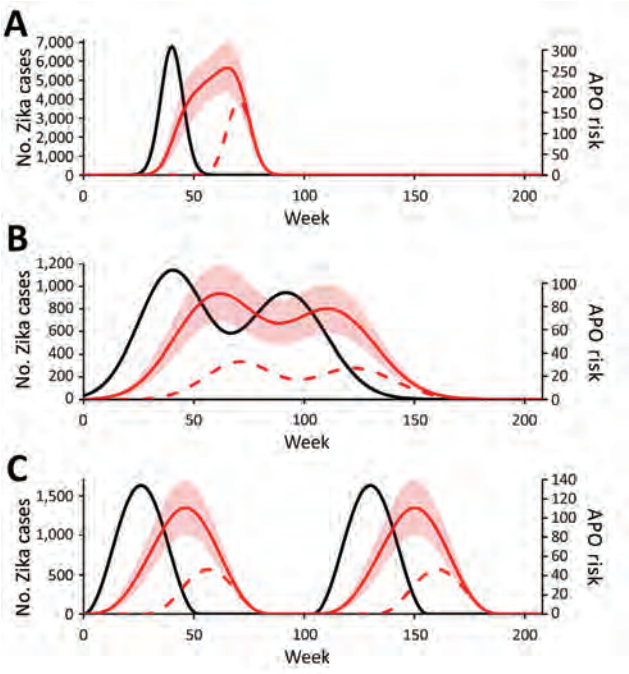

Figure 2. Expected temporal distribution of Zika virus–related adverse pregnancy outcomes under different hypothetical outbreak scenarios, Brazil, April 2015–July 2017. Black lines indicate Zika cases; red lines indicate risk (APOs/1,000 births) for Zika-associated APO in subsequent weeks based on the 2 risk distributions in panel A. Dashed lines indicate timing of outbreaks: A) short, single-peaked outbreak; B) double-peaked outbreak; C) biennial epidemics (i.e., a seasonal endemic state). A population size of 1 million, reporting of 17% of Zika infections, and a 50% attack rate during a 4-year period were assumed. APO, adverse pregnancy outcome.

infection. Why some areas of Latin America have reported more cases of microcephaly than others remains unclear (*11*). There may be unmeasured cofactors that alter the risk for APO on Zika virus infection (*12*). Another factor could be differences in the proportion of Zika cases reported, which could lead to variation in incidence of APOs. We assumed 17% of Zika infections were reported (*6,8*); if the proportion reported was larger, it would mean fewer women were infected during the epidemic, and hence fewer would be expected APOs (online Technical Appendix, Sensitivity Analysis on Fraction of Cases Reported section).

Finally, Brazil made Zika notifiable in November 2015, which might have increased reporting (*13*). In addition, the Zika incidence data varied markedly by region, which may be due to true differences in outbreak dynamics or to differences in reporting of cases (Figure 1). Although variability in weekly Zika incidence data would alter the precise relationship between Zika cases and population-level rate of APO, the general shape and duration of enhanced risk estimated in the model remains the same (online Technical Appendix, Sensitivity Analysis on Fraction of Cases Reported section).

Conclusions

Our results suggest that if fetal injury from Zika virus infection can occur across a range of gestational ages, APOs after a Zika outbreak could occur for a long time after the outbreak subsided. This duration is longer than if the risk is assumed to be in the first trimester only (*2,14*). Combined with epidemiologic reports of APOs collected in Brazil, which show an increase in microcephaly rate at a time inconsistent with first trimester–only risk, evidence is mounting to recommend extended surveillance for APOs and to include a spectrum of outcomes, not only microcephaly (*10,15*).

Our results suggest that when Zika outbreaks are identified, surveillance and planning for infection-associated APOs might need to focus on a longer period than previously thought. In addition to the potential for APOs several months after an epidemic, the risk period may begin soon after the outbreak is detected. Further studies are crucial to refine the risk for APO during gestation and to ensure pregnant women can be correctly informed of their risk, so that population-level surveillance can be effectively implemented.

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Dr. Kucharski is a mathematical modeler focused on outbreak disease dynamics at the London School of Hygiene & Tropical Medicine. His research interests include new emerging infections and immunity from individual infection history.

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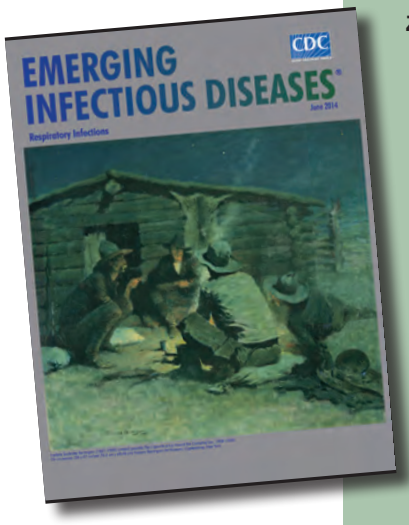
Zika Virus

Zika [zēkˈə] Virus



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Zika virus is a mosquito-borne, positive-sense, single-stranded RNA virus in the family *Flaviviridae*, genus *Flavivirus* that causes a mild, acute febrile illness similar to dengue. In 1947, scientists researching yellow fever placed a rhesus macaque in a cage in the Zika Forest (*zika* meaning "overgrown" in the Luganda language), near the East African Virus Research Institute in Entebbe, Uganda. A fever developed in the monkey, and researchers isolated from its serum a transmissible agent that was first described as Zika virus in 1952. It was subsequently isolated from a human in Nigeria in 1954. From its discovery until 2007, confirmed cases of Zika virus infection from Africa and Southeast Asia were rare. In 2007, however, a major epidemic occurred in Yap Island, Micronesia. More recently, epidemics have occurred in Polynesia, Easter Island, the Cook Islands, and New Caledonia.

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Rodent Abundance and Hantavirus Infection in Protected Area, East-Central Argentina

Malena Maroli,[1] María Victoria Vadell,[1] Paula Padula, Isabel E. Gómez Villafañe

We captured 3 hantavirus rodent hosts in Otamendi Natural Reserve, Argentina, during 2007–2012. Hantavirus antibodies were found only in *Akodon azarae* grass mice, mainly in males and old animals. Higher abundance of this species was associated with warm and rainy weather and high water levels, which peaked after a strong El Niño event.

Hantavirus pulmonary syndrome is an emerging infectious disease caused by New World hantaviruses (family *Hantaviridae*) and transmitted by rodents of the family *Cricetidae* (*1*). In Argentina, 7 native rodent species have been identified as hantavirus reservoirs (*2*). Three of these species (*Oligoryzomys flavescens* [yellow pigmy rice rat], host of Lechiguanas virus; *O. nigripes* [black-footed pigmy rice rat], host of Juquitiba virus; and *Akodon azarae* [grass mouse], host of Pergamino virus) are present in east-central Argentina. *A. azarae* mice have not been associated with cases of hantavirus pulmonary syndrome (*2*). The purpose of this long-term study was to identify factors affecting hantavirus infection and reservoir abundance.

The Study

We conducted a study in 6 habitats in the Otamendi Natural Reserve (34°10′S, 58°48′W) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-1372-Techapp1.pdf) in Buenos Aires, Argentina, an area with low anthropogenic and no rodenticide pressures. Rodents were live-trapped during September 2007–December 2012. During December 2011–December 2012, for logistic and security reasons, trapping was concentrated in lowlands, salty grasslands, and highlands containing *Ligustrum* spp. Traps were baited with a mixture of peanut butter, fat, and rolled oats, placed every 10 m on permanent grids or transects, depending on the shape of each habitat, and set for 3 consecutive nights.

We ear-tagged each captured rodent; identified its species, breeding status, body length (an indicator of age) and body mass; and obtained a blood sample from a cut on the tip of the tail to test for hantavirus antibody (*3*). Rodents were released at point of capture. We calculated trap success (number of captured rodents/number of trap-nights), species richness, abundance ratio (TS_i/TS_{total}), the Shannon-Weaver Diversity Index, and hantavirus antibody prevalence by species, habitat, and trap session. We calculated body condition as weight divided by the cube of body length.

We recorded percentages of green/dry grass and broadleaf cover 1 m in height, bare ground, and maximum vegetation height during April 2009–December 2012 by using a 1-m^2 quadrant placed around each trap station (*4*). We calculated maximum and minimum temperatures, monthly rainfall, and number of days with temperatures <0°C during the month before each trapping session. These variables were also used with time lags of 1 and 2 months. We determined anomalies in temperature by using the Oceanic Niño Index (*5*). We recorded mean, maximum, and minimum water levels in the Paraná River during the month before each trapping session and the number of months since the last flooding event for each trapping session.

We assessed associations between hantavirus infection (estimated as antibody prevalence) and vegetation, hydrologic, meteorologic, and rodent population characteristics; presence of hantavirus antibody and individual characteristics; and known hantavirus host abundance and vegetation, hydrologic, and meteorologic characteristics. Analyses were conducted by using logistic regressions with forward-stepping selection and binomial family distributions of errors and logit link or clog-log functions (*6*) in R software (multcomp and car packages) (*7*).

During the study period, we captured 650 animals 752 times during 15,833 trap-nights. We captured 3 known hantavirus rodent host species: *A. azarae* grass mice (n = 204), *O. flavescens* yellow pigmy rice rats (n = 36) and *O. nigripes* black-footed pigmy rice rats (n = 20). We also captured 6 other species: *Oxymycterus rufus* red hocicudos (n = 223), *Scapteromys aquaticus* swamp rats (n = 129), *Deltamys kempi* Kemp grass mice (n = 27), *Calomys laucha*

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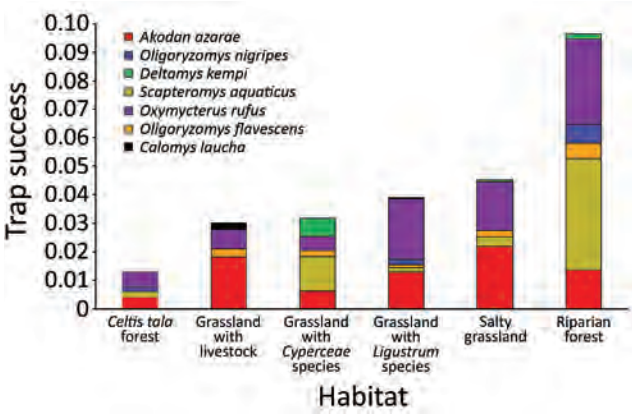

Figure 1. Trap success (no. animal captures/trap-night) by rodent species in each habitat of Otamendi Natural Reserve, Argentina, 2007–2012. *Cavia aperea* Brazilian guinea pigs and *Holochilus* sp. marsh rats were not included because too few animals were captured.

small vesper mice (n = 7), *Cavia aperea* Brazilian guinea pigs (n = 3), and *Holochilus* sp. marsh rats (n = 1) (Figure 1).

We detected hantavirus antibodies in *A. azarae* grass mice (Table 1) and in 1 *D. kempi* Kemp grass mouse. In the study area, *A. azarae* grass mice are the known reservoirs of Pergamino virus, a hantavirus variant that has not been associated with human disease (*2*,*8*). However, because virus mutations can result in infection of other hosts, constant monitoring is needed. Antibodies in the *D. kempi* Kemp grass mouse were probably caused by a spillover event (*2*). Maroli et al. (*9*) demonstrated that *A. azarae* grass mice share regions with other rodents, suggesting that these mice could promote spillover infections. Antibody prevalence in *A. azarae* grass mice was 23.9%, which exceeded prevalences reported in other areas of the Argentinean pampas (*10*).

We captured antibody-positive rodents at every site (Table 1; online Technical Appendix Figure 2) but results showed no evidence of spatial focality, in contrast to what has been reported for other hantavirus–rodent systems (*11*,*12*). Significantly greater prevalence was associated with low green grass cover (estimate –0.03721, p = 0.050). Although variation in antibody prevalence among seasons was not significant, absence of continuous trapping of antibody-positive rodents throughout the study (online Technical Appendix Figure 2) suggests that temporary local virus extinctions might be a factor in some local populations, which would later have virus reintroduced from nearby source populations (*12*). However, these results should be interpreted cautiously because they might be caused by a failure to detect low levels of antibody in these populations.

The effect of community characteristics on disease risk is a topic of current debate (*13*). Hantavirus antibody prevalence was not associated with any of the community variables analyzed. However, this lack of association with environmental variables cannot be considered conclusive because of difficulties in assessing the role of environmental factors in such a complex system.

Antibody-positive *A. azarae* grass mice were more likely to have a longer body length (mean 106 mm, estimate 0.0929; p<0.001) (Table 1) than antibody-negative mice (mean length 98 mm), suggesting that hantavirus transmission among rodents is primarily horizontal, as reported for many hantavirus–reservoir systems (*1*). In addition, male *A. azarae* grass mice (estimate 1.4440; p = 0.0027) (Table 1) were more frequently infected than female mice, probably because of aggressive encounters with other rodents (*1*). Maroli et al. (*9*) reported that longer *A. azarae* grass mice travel greater distances, increasing the probability of intraspecific encounters and potential hantavirus transmission.

Abundance of hantavirus host species in the Otamendi Natural Reserve was generally associated with warm and rainy weather and high water levels; abundance was highest after a strong El Niño event and lowest after a strong La Niña event (Figure 2; Table 2). These variables might indirectly affect rodent population abundance as proposed in the trophic cascade hypothesis (*14*).

We captured *A. azarae* grass mice in all habitats (Figure 2), showing that this species can occupy many areas and sites with low vegetation heights (Table 2). We also trapped *O. flavescens* yellow pigmy rice rats in 5 of 6 habitats but at lower rates (Figure 1; Table 2), consistent with other studies showing that this species is not dominant in the rodent community (*15*). Low abundance and short-distance movements (*9*) might restrict virus dispersal among habitats and could be the reason for the lack of detection of virus antibody. *O. nigripes* black-footed

Table 1. Characteristics of *Akodon azarae* grass mice per habitat in Otamendi Natural Reserve, Argentina

Habitat	*A. azarae* trap success rate (no.)*	Males, %	Body length, mm	Hanatavirus antibody prevalence, %
Riparian forest	0.014 (23)	70	102	35
Celtis tala forest	0.004 (8)	63	99	25
Lowland grassland with *Cyperaceae*	0.006 (22)	59	103	35
Highland grassland with *Ligustrum* spp.	0.013 (44)	61	93	2
Highland grassland with livestock	0.018 (32)	54	99	28
Salty grassland	0.022 (75)	61	99	29

*Values in parentheses are total number of *A. azarae* grass mice.

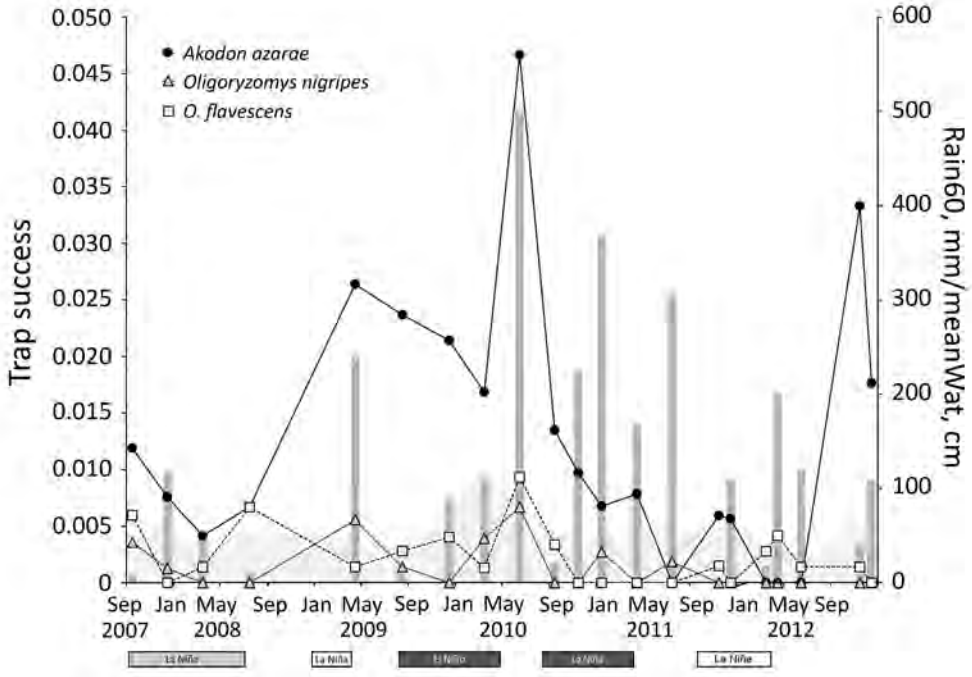

Figure 2. Trap success (average no. animal captures/trap-night) of known hantavirus rodent host species in Otamendi Natural Reserve, Argentina (lines), monthly accumulated rainfall applying time lags of 2 months (Rain60; gray bars), and mean water level during the month before each trapping session (MeanWat, gray shaded area), September 2007–December 2012. El Niño and La Niña events and their intensities (white, weak; light gray, moderate; and dark gray, strong) are shown below the x-axis.

pigmy rice rats are found in many areas, (*2*), but on the basis of our study, prefer habitats with trees (Table 2). This species was also found in areas with low green broadleaf cover (Table 2).

Table 2. Variation in abundance of 3 rodent species in relation to environmental characteristics in Otamendi Natural Reserve, Argentina*

	Akodon azarae grass mouse			*Oligoryzomys nigripes* black-footed pigmy rice rat			*O. flavescens* yellow pigmy rice rat		
Explanatory variable	Estimate	SE	p value	Estimate	SE	p value	Estimate	SE	p value
Meteorological model		(28.90%)			(35.50%)			(15.45%)	
Intercept (riparian forest)	–2.937[bc]	0.604	<0.001	6.033[a]	0.435	<0.001	–1.586	1.217	0.193
Celtis tala forest	–1.426[e]	0.415	<0.001	–1.972[b]	0.678	0.004	–	–	
Lowland grassland with *Cyperaceae* spp.	–0.923[de]	0.302	0.002	–	–	–	–0.962	0.489	0.049
Highland grassland with *Ligustrum* spp.	–0.161[cd]	0.261	0.536	–1.555[b]	0.520	0.003	–1.631	0.604	0.007
Highland grassland with livestock	0.169[ab]	0.278	0.543	–	–	–	–0.709	0.560	0.206
Salty grassland	0.390[a]	0.242	0.107	–	–	–	–0.932	0.489	0.057
Rain60	0.002	0.001	0.001	0.006	0.002	<0.001	–	–	–
MaxT	–0.049	0.018	0.005	–	–	–	–0.120	0.040	0.002
ONI	0.513	0.098	<0.001	0.408	0.384	0.289	0.305	0.270	0.258
Vegetation model		(12.34%)			(11.46%)			(6.37%)	
Intercept	–3.210	0.166	<0.001	–8.999	1.230	<0.001	–7.070	0.459	<0.001
Height	–0.255	0.038	<0.001	–	–	–	–	–	–
GBroad	–	–	–	–0.025	0.010	0.013	0.020	0.008	0.011
Hydrological model		(22.10%)			(16.85%)			–	
Intercept (riparian forest)	–5.289	0.282	<0.001	–6.068	0.636	<0.001	–	–	–
Celtis tala forest	–1.275	0.412	0.002	–1.514	0.653	0.020	–	–	–
Lowland grassland with *Cyperaceae* spp.	–0.732	0.300	0.015	–	–	–	–	–	–
Highland grassland with *Ligustrum* spp.	0.012	0.260	0.964	–1.245	0.511	0.015	–	–	–
Highland grassland with livestock	0.278	0.276	0.313	–	–	–	–	–	–
Salty grassland	0.560	0.241	0.020	–	–	–	–	–	–
MeanWat	1.149	0.199	<0.001	1.183	0.585	0.043	–	–	–

*The percentage of variance explained by each model is shown in parentheses in the first line above the explanatory variables. Superscript letters show significant differences in abundance among habitat types (p<0.05 by multiple Tuckey comparisons). *O. flavescens* yellow pigmy rice rats did not show differences in abundance among habitat types. Height, maximum vegetation height; GBroad, fresh broadleaf cover; MaxT, maximum temperature during the month before each trapping session; MeanWat, mean water level during the month before each trapping session; ONI, Oceanic Niño Index; Rain60, monthly accumulated rainfall applying a time lag of 2 months; –, not included in the final model.

Conclusions

Abundance of hantavirus reservoir rodents was influenced principally by meteorologic factors that could be used to predict host population dynamics. However, the presence of hantavirus antibody was mainly influenced by rodent sex and age. Although the prevalence of infection did not vary with environmental factors, greater abundance of hosts indicates a greater absolute number of infected rodents, and therefore, an increased risk for transmission to humans.

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Two-Center Evaluation of Disinfectant Efficacy against Ebola Virus in Clinical and Laboratory Matrices

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Ebola virus (EBOV) in body fluids poses risk for virus transmission. However, there are limited experimental data for such matrices on the disinfectant efficacy against EBOV. We evaluated the effectiveness of disinfectants against EBOV in blood on surfaces. Only 5% peracetic acid consistently reduced EBOV titers in dried blood to the assay limit of quantification.

Effective disinfection of Ebola virus (EBOV) in body fluids is critical for emergency response to outbreaks. However, for such fluids, data are scarce for disinfectant efficacy. This information is essential for informed disinfection processes, environmental decontamination, waste disposal practices, and safety practices for healthcare workers and public health responders (*1*). We investigated the efficacy of disinfectants against EBOV spiked into cell culture medium and whole blood.

The Study

Six disinfectants were tested: Purrell Advanced (GOJO Industries, Akron, OH, USA) 30 μL; Steriplex SD (sBiomed LLC, Orem, UT, USA) 100 μL; Micro-Chem Plus (National Chemical Laboratories, Inc., Winona, MN, USA) 30 μL; Micro-Chem Plus 100 μL; bleach (Clorox, Oakland, CA, USA) 30 μL and 100 μL; acidified bleach 100 μL; and peracetic acid (Sigma-Aldrich, St. Louis, MO, US) 100 μL. The 2 most effective disinfectants were evaluated at 2 laboratories: the National Biodefense Analysis and Countermeasures Center (NBACC; Frederick, MD, USA); and the Defence Science and Technology Laboratory (DSTL; Porton Down, UK). Because of local regulations and operating procedures, some methods were modified between the laboratories (Table 1).

At NBACC, material was prepared as follows. Passage 1 of Ebola virus H.sapiens-tc/GIN/2014/Makona-C05 virus (Rocky Mountain Laboratory, National Institutes of Health, Hamilton, MT, USA) was used to generate passage 2 virus stock in Vero E6 cells (Table 1). All work with viable EBOV was performed in Biosafety Level 4 laboratories.

At DSTL, material was prepared as follows. Passage 4 of Ebola virus H.sapiens-wt/GIN/2014/Makona-C07 virus (Public Health England, London, UK) was passed twice in Vero E6 cells, creating passage 6 material. All work with viable EBOV was performed in Biosafety Level 4 laboratories.

Stainless steel and aluminum coupons (≈22 mm^2) were sterilized before use. Test matrices included cell culture medium, human whole blood (NBACC), or rat whole blood (DSTL).

To test disinfectants, we spiked Ebola/Mak 1:10 into a test matrix and then deposited it onto coupons. We disinfected coupons immediately (wet) or kept them at ambient conditions until dry by visual examination (*2*) and then disinfected (dry). Disinfectants were applied for various contact times (Table 1). We performed no surface agitation or mixing before sample recovery, per the method of the American Society for Testing Materials International (West Conshohocken, PA, USA) (*2*). We performed neutralization by submersion into cell culture medium and vortexing according to recommendations of ASTM International (*2*,*3*) (Table 1). Viable virus was measured in samples as described (*4*,*5*). We performed statistical analysis of sample results (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-0504-Techapp1.pdf).

All disinfectants tested reduced virus titer to the assay lower limit of quantification (LLOQ) of the assay when evaluated with EBOV/Mak deposited on surfaces in cell culture medium (Table 2). However, only 5% peracetic acid consistently reduced the titer of EBOV/Mak in dried human blood to the assay LLOQ. These data collectively indicate that surface-dried whole blood provides a more protective matrix for EBOV/Mak than does surface-dried cell culture medium.

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Because EBOV/Mak in dried blood resists complete bleach disinfection (0.5% or 1.0% sodium hypochlorite) but this method is commonly used in outbreak and health-care settings (*6*), we sought independent confirmation. We shared protocols and surface coupons with DSTL, who verified that EBOV/Mak dried in cell culture medium was

Table 1. Study parameters for evaluation of 6 disinfectants in reducing Ebola virus titers in dry blood or cell culture medium*

Parameter	NBACC	DSTL
Virus (dilution in blood)	Ebola virus/Makona-C05 (1:10)	Ebola virus/Makona-C07 (1:10)
Cells	Vero C1008, Vero 76, clone E6, Vero E6 (ATCC CRL-1586)	Vero C1008, ECACC #85020206
Blood source	Human whole blood in EDTA (Bioreclamation IVT, Westbury, NY, USA)	Fresh whole blood in EDTA from male Porton rats
Blood droplet size (µL) and state	10, wet or dried	20, dried
Surface coupons	304 stainless steel (Diamond Perforated Metals, Visalia, CA, USA); 6061 Aluminum (Speedy Metals, New Berlin, WI, USA) used for experiments with dry medium (peracetic acid and acidified bleach) and wet blood (peracetic acid) only	3014 stainless steel
Disinfectant		
Sodium hypochlorite		
Supplier	Clorox (Oakland, CA, USA)	Sychem (Leigh On Sea, UK)
Concentration tested, vol/vol	0.5%, 10% hypochlorite	1% hypochlorite
Volume tested, µL	30 or 100	100
Contact time, min	15	15
Peracetic acid		
Supplier	Sigma-Aldrich (St. Louis, MO, USA)	Sigma-Aldrich
Concentration tested, vol/vol	5%	0.2%
Volume tested, µL	100	100
Contact time, min	5	10
Micro-Chem Plus		
Supplier	National Chemical Laboratories, Inc. (Winona, MN, USA)	ND
Concentration tested, vol/vol	1.5%	
Volume tested, µL	30 or 100	
Contact time, min	10	
Purell Advanced		
Supplier	GOJO Industries (Akron, OH, USA)	ND
Concentration tested, vol/vol	70% ethanol	
Volume tested, µL	30	
Contact time, min	2	
Steriplex SD		
Supplier	sBioMed LLC (Orem, UT, USA)	ND
Concentration tested	0.015% silver, 10.000% ethanol, 0.020% H_2O_2, 0.150% peroxyacetic acid, 0.150% acetic acid, 0.075% inert food grade ingredients proprietary, 89.590% water	
Volume tested, µL	100	
Contact time, min	5	
Acidified bleach		
Supplier	Clorox	ND
Concentration tested, vol/vol	10% bleach (0.5% sodium hypochlorite) + 1% acetic acid	
Volume tested, µL	100	
Contact time, min	15	
Method		
Neutralization	5 mL cell culture medium (10% FBS) or direct recovery using washing/filtration	2 mL cell culture medium (2% FBS)
Recovery method	30 µL bleach: none (5 mL medium only); 100 µL bleach; Steriplex SD: acidified bleach or peracetic acid; after neutralization in 5 mL medium, 2 mL was ultrafiltered in an Amicon (Bedford, MA, USA) 100-kDa NMWL unit, washed 3 times with 2 mL PBS after centrifugation at 5,000 × *g* for 10 min, and resuspended in 2 mL medium; Purell Advanced: diluted to 130 µL with PBS and recovered using a PD MultiTrap G-25 96-well microplate column array. Dilute supernatant to 5 mL with medium; Micro-Chem Plus: samples directly added to Amicon Ultra-0.5 (30- or 100-kDa NMWL) ultrafiltration unit, washed 3 times with 0.5 mL PBS by centrifugation at 5,000 × *g* for 10 min, and resuspended in 5 mL medium	Washed by centrifugation 8,000 rpm for 5 min and resuspended in 1 mL medium

*Titers were assessed by using the 50% tissue culture infectious dose assay. ATCC, American Type Culture Collection; DSTL, Defence Science and Technology Laboratory; ECACC, European Collection of Authenticated Cell Cultures; FBS, fetal bovine serum; NBACC, National Biodefense Analysis and Countermeasures Center; ND, no data; NMWL, nominal molecular weight limit; PBS, phosphate-buffered saline.

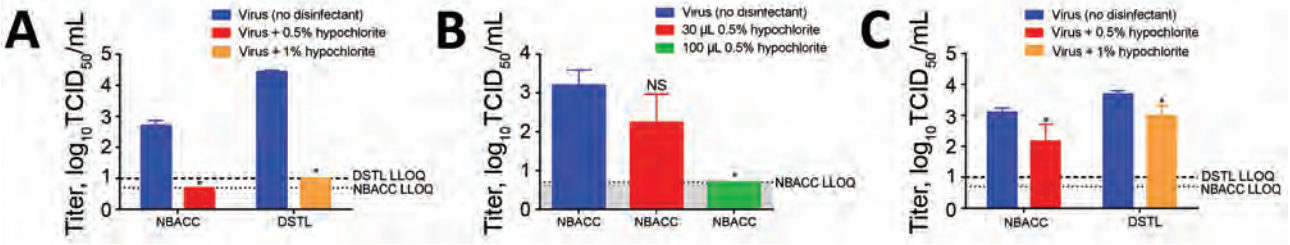

Figure 1. Effect of common bleach disinfection of Ebola virus in A) dried cell culture medium, B) wet blood, and C) dried blood. Coupons were spotted with Ebola virus/Makona (EBOV/Mak). Bleach solutions (0.5% or 1.0% hypochlorite) were effective in reducing the titer of EBOV/Mak to the assay LLOQ in dried cell culture medium or wet blood. Incomplete disinfection was observed when virus was suspended in blood and dried for 1 h before disinfection. Data were confirmed for dried cell culture medium and dried blood studies at an independent laboratory (DSTL). Error bars indicate SD. *Significant difference ($p<0.05$) between control and disinfected samples. DSTL, Defence Science and Technology Laboratory; LLOQ, lower limit of quantification; NBACC, National Biodefense Analysis and Countermeasures Center.

highly susceptible to disinfection with sodium hypochlorite (Figure 1, panel A). However, when dried blood samples were treated with 0.5% or 1.0% sodium hypochlorite, viral titer reductions of only 88.3% and 79.0%, respectively, were observed (Figure 1, panel C). Furthermore, viable virus was recovered from all samples, confirming that dried blood represents a challenging matrix for disinfection of EBOV/Mak.

We also measured effectiveness of disinfection with 10% bleach against EBOV in wet blood on coupons. When we suspended EBOV/Mak in wet blood, 10% bleach was either 89.2% (30 µL) or 99.7% (100 µL) effective in reducing viral titers, depending on the volume (and consequently the final concentrations) of bleach used. Application of 100 µL of bleach to wet blood samples resulted in a significant reduction in viral titer to the LLOQ of the assay (Figure 1, panel B), suggesting that wet blood is less challenging to disinfection than dried blood.

Of the 6 disinfectants we evaluated, only 5% peracetic acid was efficacious in disinfecting dried blood samples containing EBOV/Mak (Table 2). Concentrations of 5% peracetic acid also reduced viral titers to the LLOQ of the microtitration assay when virus was in either dried cell culture medium (Figure 2, panel A) or wet blood (Figure 2, panel B). Although studies at NBACC showed complete inactivation of EBOV/Mak in dried blood by 5% peracetic acid, complementary studies at DSTL showed that use of a lower concentration (0.2%) of peracetic acid resulted in a 94.9% reduction in viral titers in samples with viable virus still present (Figure 2, panel C). Taken together, these results suggest that there might be a concentration-dependent reduction in viral titers in dried blood when peracetic acid is used for disinfection.

Conclusions

The purpose of this study was to test products for disinfection of EBOV in a relevant clinical matrix. Previous studies showed that filoviruses remain viable in blood for extended periods (*5*,*7*,*8*). Therefore, it was imperative to identify efficacious disinfectants for this matrix. Our results indicate that although bleach, Purell Advanced, and Micro-Chem Plus effectively inactivated EBOV in cell culture medium and wet blood, they were less effective in dried blood.

Only 5% peracetic acid consistently reduced EBOV titers in dried blood to the assay LLOQ. Peracetic acid is a strong oxidant and broad-spectrum disinfectant commonly used in disinfection of a variety of pathogens in waste

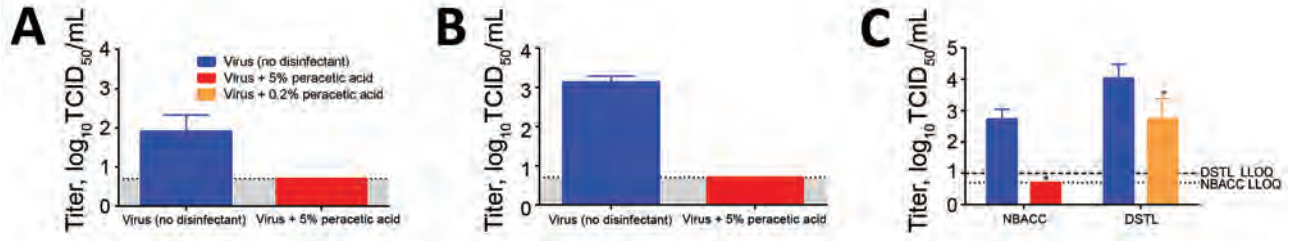

Figure 2. Effect of 5% peracetic acid disinfection of Ebola virus in 3 different matrices. Coupons were spotted with Ebola virus/Makona (EBOV/Mak) in cell culture medium (A) or blood (B, C). Peracetic acid was effective in reducing the titer of EBOV/Mak to the assay LLOQ in dried cell culture medium or wet blood. Although complete disinfection was observed when virus was suspended in blood and dried for 1 h before disinfection with 5% peracetic acid (NBACC), incomplete disinfection was observed with 0.2% peracetic acid (DSTL). Error bars indicate SD. *Significant difference ($p<0.05$) between control and disinfected samples. DSTL, Defence Science and Technology Laboratory; LLOQ, lower limit of quantification; NBACC, National Biodefense Analysis and Countermeasures Center.

Table 2. NBACC studies of evaluation of 6 disinfectants for reducing Ebola virus titers in dry blood or cell culture medium*

Disinfectant and volume, µL	Dried blood				Dried cell culture medium			
	Mean log_{10} $TCID_{50}$ (SD) disinfected	Mean log_{10} $TCID_{50}$ (SD) control	log difference	*t*-test p value	Mean log_{10} $TCID_{50}$ (SD) disinfected	Mean log_{10} $TCID_{50}$ (SD) control	log difference	*t*-test p value
Purell Advanced, 30	3.1 (0.2)	2.6 (0.3)	<0	9.5×10^{-1}	0.7 (0)	2.4 (0.3)	1.7	5.6×10^{-3}
Steriplex SD, 100	2.4 (0.2)	3.0 (0.2)	0.6	8.8×10^{-3}	0.7 (0)	3.3 (0.2)	2.6	5.9×10^{-4}
Micro-Chem Plus, 30	2.8 (0.5)	3.4 (0.5)	0.6	4.9×10^{-2}	0.7 (0)	1.5 (0.2)	0.8	3.6×10^{-5}
Micro-Chem Plus, 100	1.5 (0.1)	2.9 (0.2)	1.4	1.4×10^{-4}	ND	ND	ND	ND
Bleach, 30	2.2 (0.5)	3.1 (0.1)	0.9	1.1×10^{-3}	0.7 (0)	2.7 (0.2)	2.0	1.3×10^{-3}
Acidified bleach, 100	1.7 (0.3)	2.7 (0.3)	1.1	5.9×10^{-3}	0.7 (0)	2.6 (0.2)	1.9	2.0×10^{-3}
Peracetic acid, 100	0.7 (0)	2.7 (0.3)	2.0	4.3×10^{-3}	0.7 (0)	1.9 (0.4)	1.2	7.8×10^{-2}

*NBACC, National Biodefense Analysis and Countermeasures Center; ND, no data; $TCID_{50}$, 50% tissue culture infectious dose.

water because of its relative ease of implementation, broad-spectrum activity in the presence of heterogeneous organic matter, small pH dependence, short contact time, and lack of harmful decomposition products (*9*). Although peracetic acid has been reported to be an effective disinfectant against EBOV (*10*), use of peracetic acid for EBOV disinfection in clinical fluids has not been specifically documented.

Organic matter in clinical fluids can reduce the virucidal activity of disinfectants by a chemical reaction between the disinfectant and the organic matter, which leaves less active disinfectant available for virus inactivation. In particular, chlorine disinfectants are prone to inactivation by reactions with organic matter (*11*,*12*). Alternatively, organic matter can prevent inactivation of viruses by acting as a physical barrier (*13*,*14*). Our results suggest that under the conditions tested, dried blood inhibits effective disinfection of EBOV and might provide a protective layer of matrix not completely dissolved in disinfectant, thereby shielding virus from inactivation.

This study used the American Society for Testing Materials International standard (*2*) for testing of disinfectants on carriers and represents a worst-case scenario. However, it is possible that precleaning, agitation, or mixing would aid in the disinfection process. Additional testing is required to confirm this possibility.

In summary, our results show the difficulty in disinfecting surfaces contaminated with EBOV in dried blood. Although all disinfectants tested were effective against dried cell culture medium containing EBOV, only 5% peracetic acid reduced dried blood virus titers to undetectable levels. These findings can be used to support public health efforts, risk assessment development, remediation decisions, and response and preparedness procedures for future outbreaks of infection with EBOV.

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Melioidosis, Singapore, 2003–2014

Long Pang, Patrick N.A. Harris, Rachel L. Seiler, Peng Lim Ooi, Jeffrey Cutter, Kee Tai Goh, Alex R. Cook, Dale Fisher,[1] Louis Yi Ann Chai[1]

In contrast with northern Australia and Thailand, in Singapore the incidence of melioidosis and co-incidence of melioidosis and pneumonia have declined. *Burkholderia pseudomallei* deep abscesses increased 20.4% during 2003–2014. These trends could not be explained by the environmental and climatic factors conventionally ascribed to melioidosis.

Melioidosis is endemic throughout Southeast Asia and northern Australia. Caused by the environmental gram-negative bacillus *Burkholderia pseudomallei*, melioidosis can manifest as bacteremia, abscesses, or pneumonia. A case-fatality rate of >50% has been reported (*1*).

Australia, Thailand, and Singapore have the most reported cases of melioidosis worldwide (*2*). The geographic, climatic, and infrastructural landscapes of these 3 countries differ vastly. The population demographics and ease of accessibility to healthcare also differ and may influence disease manifestations and outcomes. Epidemiologic case studies from Singapore are limited (*3*). We describe the latest epidemiologic findings based on national melioidosis surveillance data for 2003–2014.

The Study

Melioidosis is a notifiable condition in Singapore. The laboratory and clinical criteria for notification are defined by the Ministry of Health, Singapore (*4*). Our study comprised all cases reported by medical practitioners and microbiology laboratories during 2003–2014. Public health officers collected demographic and clinical details of persons with notified cases using a standardized template. The data were extracted from the Ministry's Communicable Disease Surveillance system (*5*). We used a Poisson regression model, offset for population, to fit the annual melioidosis incidence to measure overall trend. A binomial generalized linear model with identity link was used to quantify temporal changes in the absolute proportion of melioidosis cases having each co-morbidity. We conducted a binomial test of whether the prevalence of comorbidities equaled that of the general population; the latter was derived from the last published National Health Survey 2010 (http://www.moh.gov.sg/content/moh_web/home/Publications/Reports/2011/national_health_survey2010.html) for persons 50–59 years of age (for diabetes and hypertension) and 40–54 years of age (for renal impairment). Analyses were performed with R Statistical Software version 3.3.1 (https://www.r-project.org/).

During the 12 years studied, notifications were received for 614 (range 31–96 annually) melioidosis cases (Table). The mean age of patients was 51.4 years; most (84.0%) patients were males. Most (72.1%) cases occurred in patients ≥45 years of age. The overall incidence of melioidosis was 1.1 per 100,000 population; incidence was highest among Malay and Indian populations (2.4 and 2.1/100,000, respectively). The most common manifestations of melioidosis were *B. pseudomallei* bacteremia (60.3%), abscesses (40.7%), and pneumonia (33.1%). Among the comorbidities reported (Table), diabetes (56.7%) and renal impairment (15.3%) had significantly higher prevalence among persons with melioidosis than among the general population of approximately the same age (19.3%, p<0.001 for diabetes; 2.0%, p<0.001 for renal impairment). In contrast, hypertension prevalence was consistent between melioidosis patients and the general population (35.5% vs. 31.9%; p = 0.057).

During 2003–2014, melioidosis incidence decreased by 10% annually (incidence rate ratio 0.900; 95% CI 0.878–0.923; p<0.001). The overall death rate was 18.4% and decreased by 12.3% annually (incidence rate ratio, 0.877; 95% CI 0.826–0.931; p<0.001) (Figure 1). Among melioidosis patients, there was a borderline declining trend in *B. pseudomallei* bacteremia cases annually (absolute risk reduction [ARR] 1.1%; 95% CI –2.3% to 0.1%; p = 0.076). However, the death rate for bacteremic patients did not change (ARR 0.4%; 95% CI –1.2% to 0.4%; p = 0.355) (Figure 2, panel A). In contrast, melioidosis patients with pneumonia decreased from a peak of 56 cases in 2004 to no cases in 2014 (ARR 5.1%; 95% CI –5.6% to –4.6%; p<0.001), accompanied by an ARR of 1.5% in mortality annually (95% CI –1.9% to –1.1%; p<0.001) or

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[1]These authors contributed equally to this article.

Table. Demographic characteristics of patients with melioidosis, Singapore, 2003–2014*

Characteristic	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	Total (%)†
No. cases	42	96	74	59	57	60	37	58	34	31	34	32	614
Patient age, y													
Mean	52.6	51.3	51.2	50.6	56.9	49.6	54.1	55	46.1	45.9	53.7	50	
Minimum	19	7	0	14	9	3	13	10	8	12	9	13	
Maximum	82	91	95	85	87	77	83	87	77	76	88	83	
Sex													
F	6	15	9	13	7	9	8	13	6	1	10	1	98 (16.0)
M	36	81	65	46	50	51	29	45	28	30	24	31	516 (84.0)
Incidence by race‡													
Chinese	0.9	2.3	1.3	1.3	0.9	1.3	0.7	1	0.5	0.4	0.5	0.4	1.0§
Malay	2	2.9	4.1	2.2	3.5	2.8	0.8	3.2	1.6	1.8	2.3	1.2	2.4§
Indian	0.7	3.8	3.2	2.2	4.5	2.5	1.5	0.6	1.1	1.4	1.1	2.3	2.1§
Comorbidity													
Diabetes mellitus	19	61	43	31	33	39	19	38	20	15	18	12	348 (56.7)
Hypertensive disease	10	29	23	18	27	27	8	21	17	10	13	15	218 (35.5)
Hyperlipidemia	1	2	10	7	4	11	2	22	4	5	7	12	87 (14.2)
Ischemic heart disease	3	19	5	10	4	4	1	3	2	1	3	5	60 (9.8)
Chronic liver disease/cirrhosis	0	4	1	1	1	1	0	0	1	0	0	0	9 (1.5)
Neoplasm	0	0	5	34	0	0	0	5	0	1	0	0	45 (7.3)
Renal impairment	9	23	11	10	13	12	3	3	3	1	3	3	94 (15.3)
Anemia	1	3	4	1	2	0	0	0	0	0	1	0	12 (2.0)
Tuberculosis	0	10	5	0	2	0	0	2	0	1	1	0	21 (3.4)
COPD	0	2	1	1	1	1	0	0	0	0	0	0	6 (1.0)
Asthma	0	4	6	1	1	2	0	2	1	1	0	0	18 (2.9)
No. deaths, %	6 (14.3)	25 (26.0)	12 (16.2)	9 (15.3)	12 (21.1)	12 (20.0)	5 (13.5)	14 (24.1)	6 (17.6)	2 (6.5%	8 (23.5)	2 (6.3)	113 (18.4)

*Data extracted from (*5,9*). COPD, chronic obstructive pulmonary disease.
†Values are no. (%) unless otherwise indicated.
‡These are the standard reported races/ethnicities in Singapore. Incidence is per 100,000 population.
§Mean.

18% reduction in death during the 12-year period (Figure 2, panel B).

The annual incidence of *B. pseudomallei* abscesses was unchanged during 2003–2014, but the number of patients with deep abscesses (primarily liver, spleen, or prostate) increased (absolute risk increment 1.7% annually; 95% CI 0.7%–2.8%; p = 0.001) or 20.4% increment over 12 years (Figure 2, panel C). We saw no evidence of a change in the prevalence of diabetes among persons with melioidosis (ARR 0.7%; 95% CI –1.9% to 0.5%; p = 0.277), although there was an ARR of 0.9% in mortality annually (95% CI –1.5% to –0.4%; p = 0.002) or 10.8% reduction over 12 years, for this at-risk patient group (Figure 2, panel D).

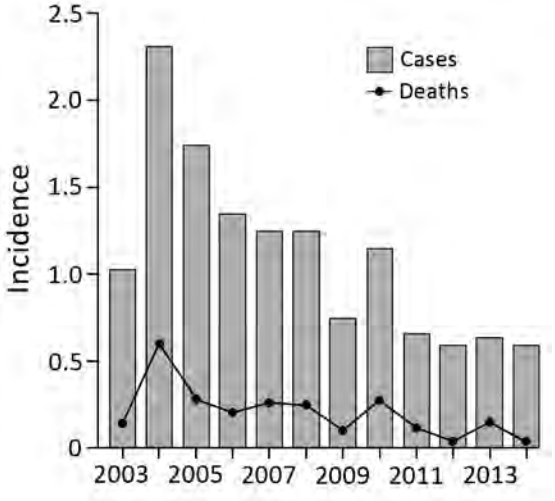

Figure 1. Annual incidence of melioidosis per 100,000 persons, Singapore, 2003–2014.

This study, comprising one of the largest melioidosis patient cohorts thus far, yielded some unanticipated disease trends from Singapore. Foremost was the decreasing melioidosis incidence, in the context of declining *B. pseudomallei* bacteremia cases and, more significantly, the steady decrease in pneumonia.

By contrast in Australia and Thailand, melioidosis numbers stayed constant or increased (*6*). Pneumonia continued to feature prominently in 50% of the cases in these countries (*7*). It would be reasonable to theorize that pneumonia would be the predominant feature following inhalation of aerosolized *B. pseudomallei* after rainfall (*8*). Several groups have reported the association between rainfall, humidity, and water exposure with melioidosis (*9*,*10*). In equatorial Singapore, however, rainfall patterns have not changed recently. In addition, our analysis of rainfall and humidity during the corresponding study period found these climatic variables to be constant on

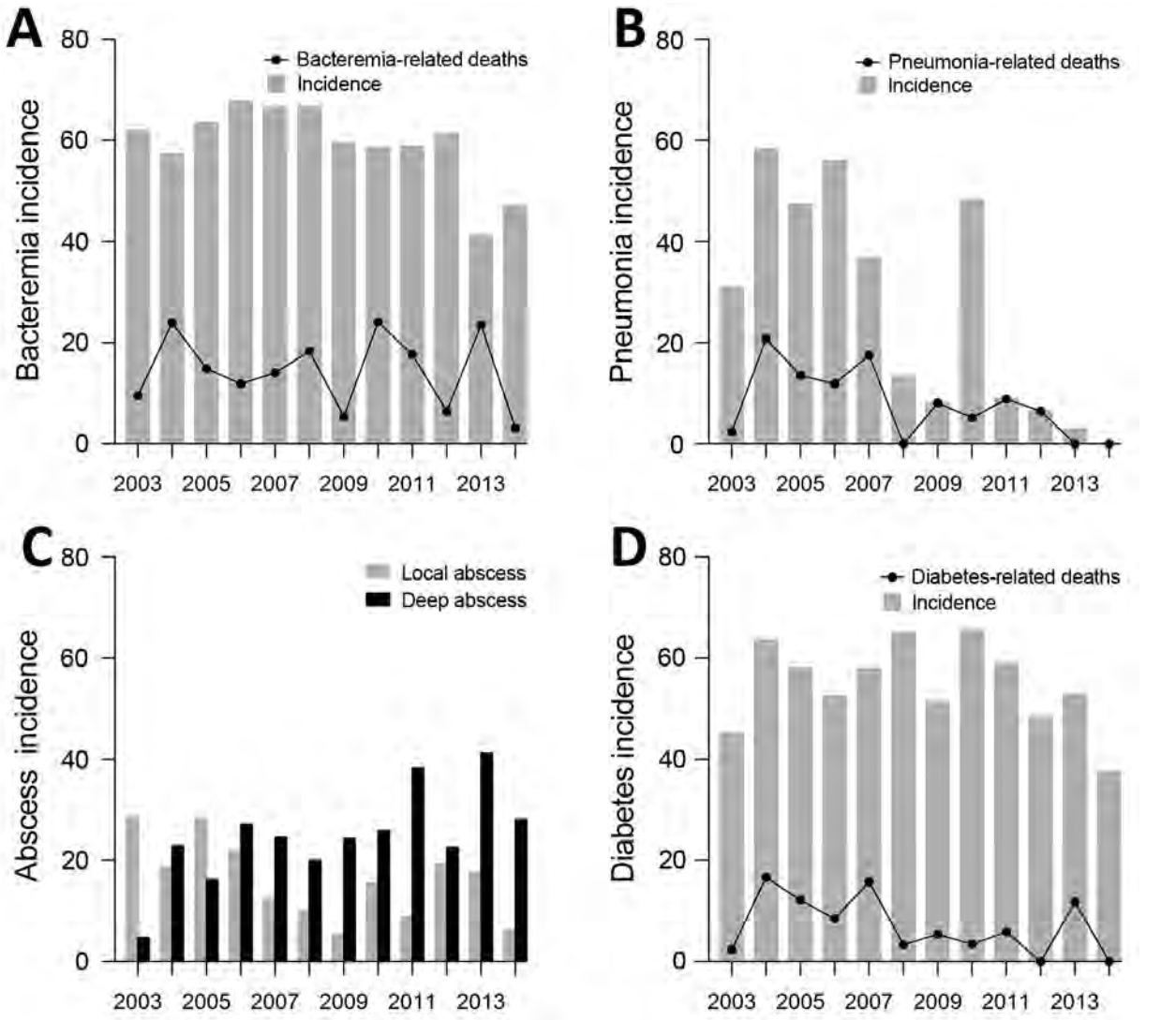

Figure 2. Melioidosis trends, Singapore, 2003–2014. A) Annual incidence of *Burkholderia pseudomallei* bacteremia and bacteremia-related deaths per 100 melioidosis cases. B) Annual incidence of melioidosis patients with pneumonia and pneumonia-related death per 100 melioidosis patients. C) Annual incidence of *B. pseudomallei* abscesses per 100 melioidosis patients. Local abscesses as per superficial or cutaneous, and deep abscess as per deep organ (primarily liver, spleen and prostate). D) Annual incidence of diabetes and diabetes-related death per 100 melioidosis patients.

a yearly scale (rainfall, p = 0.846; humidity, p = 0.815). Hence, climate is unlikely to be related to the decline in disease incidence.

Soil, in particular anthrosol and acrisol soil, encountered in irrigated agriculture, has been suggested as a likely reservoir for *B. pseudomallei* (*10*). Consequently, urbanization leading to reduction of agricultural or rural land areas ought to align with a lower incidence of melioidosis. However, the developed island state of Singapore already had reached 100% urbanization by the early 1990s (*11*); thus, urbanization could not have been the primary factor for this decreasing incidence after 2003.

Conversely, water sanitization, storm/rainwater drainage, and flood reduction have remained a major focus of ongoing infrastructural improvements of the Singapore government through the 2000s to now. The national water strategy entails a complex system encompassing optimized drainage and collection, followed by water treatment intended for consumption and use. Flood risk is managed through design and implementation of state-of-the-art water drainage systems and flood-protection measures for public infrastructures (*12*). Together with a national flood alert response plan, these measures potentially minimize direct rain or contaminated water exposure and aerosol inhalation risk; thus, they are plausible factors to account for the overall melioidosis and pneumonia case reductions, but further study is needed to investigate this possibility.

We did not anticipate the 20.4% increase of *B. pseudomallei* deep organ abscesses during the study period. We theorized that it might partly be explained by compromised host immunity to *B. pseudomallei*, attributable to the progressively higher prevalence of diabetes in Singapore (than in Australia and Thailand) (*13*). Specifically in the context of abscess development, diabetic tissue macrophages impair *B. pseudomallei* killing capacity and an impaired interleukin IL12–interferon-γ response had been implicated (*14*). Conversely, melioidosis-associated deaths in patients with diabetes had decreased by 10.8% during our study period. This improvement in outcome might have found its roots in the drive for optimization of diabetes care at the public health level in Singapore in recent years (*15*).

Conclusions

The overall death rate from melioidosis in Singapore was 18.4%, similar to that in the Northern Territory of Australia (14%). In both locations, these rates were attained on the background of similar standards and accessibility to healthcare and a low threshold for institution of treatment for melioidosis in accordance with recommendations (*1*). As efforts continue to further optimize clinical outcomes in acute melioidosis, our experience from Singapore for 2003–2014 suggests that acquisition of melioidosis and pneumonia may be curtailed through enhanced environmental and water management incorporating countrywide infrastructural improvements. In addition, enhanced management of the at-risk cohort of persons with diabetes also might prove pivotal in reducing disease.

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Phylogeny and Immunoreactivity of Norovirus GII.P16-GII.2, Japan, Winter 2016–17

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During the 2016–17 winter season in Japan, human norovirus GII.P16-GII.2 strains (2016 strains) caused large outbreaks of acute gastroenteritis. Phylogenetic analyses suggested that the 2016 strains derived from the GII.2 strains detected during 2010–12. Immunochromatography between 2016 strains and the pre-2016 GII.2 strains showed similar reactivity.

Norovirus is a major cause of acute gastroenteritis in humans and is genetically classified into 7 genogroups (GI–GVII). Among them, norovirus GI, GII, and GIV infect humans, and human norovirus (HuNoV) has many genotypes (*1*). GII viruses are common and have 22 confirmed genotypes (*1*).

In Infectious Diseases Weekly Report (https://www.niid.go.jp/niid/en/idwr-e.html), the Japanese national surveillance system reports the number of patients with acute gastroenteritis and their pathogenic agents who were examined at clinics or hospitals of the sentinel surveillance medical institutes (≈3,000 institutions). This report indicated that HuNoV GII.2 was the third or fourth most prevalent genotype during the past 5 seasons (2011–2016), but became the predominant strain during the 2016–17 season. Of note, the total number of children with acute gastroenteritis in 2016–17 was the second largest over the past 11 epidemiologic seasons (*2*,*3*).

On the basis of these observations, we used the full length of the RNA-dependent RNA polymerase (RdRp) region and capsid (VP1) gene to study the phylogeny of HuNoV strains detected during 2016–17 winter season (2016 strains). We also determined the immunoreactivities of the variant strains by various immunochromatography (IC) kits and the bioluminescent enzyme immunoassay (BLEIA).

The Study

In this study, we analyzed 26 GII.2 strains detected during October 2016–January 2017. Of those, we analyzed 19 strains for their phylogeny, and examined 7 strains by IC and BLEIA (*4*). We collected samples from the patients (children and adults) with acute gastroenteritis (mean ±SD age 9.2 ± 12.0 years). Because we could not collect adequate amounts of fecal specimens in some instances, we could not perform sequence analyses for all of them. Moreover, to compare the sensitivities of IC and BLEIA for the previous and current GII.P16-GII.2 strains, we conducted these tests using 2 fecal specimens containing GII.P16-GII.2 virus detected in 2009. These specimens, stored at –80°C, were obtained from clinics or hospitals, such as the sentinel surveillance medical institutions in Ibaraki Prefecture and Kawasaki City in Japan. We obtained written informed consent from the patients or their guardians for the samples. The study protocols were approved by the National Institute of Infectious Diseases for Public Health Ethics Committees (No. 576).

We performed viral RNA extraction and reverse transcription PCRs as described (*5*). To analyze the complete RdRp region and VP1 gene, we used primer-walking methods with primers designed by the PrimaClade server (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/1/17-0284-Techapp1.pdf) (*6*) and performed sequencing as described (*5*). To determine the genotype of

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the current virus, we used Norovirus Genotyping Tool version 1.0 (*7*). The accession numbers of the current strains are listed in Technical Appendix Table 2. To construct the phylogenetic tree, we collected complete sequences of the VP1 gene and RdRp region of HuNoV from GenBank (online Technical Appendix Table 2). We used 61 reference strains for phylogenic analysis of the VP1 gene and 70 strains for the RdRp region. We selected the best substitution models using the Bayesian information criterion (BIC) method by MEGA 6.0 (http://www.megasoftware.net (*8*). Using sequences from both regions, we inferred phylogenetic trees by the maximum-likelihood method as implemented in MEGA 6.0 (*8*).

To explore the immunoreactivities of 2016 strains, we used 7 fecal specimens from the patients with acute gastroenteritis attributed to the 2016 strains to assess relationships among these strains' genome copy numbers and the sensitivities of 5 commercial IC kits: ImmunoCatch-Noro (Eiken Chemical, Tokyo, Japan); Quick Chaser-Noro (Mizuho Medy, Tosu-shi, Japan); Quick Navi-Noro 2 (Denka Seiken, Tokyo, Japan); GE test Noro Nissui (Nissui Pharmaceutical, Tokyo, Japan); RIDA QUICK Norovirus (R-Biopharm AG, Darmstadt, Germany). In addition, we evaluated the BLEIA (Eiken Chemical, Tokyo) to compare sensitivities for the IC kits to the 2016 strains. We quantified HuNoV genome numbers by using a real-time PCR (*9*). Furthermore, to test the

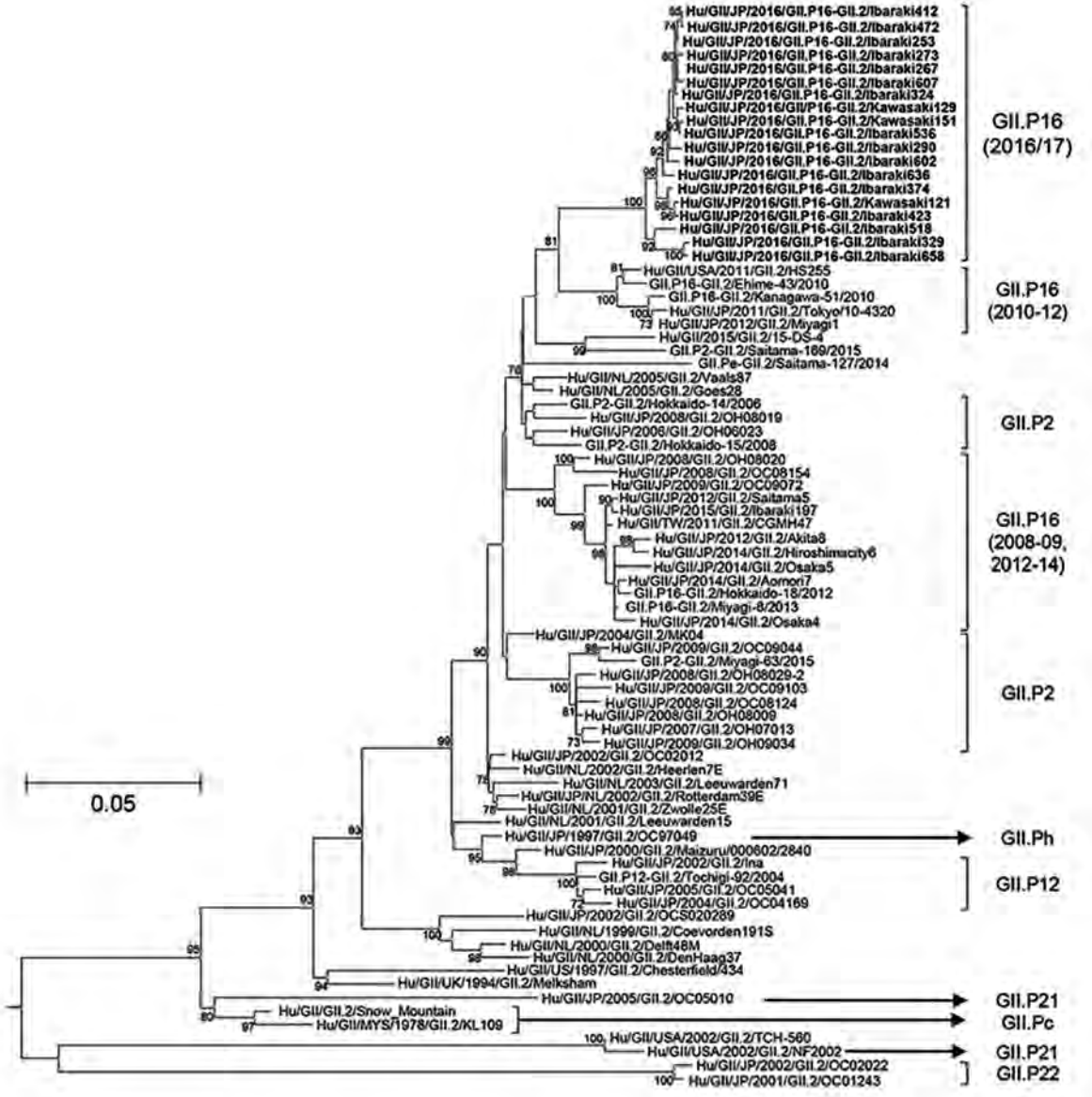

Figure 1. Phylogenetic tree for the capsid (VP1) gene in human norovirus GII strains. The tree was constructed by using the maximum-likelihood method. Bold letters denote GII.2v strains. Numbers at branch nodes show bootstrap values with ≥70% support. Scale bar represents number of nucleotide substitutions per site.

sensitivity of the IC kits and BLEIA with the GII.2 strains before and during the 2016–2017 winter season, we used 2 fecal specimens collected in 2009–10.

The genotype of HuNoV detected in the 2016–17 season was GII.P16-GII.2. The maximum-likelihood tree of the VP1 genes formed many clusters (Figure 1). The 2016 strains diverged from common ancestors of the GII.P16-GII.2 cluster detected in 2010–12. The phylogenetic tree of the RdRp coding region also formed many clusters (Figure 2). The common ancestors of GII.P16 strains diverged into 2 clusters; one contains strains with capsid genotypes GII.3, GII.4, GII.13, and GII.2 detected in 2010–12, and the other one contains strains with capsid genotypes GII.17 and GII.2 detected in 2009–10 and 2012–15. On the basis of these trees, the 2016 strains could have diverged from GII.2 detected in 2010–12. Finally, RdRp of the 2016 strains diverged from the common ancestors of the 2016 strains and GIIP16-GII.4.

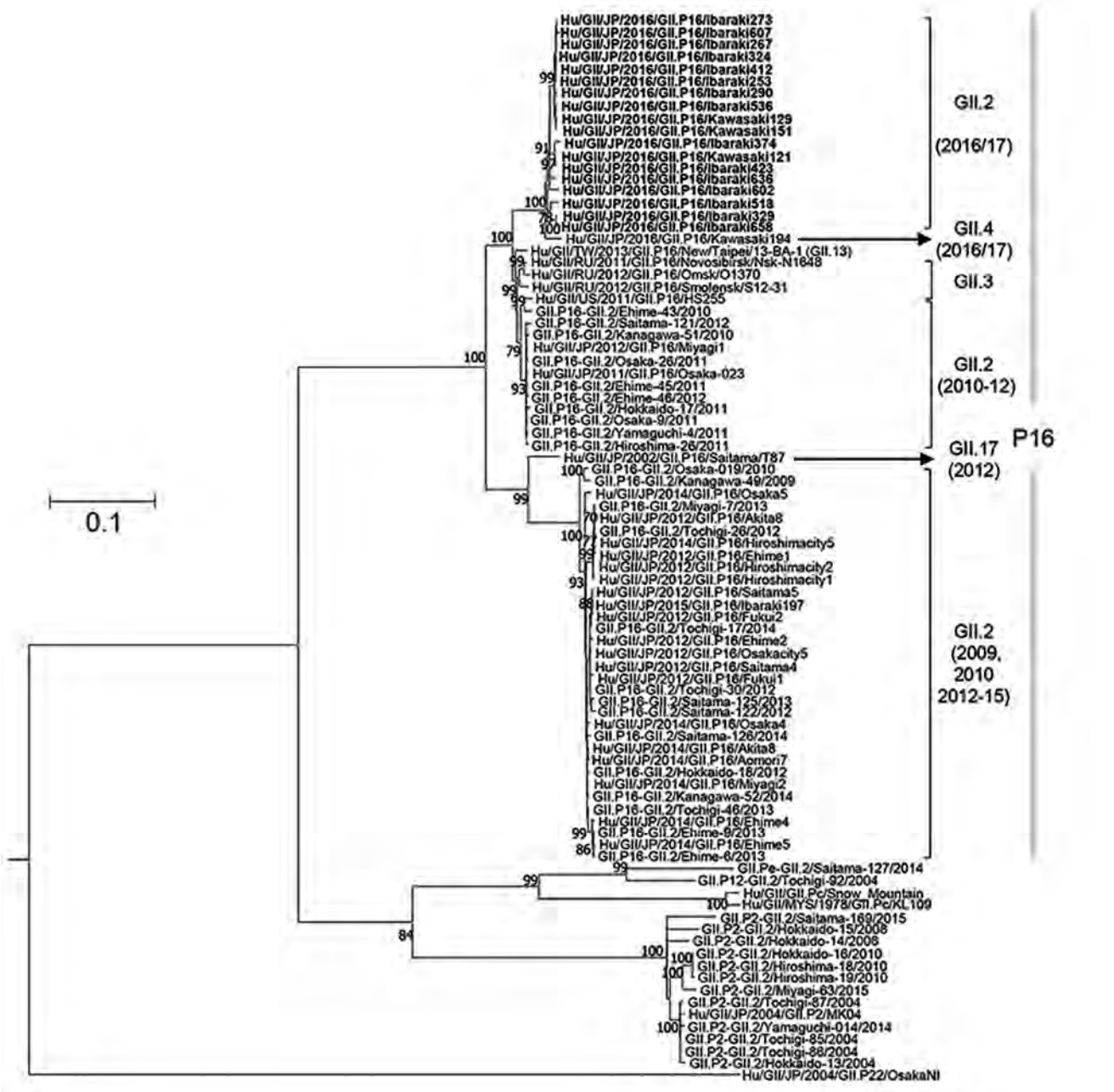

Figure 2. Phylogenetic tree for the RNA-dependent RNA polymerase (RdRp) region in human norovirus GII strains. The tree was constructed by using the maximum-likelihood method. Bold letters denote GII.2v strains. Numbers at branch nodes show bootstrap values with ≥70% support. Scale bar represents number of nucleotide substitutions per site.

Table. Sensitivities of 5 immunochromatography kits and the BLEIA for 2016 and pre-2016 GII.P16-GII2 norovirus strains*

Sample ID	Virus genome, copies/g	Norovirus immunochromatography kit test results					BLEIA result
		ImmunoCatch-Noro	Quick Chaser-Noro	Quick Navi-Noro 2	GE test Noro Nissui	RIDA QUICK	
Kawasaki181, 2016 strains	9.12×10^9	+	+	+	+	+	+
Kawasaki159, 2016 strains	7.47×10^8	+	+	+	–	+	+
Kawasaki163, 2016 strains	2.30×10^7	+	+	–	–	+	+
Kawasaki173, 2016 strains	1.31×10^7	+	–	–	–	–	+
Kawasaki125, 2016 strains	2.26×10^6	–	–	–	–	–	+
Kawasaki175, 2016 strains	1.82×10^6	–	–	–	–	–	–
Kawasaki177, 2016 strains	3.16×10^5	–	–	–	–	–	–
Ibaraki09–1095, pre-2016 GII.P16-GII.2	8.09×10^7	+	–	–	–	–	+
Ibaraki09–965, pre-2016 GII.P16-GII.2	1.40×10^7	+	–	–	–	–	+

*Kit manufacturers: ImmunoCatch-Noro, Eiken Chemical, Tokyo, Japan; Quick Chaser-Noro, Mizuho Medy, Tosu-shi, Japan; Quick Navi-Noro 2, Denka Seiken, Tokyo, Japan; GE test Noro Nissui, Nissui Pharmaceutical, Tokyo, Japan; RIDA QUICK Norovirus, R-Biopharm AG, Darmstadt, Germany. BLEIA, bioluminescent enzyme immunoassay; ID, identification information; +, positive; –, negative.

To examine the antigenicity between 2016 strains and pre-2016 GII.2 strains, we assessed the relationship between the quantities of GII.2 genome and the results of the IC kits and BLEIA (Table). The IC kits we used in this study all showed the 2016 strains positive, when $\approx 10^{10}$ copies/g of viral genome were in each sample. The reactivities of these kits against lower copy numbers of 2016 strains and pre-2016 GII.2 strains were significantly different. For example, 1 kit (ImmunoCatch-Noro) was >100 times more sensitive than another kit (GE test Noro Nissui) used in this study. BLEIA could detect samples with 2016 strains of genome copy number of $\approx 2.3 \times 10^6$ copies/g in fecal samples.

Conclusions

We describe the phylogeny and immunoreactivity of the strains that suddenly emerged in Japan in 2016. As shown in the trees, the 2016 strains diverged from the HuNoV GII.P16-GII.2 detected in 2010–12, rather than that detected in 2015. Tohma et al. showed that the GII.P16 strains circulating in the 2016–17 winter season carried 4 amino acid mutations (S293T, V332I, K357Q, and T360A) in their polymerase from pre-2016 GII.P16-GII.2 strains (*10*). Among them, all 2016 strains had amino acid mutations (S293T, K357Q, and T360A), and 17 of the 19 strains also had the V332I mutation. Moreover, some reports suggested that the GII.P16-GII.2 strains detected in 2016 were a new recombinant strain, and both the RdRp region and VP1 gene sequences of our strains and the new strains were similar (identity ≈98%–99%; data not shown), although the analyzed sequence lengths were different (*11*,*12*). However, in this study, we could not obtain clear data for the recombinant strains.

Previous reports showed that IC kits were positive for most of the HuNoV GII genotypes in clinical samples (fecal specimens), corresponding to around 10^7 copies of norovirus genome per gram in the fecal specimen (*13*,*14*). In contrast, another report showed that the reactivity of the pre-2016 GII.2 strains was lower than other genotypes (*15*). We obtained similar data on the sensitivity in each kit between the pre-2016 GII.P16-GII.2 and 2016 strains. However, we saw substantially different sensitivities among these IC kits (maximum 100-fold). Our findings show that the IC kits may be valuable for detecting the GII.P16-GII.2 strains, including pre-2016 GII.2 and 2016 strains, on a case-by-case basis or as a backup test in the laboratories with a rapid bedside norovirus diagnosis.

The 2016 NoV strains caused outbreaks of acute gastroenteritis in many countries (*2*,*10*–*12*). Additional molecular epidemiologic analyses are needed to track the epidemics and to better understand the viral evolution.

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Mammalian Pathogenesis and Transmission of Avian Influenza A(H7N9) Viruses, Tennessee, USA, 2017

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Infections with low pathogenicity and highly pathogenic avian influenza A(H7N9) viruses affected poultry in 4 states in the southeastern United States in 2017. We evaluated pathogenicity and transmission of representative viruses in mouse and ferret models and examined replication kinetics in human respiratory tract cells. These viruses can cause respiratory infections in mammalian models.

Influenza A viruses have been associated with sporadic influenza outbreaks in commercial poultry throughout North America, typically due to low pathogenic avian influenza (LPAI) H5 and H7 subtype viruses (*1*). Recent emergence and spread of highly pathogenic avian influenza (HPAI) H7N3 and H7N8 subtype viruses in North America have underscored the capability of LPAI viruses to mutate into HPAI viruses and cause devastating losses to domestic poultry (*2*). Spread of avian influenza viruses in waterfowl flyways in North America, especially those over areas of dense commercial poultry operations, necessitates constant surveillance and study (*3*). Because these viruses are reportable to the World Organisation for Animal Health, detection of these subtypes also has a major role in trade of commercial poultry products (*4*).

The Study

In March 2017, outbreaks of infection with HPAI H7N9 subtype virus were reported on 2 commercial broiler breeder farms in Lincoln County, Tennessee, USA. LPAI H7N9 subtype virus was concurrently and subsequently reported in commercial and backyard producer farms in Tennessee, Alabama, Kentucky, and Georgia (*5*,*6*). More than 270,000 birds died or were culled; no human cases were reported. Similar to previous epornitics of LPAI H7N9 subtype virus in Kentucky, Minnesota, and Nebraska in recent years, viruses isolated in Tennessee in 2017 were of the North American wild bird lineage and genetically and phenotypically distinct from the Asian lineage of avian influenza A(H7N9) virus circulating in China (*6*,*7*).

This study was approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention. We examined in 2 mammalian models the pathogenicity and transmissibility of LPAI and HPAI H7N9 subtype viruses isolated from chickens in Tennessee (ck/TN) and evaluated the capacity for these viruses to replicate in a representative human respiratory cell line. H7N9 isolates A/ck/TN/17-007147-2/2017 (HPAI) and A/ck/TN/17-007431-3/2017 (LPAI) differ by a 9-aa insertion in the hemagglutinin gene and 18 additional amino acids throughout the genome (*5*,*6*).

Table. Infection of mice and ferrets with influenza A(H7N9) ck/TN viruses, Tennessee, USA, 2017*

Characteristic	LPAI	HPAI
Mice†		
Weight loss‡	1.7	2.3
Virus titer§		
10^6 lung, day 3 pi	4.3 ± 1.1	<1.5
10^3 lung, day 3 pi	2.8 (1/3)	<1.5
10^6 lung, day 6 pi	5.6 ± 2.1	<1.5
10^3 lung, day 6 pi	<1.5	<1.5
Ferrets, days 1–10 pi¶		
Weight loss‡	3.1	4.6
Fever#	1.2	0.6
Virus in rectal swab specimen**	2/3	1/3
Virus titer at day 3 pi§		
Nasal wash	4.9 ± 0.5	3.6 ± 0.9
Nasal turbinates	5.8 ± 0.6	4.8 ± 0.8
Trachea	3.5 ± 0.4 (2/3)	<1.5
Lung	<1.5	<1.5
Olfactory bulb	3.8 (1/3)	<1.5
Brain	<1.5	<1.5
Intestine	<1.5	<1.5

*EID_{50}, 50% egg infectious dose; HPAI, highly pathogenic avian influenza virus; LPAI, low pathogenicity avian influenza virus; pi, postinoculation.
†Mice were 8 weeks of age and inoculated intranasally with 10^6 EID_{50} or 10^3 EID_{50} of virus in a volume of 50 μL.
‡Percent mean maximum weight loss after inoculation with 10^6 EID_{50} of virus (days 2–10 pi).
§Virus titers are expressed as mean ± SD $\log_{10}$ EID_{50}/mL for animals with positive virus detection (n = 3 unless otherwise denoted in parentheses). Inoculation dose was 10^6 EID_{50} (mice and ferrets) or 10^3 EID_{50} (mice). The limit of virus detection was $10^{1.5}$ EID_{50}/mL.
¶Ferrets were 7 mo of age, serologically negative for currently circulating viruses by hemagglutinin inhibition assay, and inoculated intranasally with 1 mL of virus.
#Mean maximum increase in body temperature (baseline body temperature range 38.1°C–38.9°C).
**No. ferrets with detectable virus in rectal swab specimens collected on days 1, 3, and 5 pi/total no. ferrets tested.

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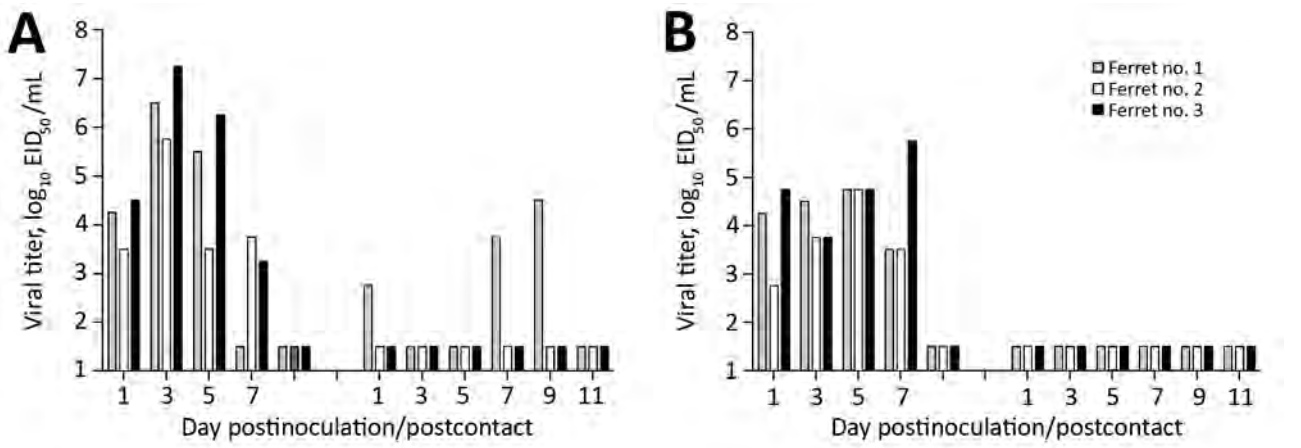

Figure 1. Transmission of avian influenza A(H7N9) virus ck/TN between ferrets in direct contact, Tennessee, USA, 2017. Three ferrets were inoculated with 10^6 EID_{50} of A) low pathogenicity avian influenza virus or B) highly pathogenic avian influenza virus, and nasal washes were collected from each ferret on the indicated days postinoculation (left bars) to assess viral replication. An immunologically naive ferret was placed in the same cage as each inoculated ferret at 24 h postinoculation, and nasal washes were collected from each contact ferret on the indicated days postcontact. Bars indicate individual ferrets. All ferrets were serologically negative for circulating influenza viruses at the start of the study. The limit of virus detection was $10^{1.5}$ EID_{50}/mL. EID_{50}, 50% egg infectious dose.

Previous investigations of H7 subtype virus pairs displaying varied pathogenicity in poultry have shown differential phenotypes in mice, ranging from mild to moderate infection, which was indistinguishable between HPAI and LPAI viruses (H7N3 subtype isolates from Chile in 2002 and British Columbia, Canada, in 2004), and severe infection with HPAI, but not LPAI, viruses (H7N8 subtype isolates from Indiana, USA, in 2016) (*8*,*9*). Among LPAI or HPAI ck/TN viruses, inoculated BALB/c mice showed mild illness (weight loss <3%) and no deaths (Table). Although LPAI virus replicated to moderate titers in lungs of mice after high-dose inoculation, HPAI virus was not detected. Infectious virus was not detected in the nose or brain of any mouse.

To examine virulence of ck/TN viruses in a mammalian species with closer physiologic similarity to humans, ferrets were inoculated with LPAI or HPAI viruses. Both viruses caused mild infection (<5% mean maximum weight loss; Table) without sustained lethargy, sneezing/nasal discharge, or high fever. LPAI and HPAI viruses were restricted to the upper respiratory tract of ferrets, and no infectious virus was detected in lungs. Both viruses were detected in nasal turbinates of all inoculated ferrets, and LPAI virus was also detected in the trachea of 2/3 ferrets and the olfactory bulb of 1/3 ferrets. Low virus titers were detected in rectal swab specimens (Table) but not in intestinal tissue or ocular samples (conjunctival washes and eye and conjunctiva tissue).

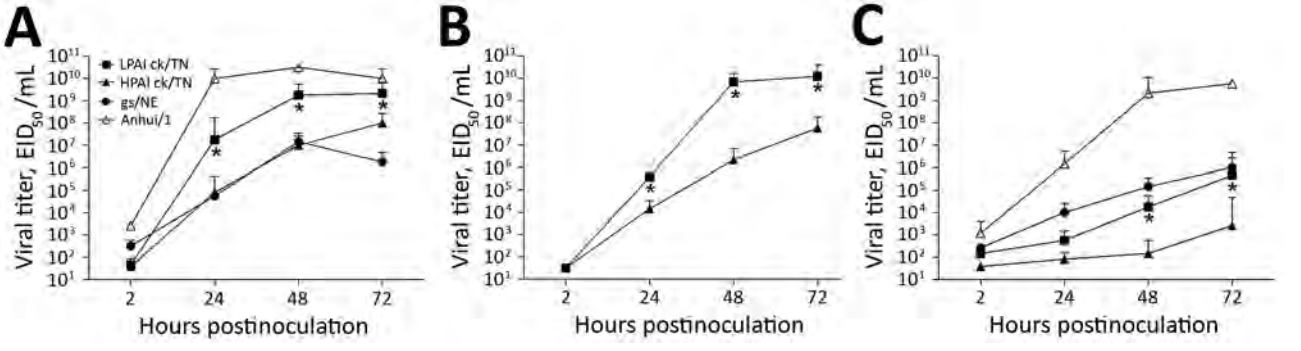

Figure 2. Replication kinetics of avian influenza A(H7N9) viruses in human respiratory tract cells, Tennessee, USA, 2017, compared with strains from Nebraska (gs/NE) and Asia (Anhui/1). Calu-3 cells (American Type Culture Collection, Manassas, VA, USA) were grown to confluence in 12-mm–diameter transwell inserts (Corning, Corning, NY, USA), infected apically with viruses shown at a multiplicity of infection of 0.01 (A and C) or 0.001 (B) 1 h, washed, and incubated at 37°C (A and B) or 33°C (C). Supernatants were removed at indicated times postinoculation, and titers of infectious virus were determined by titration in eggs. The limit of virus detection was $10^{1.5}$ EID_{50}/mL. Values are mean from triplicate independent cultures per virus. Error bars indicate SDs. *p<0.05 for HPAI vs. LPAI ck/TN viruses by 2-way analysis of variance with a Tukey posttest. EID_{50}, 50% egg infectious dose; gs, goose; HPAI, highly pathogenic avian influenza virus; LPAI, low pathogenicity avian influenza virus.

These findings indicate that HPAI and LPAI ck/TN viruses have mild virulence for 2 mammalian species.

Selected HPAI and LPAI H7 subtype influenza viruses from North America have the capacity to be transmitted between ferrets when the animals are placed in close contact (*10*). However, the transmissibility of ck/TN H7N9 viruses was not known. Ferrets inoculated with LPAI and HPAI ck/TN viruses shed virus in nasal wash specimens through days 5–7 postinfection and showed seroconversion to homologous virus (Figure 1). HPAI virus was not transmitted to immunologically naive cage mates, because all contact ferrets remained seronegative at the end of the study. However, 1/3 LPAI virus contact ferrets shed virus to titers $>10^4$ 50% egg infectious dose/mL and seroconverted to homologous virus, which demonstrated a limited capacity for virus transmission in this model.

To determine if the growth advantage of LPAI virus in mice and ferrets was maintained in human cells, we compared replication kinetics of HPAI and LPAI ck/TN viruses in a human bronchial epithelial cell line (Calu-3). Although both viruses replicated to high titers ($>10^7$ 50% egg infectious dose/mL) at a multiplicity of infection of 0.01 or 0.001 (Figure 2, panels A, B), LPAI virus replicated to a significantly higher titer than HPAI virus at either multiplicity of infection or culture temperature tested ($p<0.05$; Figure 2), despite showing a temperature sensitivity at 33°C, which was similar to that for other avian influenza viruses (*11*). Virus titers for LPAI ck/TN virus were significantly higher than those for a previously studied LPAI H7N9 virus from North America (A/goose/Nebraska/17097-4/2011) 24–72 h postinfection at 37°C ($p<0.05$), but were reduced compared with the Asian lineage H7N9 subtype A/Anhui/1/2013 virus (Figure 2, panel A).

Conclusions

Detection of HPAI and LPAI H7 viruses in the United States represents a threat to commercial poultry activities and avian health. Limited transmission in a direct contact setting of LPAI (but not HPAI) ck/TN virus is similar to virus transmission of LPAI H7N8 and H7N2 subtype viruses (*8*,*10*), and suggests that influenza A(H7N9) viruses isolated in the United States in 2017 represent a low threat for human health in their current form. However, the ability of H7 subtype viruses in North America to acquire genetic insertions at the hemagglutinin cleavage site after recombination with host RNA (*12*,*13*) and their potential to acquire mutations associated with mammalian adaptation and virulence underscores the need to monitor birds for LPAI H7 viruses. This monitoring is especially needed in and around regions with wild bird flyways and high density of poultry because these viruses can rapidly and sporadically mutate to become HPAI viruses (*3*).

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Whole-Genome Analysis of Recurrent *Staphylococcus aureus* t571/ST398 Infection in Farmer, Iowa, USA

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Staphylococcus aureus strain sequence type (ST) 398 has emerged during the last decade, largely among persons who have contact with swine or other livestock. Although colonization with ST398 is common in livestock workers, infections are not frequently documented. We report recurrent ST398-IIa infection in an Iowa farmer in contact with swine and cattle.

Livestock, especially swine, are a reservoir for *Staphylococcus aureus* sequence type 398 (ST398) (*1*). Carriage of this strain is primarily reported in persons with occupational exposure to livestock; however, less is known about the frequency and severity of ST398 infections, particularly in the United States, where surveillance for this organism is limited (*2*). We report colonization and recurrent infection with methicillin-sensitive *S. aureus* (MSSA), belonging to livestock clade ST398, in a farmer in Iowa, USA.

A participant in a longitudinal study of rural Iowans (*2*) provided swab samples of his current skin infection. Swabs were cultured, and *S. aureus* isolates were subjected to molecular analyses as previously described (*3*,*4*). Institutional review board approval was obtained from the University of Iowa.

The 61-year-old man enrolling in the Iowa study in July 2011 reported a skin infection on his foot. He had visited a doctor the previous day and received ciprofloxacin, and noted his infection was chronic. He also reported a history of heart disease and diabetes. The participant was a farmer who raised swine (900 head × 40 years) and cattle (500 head × 10 years), and owned a dog. He reported working directly with swine and cattle ≈2 hours per day and noted that he did not use personal protective equipment such as gloves or masks. His nose and throat were colonized with MSSA ST398 that was *mecA*-negative, periventricular leukomalacia–negative, and staphylococcal protein A type t571. The isolate was resistant to tetracycline, trimethoprim/sulfamethoxazole, and levofloxacin. The participant's wife was also colonized at enrollment with MSSA in her throat, showing the same molecular characteristics and susceptibility patterns as her spouse. She reported no exposure to livestock.

The isolate obtained from the participant's first culture showed the same molecular characteristics as isolates from his nasal and throat swabs and the isolate from his second infection culture received in November 2011 (Table). He described this second infection as cellulitis: he reported draining pus, a general ill feeling, and headaches. The infection was treated empirically with ciprofloxacin, neosporin, warm compresses, and incision and drainage. Isolates from his third incident infection culture in August 2012 showed the same molecular characteristics as previous samples, but 2 different susceptibility patterns: 1 isolate was the same as previous, while another showed additional phenotypic resistance to oxacillin (MIC 4 μg/mL by broth dilution), making this a borderline-resistant *S. aureus* infection. Genome sequence analyses showed that this strain had not acquired a *mec* gene and likely had become resistant from a mutation in a penicillin-binding protein. This infection was treated with mupirocin, trimethoprim/sulfamethoxazole, ciprofloxacin, warm compresses, and drainage. All of the farmer's isolates had the t571 *spa* type, which is common among livestock-independent (i.e., human-associated) ST398 lineages (*4*). *S. aureus* t571 strains have been isolated from an Iowa childcare worker (*5*), inmates from a Texas jail (*6*), and in community members in New York and New Jersey (*7*); t571 strains have been rarely isolated from Iowa livestock (*8*). However, whole-genome sequence analyses with 89 previously published clonal complex 398 genome data confirmed that the isolates from this case were from the livestock-associated ST398 lineage (CC398-IIa) (*4*).

Longitudinal follow-up complemented with whole-genome sequence analyses showed that the participant repeatedly experienced infections by the same strain over the course of 13 months. The isolates from this patient and his wife formed a distinct clade within the ST398 global phylogeny (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/1/16-1184-Techapp1.pdf). In-depth genomic analyses revealed a novel ≈265,000 bp recombinant region originating from clonal complex 9, and unique to this lineage.

It is unclear whether the farmer's recurrences arose from treatment failure, repeated acquisitions from exposure to livestock, or the result of long-term colonization and evolution. In contrast, the farmer's wife, who was colonized by the same strain, reported no direct contact

Table. Details of recurrent *Staphylococcus aureus* t571/ST398 infection in farmer, Iowa, USA*

Date	Antimicrobial drug resistance profile	Description of infection	Treatment
July 2011	TET, SXT, LVX	Cellulitis	Ciprofloxacin
November 2011	TET, SXT, LVX	Cellulitis, draining pus, headache, ill feeling	Ciprofloxacin, Neosporin, warm compress, incision, drainage
August 2012†	TET, SXT, LEVO (isolate 1); OXA, TET, SXT, LVX (isolate 2)	None provided	Mupirocin, SXT, ciprofloxacin, warm compress, drainage

*All isolates were t571/ST398 and were PVL negative. Neosporin is manufactured by Johnson & Johnson (New Brunswick, NJ, USA). LEVO, levofloxacin; LVX, levofloxacin resistance to isolate 2; OXA, oxacillin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.
†Two isolates from this infection were tested and had different antimicrobial drug resistance profiles as noted.

with livestock and may have become colonized by human-to-human transmission from her husband. Comparison with other strains collected as part of this (*2*) and other studies (*9*) identified closely related isolates from 3 other participants and 1 pig (online Technical Appendix Figure 1) distributed throughout the state of Iowa and into western Illinois (online Technical Appendix Figure 2).

This dataset does not specify whether the evolution of the borderline-resistant *S. aureus* phenotype took place in the patient or the livestock reservoir. The farmer was prescribed ciprofloxacin multiple times; this could have contributed to emergence of borderline oxacillin resistance (*10*). The farmer's first phenotypically methicillin-resistant strain was not detected until >1 year after enrollment into this study. This case demonstrates the ability of livestock-associated *S. aureus* ST398 to cause repeated skin and soft tissue infections in a manner similar to community-associated strains, and the necessity to screen for MSSA and methicillin-resistant *Staphylococcus aureus* when studying the spread of these organisms.

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Visceral Leishmaniasis in Traveler to Guyana Caused by *Leishmania siamensis*, London, UK

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The parasite *Leishmania siamensis* is a zoonotic agent of leishmaniasis; infection in animals has been documented in Europe and the United States. Reported authochthonous human infections have been limited to Thailand. We report a case of human visceral *Leishmania siamensis* infection acquired in Guyana, suggesting colonization in South America.

A 65-year-old woman was admitted to a hospital in London, UK, during March 2014 after collapsing in the street. She was anemic and mildly thrombocytopenic (hemoglobin level 8.1 g/dL, leukocyte count 4.62 × 10^9/L, platelet count 143 × 10^9/L). She had been unwell for 14 months, experiencing night sweats and a steady loss of energy but was otherwise asymptomatic. She reported no fever. On examination, she had hepatic enlargement and lymphadenopathy, was afebrile, and had normal liver function test results. She also had negative serologic test results for HIV, hepatitis B, hepatitis C, and the parasitic nematode spp. *Strongyloides*. Her CD4 count was 790 and rheumatoid factor was weakly positive; her immunoglobulin levels were within reference ranges.

The patient was from Guyana and migrated to the United Kingdom in 1967. Her relevant travel history comprised 2 recent visits to Guyana (Georgetown in 2012 and Freetown in 2013), Caribbean Grenada in 2012, Ghana in 2005, and France (Paris and Marseille) in 2003. We investigated for hematologic malignancy by bone marrow aspiration; *Leishmania* amastigotes were visible.

Results of serologic testing for *Leishmania* antibodies were negative by using a Rapydtest (rK39 RDT; Apacor Ltd., Berkshire, UK) and weakly positive by using the direct agglutination test (1:3,200; cutoff 1:1,600). *Leishmania* DNA was extracted from the sample. *Leishmania*–specific PCR amplification produced positive amplicons from kinetoplast minicircle, genomic heat shock protein 70, and internal transcribed spacer 1 (ITS1) targets. The kinetoplast–specific amplicon size appeared nearest to that of *Leishmania major*, which is not considered an agent of visceral leishmaniasis among humans.

The size and restriction fragment length polymorphism banding pattern of the ITS1 amplicon was distinct from all previously sampled human *Leishmania* species. Sequencing of the ITS1 amplicon revealed either 99% or 100% identity to ITS1 sequences from *Leishmania siamensis*. Lower levels of identity were seen in homologs from other human *Leishmania* species. Phylogenetic analysis of these sequences against reference sequences from other *Leishmania* species confirmed they clustered with *L. siamensis* sequences as a monophyletic group, supported by bootstrap values of 100% (Figure). We saw a major divergence from other *Leishmania* sequences.

We treated the patient with liposomal amphotericin B (AmBisome [Gilead Sciences Ltd., London, UK) at a dose of 3 mg/kg given on days 1–5, and 7-day courses beginning on days 10 and 20. She responded well and her blood test results returned to reference values. She had some mild reversible renal impairment during treatment; she recovered and did not relapse during a 10-month follow-up period.

Autochthonous human visceral leishmaniasis caused by *L. siamensis* was thought to be geographically confined to Thailand (*1–5*). Many of those case-patients also showed evidence of immune deficiency, such as HIV

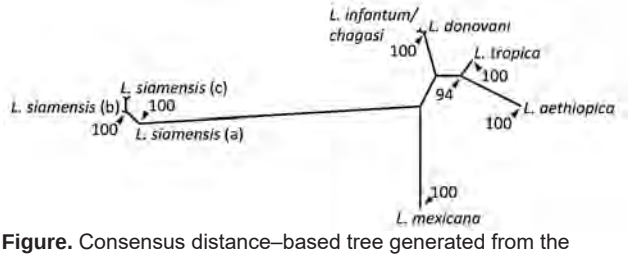

Figure. Consensus distance–based tree generated from the infecting amastigote's internal transcribed spacer 1 sequence and homologous sequences from other related human *Leishmania*-infected samples. Posterior bootstrap values are presented as the percentage of trees from 100 pseudorandomly sampled datasets which supported a given node with a value >90%. The sequences for the various terminal nodes, chosen for nearest identity to the derived sequence (EMBL-LT577674) by a BLASTn search are as follows: *L. aethiopica*, GQ920674, GQ920676, GQ920673; *L. tropica*, FJ948454, FJ948450, FJ948456; *L. mexicana*, AJ00313, AF466381; *L. donovani*, FJ753386, AM901452, AM901453; *L. infantum/chagasi* GU045592, FN398343, GU045591; *L. siamensis* (a), EF200012, (b), JX195637, GQ28127, JQ617283, JQ001751, GQ293226, (c), JQ866907, GQ226034. Branch lengths are proportional to the intersequence divergence, calculated by using the Fitch-Margoliash method of measuring pairwise distances derived from the F84 model.

infection (*1*,*3*). This patient had no evidence of immune deficiency, nor had one manifest during the follow-up period after her illness, yet she had a negative rK39 test result (which detects *Leishmania* antibodies) despite a visceral infection. Another patient with *L. siamensis* visceral leishmaniasis also had a negative rK39 test result (*2*); therefore, *L. siamensis* infections may not be detectable by rK39 testing.

The phlebotomine sand fly *Sergentomyia* (*Neophlebotomus*) *gemmea* is a possible vector for *L. siamensis* in Thailand (*6*–*8*). Sand flies from the *Sergentomyia* genus are generally zoophilic and therefore discounted as vectors of medically consequential *Leishmania* spp. Several *Sergentomyia* species are present in Europe and in South and North America, which may explain the presence of autochthonous zoonotic *L. siamensis* in these locations. Human infection with *L. siamensis* outside Thailand raises questions concerning transmission of this species to humans by anthropophilic phlebotomine sand flies or other species generally categorized as zoophilic.

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Investigation of Canine-Mediated Human Rabies Death, Haiti, 2015

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In Haiti, an investigation occurred after the death of a 4-year-old girl with suspected rabies. With tips provided by community members, the investigation led to the identification of 2 probable rabies-related deaths and 16 persons bitten by rabid dogs, 75% of which chose postexposure prophylaxis. Community engagement can bolster rabies control.

Haiti is one of the few countries in the Western Hemisphere where canine rabies control has not successfully eliminated canine-mediated human rabies deaths (*1*). During 2010–2012, an average of 4 canine and 7 human rabies cases were reported annually in Haiti; however, this rate is widely recognized as an underestimate of the true burden (*2*–*4*).

On September 22, 2015, public health officials of Haiti requested assistance with the investigation of a suspected human rabies death in Platfon, a rural part (human population 769) of the Gonaives Arrondissement. The case involved a 4-year-old girl who was bitten on the stomach by a dog 3 months before the onset of illness in late August. She had clinical symptoms consistent with rabies before death: hydrophobia, agitation, and localized paralysis. The child was not taken to the hospital for treatment after the bite or during her illness and died at home on September 8. In response to this probable human rabies case (according to the World Health Organization case definition) (*1*) and reports of increased rabies-like illnesses among dogs in the community, an international multiagency team was assembled to identify potential additional persons bitten for postexposure prophylaxis, conduct a dog vaccination campaign, and characterize healthcare-seeking and dog ownership behaviors.

We identified 4 additional persons bitten by the same dog responsible for the initial probable rabies case. During the investigation, community members requested the team investigate 2 additional events of suspected rabies transmission. The first event involved 10 persons bitten by a dog in the city of Gonaives (Figure, panel A). Brain samples from the dog were laboratory-confirmed positive for rabies virus. The second event involved 3 persons bitten by a dog, 1 of which subsequently died in July. In total, 2 probable human rabies deaths and 16 bitten persons were identified during the 3-week investigation (Figure, panel B). Although educational outreach and postexposure prophylaxis were offered at no cost to all persons bitten, 25% (n = 4) refused vaccination because of religious beliefs. Their current health status is unknown.

We surveyed 82 dog owners (mean 35.6 [range 18–60] years of age) attending the canine vaccination campaigns in Platfon and the city of Gonaives. The owners reported that 24 (4.3%) of 559 persons living in their households (median persons/household 6.9), including themselves, were bitten by dogs within the past year. Although 70% of survey respondents were aware that rabies can be transmitted through animal bites, only 53% knew that these bites could result in death. Approximately 83%

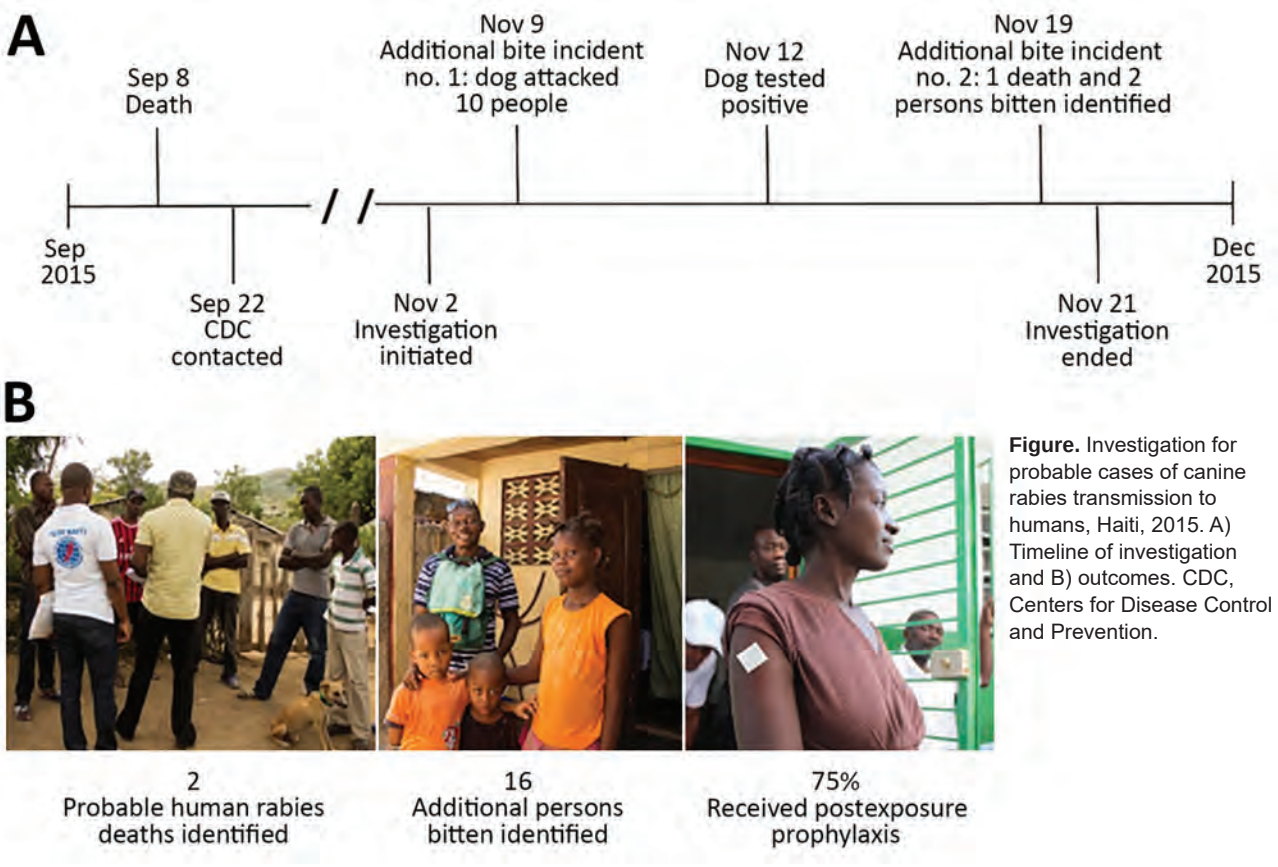

Figure. Investigation for probable cases of canine rabies transmission to humans, Haiti, 2015. A) Timeline of investigation and B) outcomes. CDC, Centers for Disease Control and Prevention.

of respondents stated they would seek medical care after an exposure but reported barriers to obtaining treatment: financial obstacles associated with transportation and treatment (47%) and absence of a local medical facility and trained personnel in the community (28%). Respondents stated it took 29 (range 5–180) minutes on average to travel to the nearest medical facility.

We also conducted animal health screenings during the vaccination campaign and estimated the local dog population (*5*) to assess vaccination coverage level. No animals were exhibiting signs consistent with rabies at the time of the investigation. A 2-day count identified 41 dogs, 21 of which had evidence of vaccination from our campaign (51.2% coverage). We estimated 87 dogs in this community and a 9:1 human-to-dog ratio, which is more similar to the ratio in Africa than Latin America, where the human-to-dog ratio is lower (*6*,*7*). Dog owners reported this was the first mass vaccination campaign available to them in the past 5 years. Given Platfon's remoteness, we believe many of these dogs were never vaccinated before this response.

Our investigation indicates that the rabies burden in Haiti is much greater than detected through passive surveillance because of the lack of healthcare-seeking behavior among persons bitten by dogs. We estimate the rabies-associated mortality rate as 0.67 cases/100,000 population, which is much higher than Haiti's health facility–based surveillance system estimates (average 0.07 cases/100,000 population) (*2*–*4*,*8*). The 2 probable human rabies cases and 16 persons bitten identified in this study were not originally reported to health officials because they did not seek care for their exposures and, thereby, were not captured in the national surveillance system.

Furthermore, this underreporting negatively affects proper allocation and stockpiling of nationally procured rabies biologics for animals. Intermittent dog vaccination campaigns are insufficient to break the cycle of rabies transmission. Sustained campaigns targeting 70%–80% of the dog population will be necessary to eliminate canine rabies (*1*,*9*). Anecdotal evidence suggests that social mobilization campaigns to increase healthcare-seeking behavior might be effective; most of the cases investigated in our study were reported by a community member in Platfon who alerted health officials after attending a rabies educator's workshop. We hope these findings will prompt a discussion for improvements in rabies prevention and control measures that empower community members with knowledge to notify officials of suspected rabies transmission events.

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Epidemiology of Cutaneous Leishmaniasis Outbreak, Waziristan, Pakistan

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During 2013–2015, prevalence of cutaneous leishmaniasis in war-affected Waziristan areas was 3.61% by PCR. Youths (1–15 years of age) were more susceptible. Internal transcribed spacer 1 PCR followed by restriction fragment length polymorphism analysis identified *Leishmania tropica* in 215 samples and *Leishmania major* in 6 samples.

Cutaneous leishmaniasis (CL), the most widespread form of leishmaniasis, caused by *Leishmania tropica* and *L. major* (*1*,*2*), has emerged as an endemic disease in Khyber Pakhtunkhwa, Pakistan (*2*–*5*), owing to frequent movement of internally displaced persons (IDPs) from Waziristan in response to surgical strikes and military operations against terrorists by Pakistani armed forces. Keeping in view the impact of the frequent outbreaks of CL in settled areas, we studied the prevalence of CL in war-affected North and South Waziristan, with the help of health department and local government authorities.

We surveyed 7,548 persons from the different endemic areas and collected samples from ulcerating skin lesions from 538 suspected CL patients, 244 in North Waziristan and 294 in South Waziristan, during April 2013–January 2015. To sort out the reservoir, we captured 72 rodents from different locations in Waziristan, such as around houses, in cornfields, and in wild plantations surrounding the houses of CL patients, and analyzed liver and spleen samples by PCR. In addition, we collected sand flies from within 1.5 km of CL patients' houses for molecular analysis (*6*). We determined the prevalence rate of CL by a formula described previously (*7*) and performed statistical analyses using statistical software SAS Enterprise Guide (version 4.2; SAS Institute, Cary, NC, USA) by univariate analysis of variance with statistical significance at $p<0.05$.

Prevalence according to sex was consistent with previous findings, indicating that CL infections were more prone to develop in males **(**Table**)** because of more social activity and interaction with IDPs, whereas females always remain covered because of Islamic rules and thus are less prone to sand fly bites. Agewise, we observed a higher prevalence rate in children 0–15 years of age (1.61%) compared with other age groups. Sand flies and rodents were also collected from different endemic villages; none of the trapped rodent samples tested positive for leishmaniasis, but samples from sand flies from 2 endemic villages of North Waziristan (Razmak and Shewa) and 1 endemic village of South Waziristan (Sreykhoray) tested positive by kinetoplast DNA PCR. Samples collected from domestic animals (sheep, goat, cattle, donkey, dogs, mules) were negative for leishmaniasis.

For this report, we performed internal transcribed spacer 1 PCR followed by restriction fragment length polymorphism analysis for identification of different species of *Leishmania*. For North Waziristan, we observed 63.0% of *L. tropica* and 8% of *L. major* specific bands by this analysis. Similarly, restriction fragment length polymorphism analysis of South Waziristan showed 54% *L. tropica* and 4% *L. major* specific bands. No *L. infantum*–positive cases were found in any human or animal (dog) samples. Moreover, different species of *Phlebotomus* and *Sergentomya* sand fly genera were identified in both North and South Waziristan; *P. sergenti* was the most abundant species, followed by *P. papatasi*. We reported leishmaniasis infection in 6 female *P. sergenti* sand flies. *P. papatasi* is also susceptible to carry *L. tropica* and is widely distributed in different parts of Pakistan, including Khyber Pakhtunkhwa Province (*8*). A previous team had reported *L. infantum* in 2 army personnel deployed in Waziristan, (*9*) but the present detailed study ruled out its presence.

We conclude that CL is prevalent in Waziristan and new cases are increasing day by day. The present study also confirms that *L. tropica* is the causative agent of CL in Waziristan. This study also confirmed that anthroponotic CL caused by *L. tropica* is the main causative agent of CL in Waziristan. All the patients whose specimens tested positive for *L. major* had a history of traveling to zoonotic CL endemic areas of Mezar Sharif (Afghanistan) and Sindh and Balochistan (Pakistan) (*10*). Because of limited access in the study area for security reasons, sampling could not be performed in other endemic parts of Waziristan, so further molecular epidemiologic studies on animal reservoirs and sand flies should be conducted in wider areas of Waziristan, including neighboring tribal and settled areas, to map the complete distribution of the disease.

Table. Areawise prevalence of CL in Waziristan, Pakistan, by microscopy and PCR

Area	No. tested	CL-positive samples, no.*		CL prevalence, %	
		Microscopy	PCR	Microscopy	PCR
North Waziristan					
Shewa	966	29	35	3†	3.62†
Spinwam	530	17	21	3.2†	3.96†
Mirali	320	6	7	1.87	2.18
Edaky	463	9	11	1.94	2.37
Darpakheil	450	11	14	2.44	3.11†
Hasankheil	373	9	11	2.41	2.94
Dosali	512	19	24	3.71†	4.68†
Miranshah	455	15	17	3.29†	3.73†
Razmak	367	14	16	3.81†	4.35†
Subtotal	4,436	129	156	2.91	3.51†
South Waziristan					
Wanna	412	14	28	3.39	6.79†
Shekai	463	12	18	2.59	3.88†
Jandola	516	10	10	1.93	1.93
Sra Roha	253	8	11	3.16†	4.34†
Makeen	169	4	9	2.36	5.32†
Janata	448	6	11	1.33	2.45
Sreykhoray	195	6	12	3.07†	6.15†
Kotkai	755	12	18	1.58	2.38
Subtotal	3,112	72	117	2.3	3.75†
Total	7,548	201	273	2.66	3.61

*CL, cutaneous leishmaniasis.
†Denotes statistically significant difference ($p<0.05$) analyzed by χ^2 test. In South Waziristan, the highest prevalence rate (6.79% by PCR), was found in Wanna district; in North Waziristan, Dosali had the highest prevalence (4.68% by PCR). However, South Waziristan showed a higher prevalence rate, 3.75% (117/3,112), than North Waziristan's 3.51% (156/4,436).

A leishmaniasis control committee should be established by health authorities in association with the Ministry of Health. It is strongly recommended that proper record-keeping and documentation systems for leishmaniasis be initiated by health authorities at the local, provincial, and national levels and be well maintained to identify leishmaniasis outbreaks so that control measures can be started in time. Further, IDP camps must be monitored regularly to minimize the risk that nonendemic areas will be exposed to the disease by infected IDPs.

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Ocular Vaccinia Infection in Dairy Worker, Brazil

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We studied a clinical case of vaccinia virus that caused an ocular manifestation in a dairy worker in Brazil. Biologic and molecular analyses identified a co-infection with 2 isolates from different Brazilian vaccinia virus phylogenetic groups.

Detection of co-infections for various viral pathogens recently has increased (*1*,*2*). However, the presence of ≥2 viral etiologic agents often is not considered (*1*). The range of pathogens that can present co-infections and the association of these infections with the occurrence and severity of disease remain unclear (*1*,*2*). Vaccinia virus (VACV), the prototype virus of the genus *Orthopoxvirus*, has been associated with exanthematic outbreaks in Asia and South America that affect mainly dairy cattle and rural workers (*3*,*4*). In Brazil, several Brazilian VACV (VACV-BR) have been isolated and characterized biologically and phylogenetically. These studies demonstrated that circulating viruses belonged to at least 2 distinct genetic clusters (*4*–*8*). Previous studies have demonstrated the co-circulation of distinct VACV isolates during the same outbreak and VACV co-infecting horses and cattle (*6*–*8*).

We obtained 2 distinct VACV isolates from the same clinical sample from 1 eye of a rural worker. Our data show that the eye was co-infected with 2 VACV and demonstrates the detection and isolation of VACV from a natural case of ocular vaccinia infection.

In September 2015, an unvaccinated 45-year-old man who worked on a farm in Carangola County, Minas Gerais State, Brazil (20°44′06″S, 42°01′52″W), showed development of typical manifestations of vaccinia infection, including fever and painful vesiculopustular lesions (online Technical Appendix Figure, panel A, https://wwwnc.cdc.gov/EID/article/24/1/17-0430-Techapp1.pdf). He had lesions on the left hand, right arm, and nose and an atypical manifestation in the left eye with aches in the ocular globe and periorbital region. The clinical condition progressed to major visual acuity losses in the affected eye. He reported recent contact with sick cows on the farm during milking.

Dried swab specimens from his lesions were soaked in 200 μL phosphate-buffered saline containing amphotericin B (4 μg/mL), penicillin (200 U/mL), and streptomycin (100 μg/mL); homogenized; and centrifuged at 3,000 × *g* for 5 min (*4*). The supernatants were used for molecular diagnosis using orthopoxvirus-specific PCR that targeted the C11R gene, which encodes viral growth factor, and the A56R gene, which encodes viral hemagglutinin protein (*4*). All samples were positive for both orthopoxvirus targets.

Vero cells were cultured in 25-cm^2 culture flasks and infected with the specimen supernatants to isolate the virus at 37°C until a cytopathic effect was detected (*4*). VACV was isolated from the hand, nose, and eye samples. These isolates were tested for their plaque phenotypes in BSC-40 cells incubated at 37°C for 48 h (*4*), which demonstrated the presence of at least 2 types of viral populations comprising small and large plaques in an estimated ratio 2:1. Two viral plaques (1 forming large and 1 forming small plaques) were obtained from the eye sample after 3 additional rounds of plaque purification in BSC-40 cells (online Technical Appendix Figure, panel B) (*4*). The viral plaques were propagated and titrated by plaque assay in Vero cells, and their DNA was extracted (*4*).

We obtained the complete genomes using the Illumina MiSeq instrument (Illumina, San Diego, CA, USA) with the paired-end application. The sequence reads were assembled de novo using ABYSS software (http://www.bcgsc.ca/platform/bioinfo/software/abyss), and the resulting contigs were ordered by the python-based CONTIGuator.py software (http://contiguator.sourceforge.net). The GenBank accession numbers are MG012795 (small) and MG012796 (large).

Analysis of the complete genome revealed 92% similarity between the 2 isolates, and some genes confirm a remarkable variability (online Technical Appendix Figure, panel C). We constructed a phylogenetic tree (online Technical Appendix Figure, panel D) using the A56R gene sequence by the maximum-likelihood method and 1,000 bootstrap replicates in MEGA 6.02 (http://www.megasoftware.net). The analysis demonstrated a co-infection with viruses from both VACV-BR groups, such that the large-plaque clone clustered with group 2 VACV-BR isolates and the small-plaque clone clustered with group 1 VACV-BR isolates. We named these isolates Carangola eye virus 1 (small) and Carangola eye virus 2 (large).

Our study demonstrated the genetic and phenotypic variability between 2 viruses isolated from the same sample in a natural human co-infection with VACV. The viruses belong to 2 distinct VACV-BR groups, reinforcing and expanding previous work with other hosts (*6*–*8*). These results raise new questions about how co-infections with these viruses might change the aspects of an infection and its signs and symptoms, such as development of ocular vaccinia. Although cases of ocular vaccinia have been reported after vaccination and accidental laboratory infection (*9*,*10*), we proved the association and isolate VACV samples from a natural ocular vaccinia infection. The effort to understand singular aspects of VACV-BR co-infections should be increased, and further molecular and biologic characterizations of these samples should be conducted to identify and better understand the natural dynamics and signs and symptoms caused by VACV-BR.

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Estimation of Undiagnosed *Naegleria fowleri* Primary Amebic Meningoencephalitis, United States[1]

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Primary amebic meningoencephalitis is an acute, rare, typically fatal disease. We used epidemiologic risk factors and multiple cause-of-death mortality data to estimate the number of deaths that fit the typical pattern for primary amebic meningoencephalitis; we estimated an annual average of 16 deaths (8 male, 8 female) in the United States.

[1]Preliminary results of this study were presented at the Infectious Diseases Society of America Conference; October 8–12, 2014, Philadelphia, Pennsylvania, USA.

Naegleria fowleri causes primary amebic meningoencephalitis (PAM); 0–8 laboratory-confirmed cases per year are documented in the United States) (*1*). PAM causes <0.5% of diagnosed encephalitis deaths in the United States (*2*). Laboratory-confirmed PAM case-patients in the United States are a median age of 12 years and are identified primarily in southern states during July–September, and 79% are male (*1*,*3*). Many case-patients are identified postmortem; 4 known survivors have been reported in the United States (*1*,*4*). The signs and symptoms of PAM can be mistaken for other more common neuroinfections, such as bacterial meningitis and viral encephalitis (*1*,*4*). Because more than half of neuroinfectious deaths are unspecified (*2*), clinical expertise and diagnostic testing availability are limited, and true PAM incidence is unknown, concern is reasonable that PAM cases might not be diagnosed. In this study, we estimate the magnitude of potentially undiagnosed cases of PAM by applying previously identified epidemiologic risk factors to unspecified neuroinfectious deaths.

We created a list of codes from the International Classification of Disease, 10th revision (ICD-10), for unspecified possible neuroinfectious deaths by using previously published data (*2*), ICD-10 codes from death certificates of known PAM case-patients, and expert opinion. We selected codes from any location on the death record, not strictly the primary or immediate cause of death (http://www.cdc.gov/nchs/deaths.htm). We chose to start in 1999 when death certificate data were first coded by using ICD-10 and ended in 2010, using the most updated data at the time of this analysis. Persons 2–22 years of age were included (±10 years from the average age of 12 years), excluding infants and older adults, who are more susceptible to bacterial meningitis. We applied known risk factors for PAM: 1) geographic location, i.e., states that reported diagnosed cases as of 2010; 2) summer seasons; and 3) sex (*3*). Within this narrowed subset of unspecified neuroinfectious deaths, we reviewed associated ICD-10 codes and removed death records that had more definitive diagnoses.

During 1999–2010, there were 1,676 unspecified neuroinfectious disease deaths among persons 2–22 years old; 49% (826/1,676) occurred during July–September of each year studied, and of those, 23% (192/826) were reported from an included state in the southern United States; 52% (100/192) were male and 48% (92/192) female. An average of 16 (8 male, 8 female) unspecified neuroinfectious deaths per year fit the typical pattern of PAM, in addition to the average 3 laboratory-confirmed cases annually during this time period.

Among all unspecified neuroinfectious deaths, the most common unspecified neuroinfectious death code used was G03.9 meningitis unspecified (n = 505) (Table). For the top 5 codes, 8%–16% of cases matched all the risk factors and 2 had a sex ratio of exactly 50%. We did not have access to death certificates for 20 known laboratory-confirmed case-patients to determine what ICD-10 codes were used in these cases.

Our estimate of annual undiagnosed PAM cases shows that unspecified neuroinfectious deaths that fit the epidemiologic pattern of PAM occur infrequently. This estimate likely includes unspecified neuroinfectious death caused by other pathogens. We have no method to differentiate cases that fit the pattern of PAM, but are caused by another pathogen. Bacterial meningitis, which can be mistaken for PAM (*1*), has decreased over approximately the same time period as this study, but does not have the epidemiologic pattern of PAM

Table. Unspecified neuroinfectious death ICD-10 codes by epidemiologic risk factor for primary amebic meningoencephalitis among persons 2–22 years of age, United States, 1999–2010*†

ICD-10 code, disease	Total	In high-incidence states‡ (%)	July–Sept (%)	In high-incidence states,‡ July–Sept	
				Male patients (%)	Female patients (%)
G03.9, Meningitis unspecified	505	257 (51)	96 (19)	28 (6)	28 (6)
G04.9, Encephalitis, myelitis and encephalomyelitis, unspecified	479	222 (46)	135 (28)	33 (7)	26 (5)
R29.8, Other and unspecified symptoms and signs involving the nervous and musculoskeletal systems	264	112 (42)	63 (24)	8 (3)	13 (5)
G00.9, Bacterial meningitis, unspecified	222	105 (47)	41 (18)	11 (5)	11 (5)
A86, Unspecified viral encephalitis	154	92 (60)	40 (26)	15 (10)	9 (6)
G06.2, Extradural and subdural abscess, unspecified	59	31 (53)	12 (20)	4 (7)	0
A87.9, Viral meningitis, unspecified	38	25 (66)	13 (34)	4 (11)	4 (11)
A89, Unspecified viral infection of the central nervous system	6	2 (33)	3 (50)	0	1 (17)
A83.9, Mosquito-borne viral encephalitis, unspecified	1	1 (100)	1 (100)	0	1 (100)

*Codes without cases (R83.5, A92.9, A85.2, A84.9, A81.9, A94, A06.6) not listed. ICD-10, International Classification of Disease, 10th revision.
†The total provided is greater than the total number of cases because each case may have >1 ICD-10 code.
‡Arizona, Arkansas, California, Florida, Georgia, Louisiana, Mississippi, Missouri, Nevada, New Mexico, North Carolina, Oklahoma, South Carolina, Texas, and Virginia.

(*5*). Viral causes (i.e., La Crosse and West Nile viruses) have a similar pattern, occurring during July–September (>80% of cases) and more commonly in males (3:2 male:female ratio) (*6*). An ICD-10 code for West Nile virus, A92.3, was added in 2005. There was only 1 case in our estimate that had the code Mosquito-borne viral encephalitis, unspecified (A83.9). Similar to PAM, cases of arbovirus disease could be included in even less-specific meningitis and encephalitis codes, illustrating that unspecified neuroinfectious deaths are likely caused by several pathogens.

Medical chart review and autopsies, not available for this study, would provide further information about the cause of death. Although this estimate likely captures more than just PAM cases for the reasons we have outlined, it might not capture all potential PAM cases. Reasons for an underestimate include inaccurate ICD-10 coding (*7*) and PAM cases that are outside the typical epidemiologic pattern (e.g., 2 cases in Minnesota [(*8*)] and out of season, such as adult cases linked to ritual nasal rinsing and sinus irrigation [(*9*,*10*)].

Although all available evidence points to PAM being a low-incidence disease in the United States, PAM remains a devastating and nearly universally fatal infection that erodes public confidence in the safety of everyday activities (swimming, using public drinking water) and increased stress on local public health departments that are already overextended. The reports of recent survivors indicate that timely diagnosis and early initiation of anti-amebic therapy may be instrumental in combating this deadly infection (*4*). Therefore awareness, evaluation of risk factors, testing, and early anti-amebic therapy provide the best opportunity for survival (*1*).

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Leprosy in Nonimmigrant Canadian Man without Travel outside North America, 2014

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In Canada, Hansen disease (leprosy) is rare and not considered in diagnoses for nonimmigrant patients. We report *Mycobacterium leprae* infection in a Canadian man whose sole travel was to Florida, USA. The *M. leprae* isolate was identified as armadillo-associated genotype 3I-2-v1. Travelers to the southern United States should avoid contact with armadillos.

In 2014, a 69-year-old nonimmigrant man from Atlantic Canada who had a 10-month history of nonscaly, annular, polycyclic plaques over his trunk and extremities sought treatment. His lesions were red-brown and 4–5 cm in diameter (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/1/17-0547-Techapp1.pdf). He also had innumerable erythematous papules and plaques measuring 0.5–2 cm. These lesions were predominantly symmetric with poorly defined borders; skin was normal between lesions. His condition did not improve with a 30-day trial of triamcinolone cream and doxycycline. He had no sensation abnormalities, thickened peripheral nerves, motor neuropathy, or alopecia. His medical history included coronary artery disease, diabetes, dyslipidemia, and hypertension. His only travel consisted of yearly visits to Davenport, Florida, USA, for the past 8 years. He had no contact with animals, including armadillos, or with persons with similar lesions. The patient had always lived in Atlantic Canada and was a retired farmer who had never employed foreign workers.

An earlier punch biopsy, taken at another center and unavailable for review, was interpreted as suggestive of erythema annulare centrifugum. A second sample, obtained 10 months after disease onset, revealed a light perivascular lymphohistiocytic infiltrate, as seen in erythema annulare centrifugum. However, scant, interspersed foamy histiocytes consistent with Virchow-globi cells were noted. Fite staining confirmed their cytoplasmic content of abundant and focally clumped acid-fast bacilli. A slit skin examination before treatment had a bacteriologic index of 4+. A *Mycobacterium leprae*–specific PCR targeting the *RLEP* gene (*1*) from skin tissue was positive, and 16S rRNA gene (GenBank accession no. NC_002677, region 1341144–1342692 bp) sequencing also confirmed *M. leprae* infection. On the basis of the World Health Organization's disease classification, the patient (having >5 skin lesions and positive skin slits) was given a diagnosis of multibacillary Hansen disease (HD). The Ridley-Jopling classification of borderline lepromatous leprosy was based on the absence of granulomas; numerous bacteria on staining; and symmetric, poorly defined skin lesions.

The patient was started on a 24-month course of ofloxacin, rifampin, and dapsone. Initially, his skin lesions improved, but after 10 months of treatment, he had nondermatomal sensory changes in his hands and erythema with induration of many preexisting lesions, indicating a reversal (type 1) reaction. He was promptly started on prednisone, and a repeat biopsy showed granuloma formation and a bacteriologic index of 1+ acid-fast bacilli, consistent with enhanced cell-mediated immunity. Two months after therapy, he had residual pigmentation without active inflammation and improving hand sensation.

To identify the probable source of infection, we genotyped the *M. leprae* from this patient using a single-nucleotide polymorphism and variable number tandem repeat (VNTR)–based algorithm for identifying zoonotic *M. leprae* strains (*2*,*3*). We amplified and sequenced genomic regions spanning the markers (online Technical Appendix Table). All the single-nucleotide polymorphisms and VNTR loci sequences were identical to the first zoonotic strain (3I-2-v1) of leprosy. The probability of an identical pattern in the random assortment of the 10 VNTRs alone is 1 in 10,000 (*2*), implying infection with a zoonotic strain of *M. leprae*.

HD, caused by *M. leprae*, is rare in Canada. In 2014, four cases were reported in Canada, and in 2015, a total of 178 new cases were reported in the United States (*4*,*5*). The diagnosis is challenging in low-incidence countries and often delayed for years (*6*). Neurologic dysfunction is a clue for the diagnosis but is often absent (*6*). Unrecognized source contact is common among HD patients (*7*), but exposure is doubtful to have occurred with this patient who had not traveled to an area considered endemic. In the southern United States, autochthonous cases of HD have occurred among native-born persons. In 2015, a total of 63 of the 96 cases reported from Texas, Louisiana, Arkansas, Mississippi, Alabama, Georgia, and Florida occurred among persons born in the United States who had never resided outside the

country (*5*). These cases might be secondary to exposure to the 9-banded armadillo (*Dasypus novemcinctus*), which lives in the area (*2*). Most HD patients in Louisiana, Texas, and Florida who had not traveled outside the United States were infected with the *M. leprae* strain 3I-2-v1, which is found in most infected armadillos (*2*). Although direct contact with armadillo blood or flesh poses the highest risk, HD has been reported in persons without direct exposure (*8*). These patients might have had exposure to contaminated soil (*9*). Alternatively, other environmental reservoirs might be responsible, exemplified by the discovery of infected Eurasian red squirrels (*Sciurus vulgaris*) in the British Isles (*10*).

M. leprae genomic analysis strongly suggests that our patient acquired the infection with the armadillo-associated *M. leprae* strain during a trip to Florida. This case highlights the possibility of HD being acquired within North America without obvious exposure to known animal reservoirs. Travelers to the southern United States should be advised to avoid contact with armadillos.

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About the Author

Dr. Bonnar, an infectious diseases graduate, completed a clinical fellowship in antimicrobial stewardship at the University of Toronto in Toronto, Ontario, Canada. His primary research interests include quality improvement projects to optimize antimicrobial use.

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Emmonsia helica Infection in HIV-Infected Man, California, USA

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Emmonsia-like fungi have rarely been reported from North America. We report a fatal case of E. helica infection in a man with advanced HIV infection from California, USA, who had progressive respiratory failure and a brain abscess.

In January 2016, a 40-year-old man sought care at a hospital in Alameda County, California, USA, with a 2-week history of progressive cough, dyspnea, pleuritic chest pain, and headache associated with fevers, chills, and night sweats. He had lost 45 kg during the past month. He had a history of inconsistently treated HIV infection and a 10-pack-per-year smoking history. He had emigrated from Mexico 10 years earlier, lived in the East Bay, and had traveled to the Central Valley of California.

On examination, he was cachectic, afebrile, hypoxic, and tachypneic. He had oral thrush, bilateral lower lobe crackles, and decreased breath sounds. The rest of the examination, including neurologic and skin assessments, was unremarkable.

Chest radiograph and computerized tomographic scan showed diffuse micronodularities with cavitary lesions in both lungs (Figure, panels A, B). Magnetic resonance imaging of the brain revealed a 6-mm ring-enhancing cerebellar lesion (Figure, panel C).

Laboratory results revealed a CD4 count and viral load of 5 cells/μL and 15,000 copies/mL, respectively. Blood leukocyte count was 14.9×10^9 cells/μL (reference 4.5–11 $\times 10^9$ cells/μL), and hemoglobin was 11 g/dL (reference 13.5–17.5 g/dL). Lactate dehydrogenase, alkaline phosphatase, and γ-glutamyl transferase were 284 units/L (reference 125–243 U/L), 148 units/L (reference 38–126 U/L), and 242 units/L (reference 3–95 U/L), respectively. Cerebrospinal fluid analyses, including cell count, protein, and glucose measurements, showed standard results.

Molecular testing of bronchoalveolar lavage (BAL) fluid for respiratory viruses showed negative results. Serologic test results for blood and urine for coccidioidomycosis, cryptococcosis, toxoplasmosis, and tuberculosis were negative, but the *Histoplasma* galactomannan urine antigen test (MiraVista, Indianapolis, IN, USA) result was positive at >25 ng/mL (reference <0.5 ng/mL). Examination of cerebrospinal fluid with Gram, acid-fast, and India ink stains and aerobic, mycobacterial, and fungal cultures were negative for organisms. Cytopathologic examination of BAL fluid and lung biopsy samples showed nonnecrotizing granulomas with hematoxylin and eosin stain and both hyphal and yeast forms with fungal stains. The yeasts exhibited multiple budding with broad bases (Figure, panel D). No organisms were seen with an acid-fast stain. Multiple sputum cultures were negative for mycobacteria.

Empiric treatment was initiated for coccidioidomycosis, *Pneumocystis jirovecii* pneumonia, tuberculosis, and bacterial sepsis with intravenous fluconazole; trimethoprim–sulfamethoxazole with steroids; rifampin, isoniazid, pyrazinamide, and ethambutol; and broad-spectrum antimicrobial drugs. On hospital day 2, the patient had hypoxic respiratory arrest and was intubated. Antifungal drugs were changed to micafungin on day 3 and then to liposomal amphotericin B (5 mg/kg/day) on day 6, and he was extubated later that day. Tuberculosis therapy was discontinued on day 15. Antiretroviral therapy was held because of concern that immune reconstitution might worsen the patient's cerebellar lesion. A spontaneous pneumothorax and respiratory failure developed that required reintubation on day 31. His family chose comfort care, and antifungal therapy was stopped on day 41. He died on day 43; autopsy was declined.

We sent a mold grown from BAL to a mycology reference laboratory for identification. On microscopic examination of the mold phase, conidia were absent in all subcultures. DNA sequences of the D1/D2 region of the large subunit and internal transcribed spacer region of the ribosomal RNA gene were compared with GenBank nBLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch); sequence similarities of 100% and 99%, respectively, were demonstrated for *Emmonsia helica* strains (including UAMH 3398, UAMH 10539, and UAMH 10593; UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Toronto, Ontario, Canada).

We conducted antifungal susceptibility testing of the mold phase. MICs (μg/mL) to fluconazole, itraconazole, posaconazole, voriconazole, and amphotericin B were 8, 0.125, 0.125, ≤0.03, and 0.06, respectively.

Emmonsia-like fungi are an emerging group of pathogens reported globally, which predominantly cause disseminated disease of immunocompromised persons (*1*). One of these, *E. helica*, was originally recorded from North America (*2*). The first reported case occurred in Alberta, Canada, in 1970 in a farmer with a fatal pneumonia and encephalitis syndrome (*3*). A fungal pathogen isolated postmortem from brain and lung tissue was initially identified as *Blastomyces dermatitidis* on the basis of serologic and histopathologic findings, but

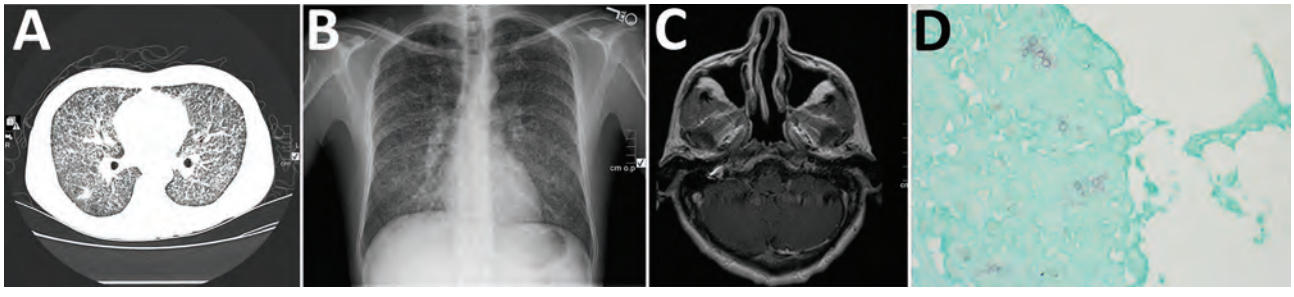

Figure. *Emmonsia helica* infection in an immunocompromised man, California, USA, 2016. A) Chest radiograph with diffuse micronodularities throughout both lung fields. B) Computed tomographic scan with diffuse micronodular pulmonary disease. C) Axial magnetic resonance image with 6-mm ring-enhancing lesion in the right cerebellum adjacent to the fourth ventricle. D) Grocott's methenamine-silver stain showing broad-based budding yeast. Original magnification ×400.

its features in culture were atypical (*3*). In 2015, Sigler determined that this isolate belonged to a new *Emmonsia*-like species, which she described as *E. helica* (*3*). Another fatal case of *Emmonsia* infection was reported from California in a patient after an orthotopic liver transplant (*4*). An isolate from that patient also was confirmed as *E. helica* (I. Schwartz et al., unpub. data).

Although the travel history for the second case-patient was not reported (*4*) and the patient in this report had resided in Mexico, these cases suggest that the area of endemicity of *E. helica* may include California. This finding is further supported by 2 other fatal cases of atypical mycoses reported in HIV-infected men from California (*5*); histopathologic findings of hyphae and multiple budding yeasts were consistent with *E. helica* (I. Schwartz et al., unpub. data). Investigations are under way to characterize the geographic and host range of *E. helica* and to clarify the phylogenetic relationships among members of the family *Ajellomycetaceae* comprising the genera *Emmonsia*, *Blastomyces*, *Histoplasma* and others because recent studies have uncovered far greater complexity than previously supposed (*1*,*6*).

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Costs of Conjunctivitis Outbreak, Réunion Island, France

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During January–April 2015, a major outbreak of conjunctivitis on Réunion Island caused a large public health impact. On the basis of general practitioner consultations, emergency department visits, and eye medication sales during the 13-week epidemic, we estimated a total healthcare cost of €3,341,191 from the outbreak.

During January–April 2015, a major outbreak of acute hemorrhagic conjunctivitis occurred on Réunion Island, causing a heavy impact on the national healthcare system of France (*1*). Réunion Island, a French overseas administrated territory, is located in the Indian Ocean between Madagascar and Mauritius; it has a surface area of 2,512 km^2 and a population of ≈840,000 (1.3% of France's population, including the nation's overseas territories; https://www.insee.fr/fr/statistiques/2119468).

The island is included in the national health insurance (NHI) program of France. Réunion Island's health system is similar to that of France; however, most patients on the island do not pay provider health fees directly. NHI pays the general practitioner (GP), the pharmacist, or hospital. Rarely, the patients pay for the GP consultations and emergency department (ED) visits, but these costs will be refunded to the patients by the NHI. Healthcare costs are higher (≈30%) on the island than in mainland France. In 2015, total healthcare expenditures in Réunion Island were

€2.561 billion; which is 1.6% of France's healthcare spending (≈€163 billion) for that year.

A syndromic surveillance system, the Organisation de la surveillance coordonnée des urgences (Organization of coordinated emergency surveillance [OSCOUR]) network, is based on data collected by all EDs across the country, including in French overseas territories (*2*). Data are collected daily directly from patients' computerized medical files that are completed during medical consultations. For each ED visit, patient age, sex, city of residence, and the diagnosis are recorded. This enables analysis by syndromic groups, age groups, and geographic areas. The diagnosis is categorized according to the International Classification of Diseases, 10th edition (ICD-10; http://www.icd10data.com/). Public health indicators are routinely monitored by using temporal and spatiotemporal analyses, including the number of ED visits for conjunctivitis (ICD-10 code B30 and subcodes, code H10 and subcodes, and code H11 and subcodes).

At the end of January 2015, by using spatiotemporal analysis of data from the OSCOUR network, we detected a cluster of conjunctivitis cases in the western part of the island that occurred during January 26–February 1 (week 5 of 2015). We organized conjunctivitis surveillance within the framework of an existing sentinel project involving 56 volunteer GPs located throughout the island who reported weekly to the Indian Ocean regional institute for public health surveillance agency, known as Cire OI (*3*).

The outbreak on Réunion Island began during week 5 then quickly spread throughout the island and ended in week 17 (end of April) of 2015. Data from ED visits show that all age groups were affected. By using the GP sentinel network and NHI data (*1*), we estimated the total number of GP consultations for conjunctivitis on the island to be 100,094. During this outbreak, we sent regular epidemiologic updates to health professionals to inform them of the ongoing epidemiologic situation and available preventive measures. Health authorities also published a press release for the general public.

On the basis of these data and the major impact for public health, we estimated the cost of this outbreak. We compiled the cost of different indicators: GP consultations, ED visits, and eye medication sales. On Réunion Island, a GP consultation fee of €27.60 and an ED visit fee of €52.60 are reimbursed by NHI. For medicated eye drop sales, we extracted data (number of sales by week and cost) from France's NHI information system, SNIIR-AM (*4*). During the outbreak period, 187,126 medicated eye drop kits were purchased and reimbursed, at a total cost of €566,443. For activity related to conjunctivitis, the cost for GP consultations was €2,762,597 and for ED visits was €12,151 (Table). During weeks 5–17, the healthcare cost was estimated at €3,341,191. The total cost is underestimated, however, because it did not include costs to individuals and businesses, including sick leave, work absenteeism of parents for sick children, and some persons who had conjunctivitis but did not consult a physician.

These data demonstrate that acute outbreaks of illness caused by nonfatal agents can have substantive public health and economic impact. In France, where medical costs are reimbursed by the state, an outbreak of this magnitude, even if virulence is negligible, should be examined thoroughly. Information for the public and health professionals should be strengthened by recurring prevention campaigns with a focus on hygiene, such as washing hands frequently; avoiding rubbing the eyes; covering one's mouth and nose when coughing or sneezing; and avoiding sharing linen, towels, or any objects owned by affected persons.

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We acknowledge the sentinel general practitioners of Réunion, the emergency departments of Réunion, and the supplier of pharmacies CERP Réunion SAS.

Table. Weekly volume and total costs of medicated eye drop sales, consultations with GPs, and ED visits for conjunctivitis outbreak, Réunion Island, France, January–April, 2015*

Epidemiologic week	No. eye drop sales	No. GP consultations for conjunctivitis	No. ED visits for conjunctivitis	Total cost of eye drop sales, €	Total cost of GP consultations, €	Total cost of ED visits, €
5	7,126	2,641	19	21,206	72,887	999
6	6,818	1,937	9	20,198	53,453	473
7	10,379	3,537	17	31,199	97,617	894
8	14,079	7,439	17	42,646	205,326	894
9	25,831	13,845	33	78,083	382,108	1,736
10	27,345	20,895	41	82,198	576,711	2,157
11	31,866	20,648	21	101,453	569,892	1,105
12	15,339	9,141	15	46,990	252,279	789
13	14,726	6,954	17	43,034	191,921	894
14	11,049	4,832	13	32,717	133,371	684
15	8,180	3,369	17	24,166	92,977	894
16	8,109	2,593	9	24,040	71,574	473
17	6,279	2,264	3	18,514	62,479	158
Total by category	187,126	100,094	231	566,443	2,762,597	12,151
Total costs					3,341,191	

*ED, emergency department; GP, general practitioners.

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Dengue Fever in Burkina Faso, 2016

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We report 1,327 probable cases of dengue in Burkina Faso in 2016. Of 35 serum samples tested by a trioplex test, 19 were confirmed dengue virus (DENV)–positive: 11 DENV-2, 6 DENV-3, 2 nontypeable, and 1 DENV-2/DENV-3 coinfection. Molecular testing should be conducted to correctly identify causative agents in this complex infectious disease landscape.

Dengue is an emerging viral disease mainly found in the tropical and subtropical zones, and a major public health concern worldwide (*1*–*3*). Dengue fever is a mosquitoborne viral infection caused by 4 distinct dengue viruses (DENVs): DENV-1–4. In some countries of sub-Saharan Africa, the circulation of all 4 viruses has been reported (*4*). However, availability of rapid tests and molecular diagnosis by reverse transcription PCR (RT-PCR) in resource-limited settings remains a challenge.

During October 29, 2016–November 21, 2016, we screened 1,947 suspected dengue cases using a rapid diagnostic test (SD BIOLINE Dengue Duo, Standard Diagnostics, Seoul, South Korea), which detects DENV nonstructural protein 1 (NS1) and dengue-specific antibodies (IgM and IgG), in response to an outbreak of acute febrile illness in Burkina Faso. All patients with acute febrile illness during this period were suspected to have dengue; notably, some patients had biphasic fever with severe headache, myalgia, arthralgia, and rash. Patients who tested positive for NS1 or DENV antibodies were considered to have a probable DENV infection. All participants provided informed consent as specified by the Declaration of Helsinki, and approval of this study was obtained from the national ethics committee.

Of the 1,947 blood samples tested, 1,327 were positive for NS1, DENV antibodies, or both. Of the 13 country regions investigated, the central region, which includes the city of Ouagadougou, was the most affected, having 1,679 of the 1,947 suspected cases (case fatality ratio 1.2% [20/1,679]) and 1,307 of the 1,327 probable cases. Of the 20 deceased patients, 18 were positive for NS1 and 2 were positive for NS1 and DENV IgM. The outbreak peaked November 11–14. Blood samples from 35 randomly selected patients were sent to the National Reference Laboratory for Influenza (Bobo-Dioulasso, Burkina Faso) for confirmation using the Centers for Disease Control and Prevention trioplex real-time RT-PCR protocol (*5*) followed by singleplex to identify the infecting DENV serotype. Of the 35 patient samples that were selected, 22 were positive for NS1, 3 were positive for both NS1 and IgG, 3 were positive for IgG, 2 were

positive for both NS1 and IgM, 1 was positive for both IgM and IgG, and 4 were negative. Nineteen (54.3%) cases were positive for DENV, and no cases were positive for Zika or chikungunya viruses (Table). Eleven patients were infected with DENV-2, 6 were infected with DENV-3, and 1 patient was co-infected with DENV-2 and DENV-3. We submitted our samples to the World Health Organization Collaborating Centre for Arbovirus Reference and Research, Institut Pasteur de Dakar (Dakar, Senegal), which confirmed our results.

In Burkina Faso, dengue represents an added burden to an infectious disease landscape dominated by malaria; therefore, implementation of molecular diagnostic testing is urgently needed to identify the correct etiologic agent associated with the disease. The trioplex real-time RT-PCR detected 19 cases of DENV. A total of 3 serum samples positive for NS1 were negative by this assay. These negative results can be explained in part by declining viremia levels that became undetectable around the time of molecular testing, although testing with a larger representative sample size could have provided more information.

We found DENV-2 to be the dominant serotype in this outbreak, followed by DENV-3. No cases of DENV-1 or DENV-4 were found, although testing a larger number of specimens might have revealed the co-circulation of these DENV serotypes. Human cases of DENV-2 in Burkina Faso is supported by previous reports of DENV-2 circulating in mosquitoes (*6*). The presence of DENV-3 in Burkina Faso is not surprising, considering this serotype has been previously reported in the region; in 2009, DENV-3 was the main etiologic virus of the outbreak in Cape Verde, which affected >17,000 persons, and was reported in 6 persons in Senegal who traveled to Italy and died (*7*). DENV-3 was also detected in the DENV outbreak in Côte d'Ivoire in 2008 (*8*).

We speculate that increased international travel between neighboring countries and mosquito circulation has led to DENV-2 and DENV-3 successfully crossing the border into Burkina Faso. This pilot study shows DENV-2 and DENV-3 are both circulating in Burkina Faso and causing human disease. Molecular diagnostics, vector control strategies, and risk communication should be implemented in Burkina Faso in preparation for future outbreaks.

Table. Characteristics and rRT-PCR results of patients with dengue fever, Burkina Faso, 2016*

Variable	No. (%)	95% CI
Age, y		
2–9	2/35 (5.71)	NA
10–19	3/35 (8.57)	NA
20–29	8/35 (22.85)	NA
30–39	9/35 (25.71)	NA
40–49	9/35 (25.71)	NA
≥50	2/35 (5.71)	NA
Unknown	2/35 (5.71)	NA
Sex		
M	24/35 (68.57)	NA
F	8/35 (22.85)	NA
Unknown	3/35 (8.57)	NA
Molecular diagnostic		
rRT-PCR trioplex	19/35 (54.3)	34.78–70.78
rRT-PCR DENV-1–4	17/19 (89.4)	75.68–103.26
DENV-1	0 (0)	NA
DENV-2	11/19 (57.8)	35.69–80.09
DENV-3	6/19 (31.5)	11.57–51.57
DENV-4	0 (0)	NA
DENV-2 + DENV-3	1/19 (5.26)	4.74–15.26
Unknown serotype	2/19 (10.5)	7.2–23.52

*Of the 35 patient samples used, 31 were positive and 4 were negative by SD BIOLINE Dengue Duo (Standard Diagnostics, Seoul, South Korea). DENV, dengue virus; NA, not applicable; rRT-PCR, real-time reverse transcription PCR.

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Increasing Number of Scarlet Fever Cases, South Korea, 2011–2016

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The increasing number of reported scarlet fever cases during 2011–2016 in the National Notifiable Infectious Disease database in South Korea occurred because of increased overall reporting and expanded reporting criteria rather than because of increasing scarlet fever incidence. Further increases are anticipated because of other expansions in reporting requirements.

Studies suggest that scarlet fever incidence has been increasing in South Korea (Republic of Korea) and other countries of East Asia since 2011 (*1*–*3*). The report describing increased numbers of scarlet fever cases in South Korea was based on National Notifiable Infectious Disease (NNID) surveillance data, which comprises cases reported through an electronic system to the Korea Centers for Disease Control and Prevention (*4*). In South Korea, scarlet fever is categorized as a group 3 NNID, which requires continuous surveillance and the establishment of control measures against possible outbreaks because of the risk for intermittent epidemics. Medical professionals who work for medical institutions are required to report cases to the local public health office. Reported data are reviewed by the local health center staff and submitted to the health authority of the province and Korea Centers for Disease Control and Prevention through an electronic reporting system (*4*). However, despite the law, the reporting rate of infectious disease by medical institutions has been low. Assessing the sensitivity of this reporting system for detecting scarlet fever cases was the goal of this report.

South Korea has a single-payer public health insurance system with universal coverage; the National Health Insurance Service is the insurer, and the Health Insurance Review and Assessment Service (HIRA) reviews payments. Using HIRA data, institutions can obtain information on the diagnoses of diseases and treatments for the entire population (*5*).

According to HIRA data, 14,550 patients in 2011 and 15,533 patients in 2013 had scarlet fever diagnoses (International Statistical Classification of Disease and Related Health Problems, Tenth Revision code A38) (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/1/17-1027-Techapp1.pdf). The number of scarlet fever cases decreased in the following 2 years and then increased again in 2016 to 13,261 cases (*6*). However, according to the NNID surveillance data, 406 cases of scarlet fever were reported in 2011, with the number of cases increasing with time, ending with 11,911 cases in 2016 (Figure), and most patients being young (<10 years of age; online Technical Appendix Table 2) (*7*). The inconsistencies between these 2 databases indicates that the number of in-hospital diagnoses of scarlet fever has been roughly constant, but the reporting rate of diagnosed scarlet fever increased markedly from 2.8% in 2011 to 89.8% in 2016.

Patients with NNIDs fall into the following 3 categories: confirmed case-patient, a person with compatible clinical symptoms who was positive for a group A *Streptococcus* by laboratory tests; suspected case-patient, a person with compatible clinical symptoms who was not tested or not positive for the pathogen by laboratory tests; and pathogen carrier. In South Korea, the reporting criteria for scarlet fever were limited to confirmed case-patients until September 2012, after which the criteria expanded to include suspected case-patients. This change contributed to the sharp increase of reported scarlet fever cases in the South Korea NNID database in 2013. This sharp increase was also facilitated by another factor: medical institutions and government agencies were aware of the poor NNID reporting rate and made efforts to improve them around this time. In a previous study, the incidence of scarlet fever in South Korea was reported to have increased rapidly on the basis of NNID data (*3*). However, considering the background of the reporting system, we believe that HIRA data, rather than the NNID database, better reflect the rate of infection in South Korea.

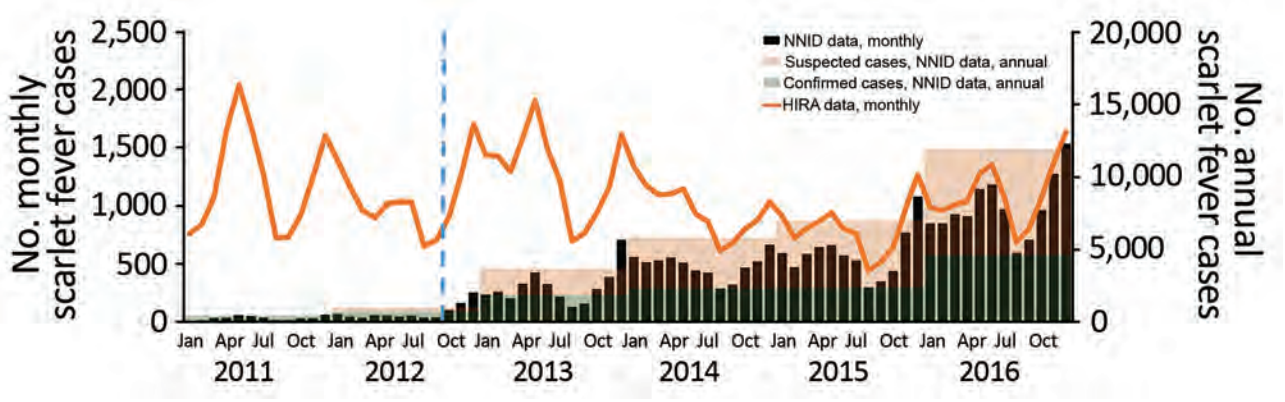

Figure. Incidence of scarlet fever determined using NNID and HIRA data, South Korea, 2011–2016. The blue dashed line indicates the starting point for NNID reporting using the expanded criteria. HIRA, Health Insurance Review and Assessment Service; NNID, National Notifiable Infectious Disease.

We report a discrepancy between the number of patients with scarlet fever diagnoses in hospitals and the numbers reported in the NNID system. We speculate that the rapid increase in the number of reported cases of scarlet fever in South Korea was not caused by an increase in the number of cases but was due to external factors, such as a drastically increased reporting rate and the inclusion of suspected cases due to the expanded reporting criteria. However, we do not completely exclude the possibility that the increased number of confirmed cases reflects an increased number of patients with scarlet fever because the number of confirmed cases, not just the overall cases, in the NNID database has been steadily increasing. Rapidly expanding private health insurance coverage, which provides additional funding, might be increasing access to healthcare for some patients and, thus, the number of persons seeking treatment for scarlet fever. In 2016, the Infectious Diseases Control and Prevention Act was amended to expand the reporting institutions for NNID from medical institutions to inspection agencies, concurrent with the government's efforts to increase the reporting rate of NNID. Therefore, the reporting rate of NNID in South Korea is expected to be much higher in the future.

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Antimicrobial Drug Resistance in Blood Culture Isolates at a Tertiary Hospital, Uganda

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We summarize antimicrobial drug resistance (AMR) patterns from blood cultures at a tertiary hospital in Uganda. High rates of resistance to first-line antibiotic drugs were observed among *Staphylococcus aureus* and gram-negative organisms. Microbiology services with susceptibility testing should be strengthened to support standardized reporting of AMR data in sub-Saharan Africa.

Antimicrobial drug resistance (AMR) is a global problem (*1*,*2*). However, because of a lack of routine surveillance systems in sub-Saharan Africa, limited data are available describing local AMR patterns (*2*,*3*). In 2015, the World Health Organization adopted an AMR global action plan that called for strengthening knowledge of AMR through surveillance and research (*1*). Understanding local AMR patterns is essential to guide clinical management of infections and to inform health policy. Here we highlight common pathogens and antimicrobial susceptibility patterns from blood culture isolates and their susceptibility patterns at Mulago National Referral Hospital, a tertiary hospital in Kampala, Uganda.

Blood cultures were collected during June 2013–October 2014 as part of patient care and research studies and processed at 2 microbiology laboratories, Makerere University College of Health Sciences (n = 345) and Mulago Hospital (n = 117). Bacterial identification and susceptibility testing were performed according to 2011 Clinical Laboratory Standards Institute M100 S21 guidelines (https://clsi.org/). Susceptibilities were summarized into a hospital antibiogram using Stata version 12 (https://www.stata.com/stata12/) and Microsoft Excel (Microsoft Corp., Redmond, WA, USA). For organisms with <10 positive isolates, raw numbers were compiled rather than percentages.

In total, 3,197 blood specimens were collected and processed, of which 462 (14%) grew an organism. Gram-positive cocci constituted 60% (279/462) of all isolates. Nearly half (127/279) of these were *Staphylococcus aureus*, of which 32% (41/127) were methicillin resistant, 43% (54/127) were methicillin sensitive, and 25% (32/127) did not have susceptibility data. In our study, 14% of the MRSA isolates (n = 41) were fully susceptible to ciprofloxacin, 55% to clindamycin, 25% to gentamicin, and 4% to trimethoprim/sulfamethoxazole. The remaining susceptibility results are described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/1/17-1112-Techapp1.xlsx).

Enterobacteriaceae were the most commonly isolated gram-negative organisms, constituting 67% (122/184) of all gram-negative bacilli, including 26% (47/184) *Escherichia coli*, 20% (36/184) *Klebsiella pneumoniae*, 9% (17/183) *Enterobacter* spp., and 3% (5/183) *Citrobacter* spp. We isolated 17 *Salmonella* isolates, 5 *S. enterica* serovar Typhi, and 12 nontyphiodal *Salmonella*. Sensitivity rates of *E. coli* to antimicrobial drugs were as follows: ceftriaxone 33%, ciprofloxacin 39%, chloramphenicol 56%, piperacillin/tazobactam 80%, and imipenem 81%. Sensitivity rates were similar, but lower overall, for *Klebsiella pneumoniae*: ceftriaxone 15%, ciprofloxacin 23%, chloramphenicol 17%, piperacillin-tazobactam 64%, and imipenem 80%. *Pseudomonas aeruginosa* was rarely isolated (n = 3).

We found that a substantial proportion of pathogens isolated from blood demonstrated AMR, principally among methicillin-resistant *S. aureus* and gram-negative organisms. These pathogens were commonly resistant to first-line antibiotic drugs (e.g., fluoroquinolones, penicillins, ceftriaxone) at rates that were much higher than those reported in high-income countries (*4*).

In Uganda, most of the literature on AMR has focused on single pathogens or subgroups of infections and not on broader, routine surveillance (*5*,*6*). Similar surveillance efforts have been undertaken in other countries in Africa, such as Kenya (*4*). In general, the most common etiologies of bacteremia there, as well as in the United States and several countries in Europe, were similar

[1]These authors contributed equally to this article.

to those at Mulago Hospital (*4*). The exception was *Salmonella*, which was isolated more frequently in Uganda and Kenya (*4*,*7*).

Although we isolated *Salmonella* spp. in only 17 specimens, we found multidrug resistance (MDR; resistance or nonsusceptibility to ≥3 different antimicrobial classes) among 3 of 5 *Salmonella* Typhi and 6 of 12 nontyphoidal *Salmonella* isolates. The emergence of MDR *Salmonella* Typhi is well documented in Southeast Asia; however, resistance has been less studied in Africa (*8*). The finding of MDR *Salmonella* Typhi in Uganda aligns with findings from the 2016 Typhoid Fever Surveillance in Africa Program, which found MDR *Salmonella* Typhi in Kenya and Tanzania (*7*).

Limitations of this study include the small sample size of individual species and an urban, tertiary hospital setting with potential selection bias for higher AMR rates. The 3,197 blood cultures collected reflect an average of ≈190 blood cultures/month in an ≈1,800-bed hospital, representing a small fraction of hospitalized patients with infectious diseases. The limited sampling is likely due to patient-incurred costs of blood cultures and limited availability of microbiology supplies. Attempts at laboratory-based AMR surveillance have been attempted in other countries in Africa, including Ghana, Rwanda, and Kenya, with similar challenges (*4*,*9*,*10*). The cost and resource availability in microbiology laboratories are major barriers to large-scale culture testing (*3*), and increased funding for microbiology laboratories is necessary to ensure AMR surveillance. Rapid, non–culture-based technology may be an AMR surveillance option.

In summary, we identified noticeable AMR to commonly available antimicrobial drugs among organisms isolated from bloodstream infections at Mulago Hospital in Uganda. Surveillance using blood culture data can facilitate selecting appropriate therapy and provide hospital antibiograms that can be used for empiric therapy of the very ill. These advantages outweigh the perceived disadvantages of high cost, low yields of recovery, and turnaround time that are frequently considered barriers to performing blood cultures in resource-limited settings. National programs should consider these benefits and prioritize funding for AMR surveillance, and from those data, develop clinical guidelines and national policies to slow the spread of AMR in sub-Saharan Africa.

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Yellow Fever Virus RNA in Urine and Semen of Convalescent Patient, Brazil

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Yellow fever virus RNA is usually detected in blood of infected humans. We detected virus RNA in urine and semen samples from a convalescent patient. A complete virus genome was sequenced for an isolate from a urine sample. This virus had a South American I genotype and unique synapomorphic changes.

Yellow fever virus (YFV) is a member of the genus *Flavivirus* and causes yellow fever in humans, characterized by fever, prostration, and hepatic, renal, and myocardial complications that lead to death in 20%–50% of cases (*1*). Clinical confirmation of YFV infections is based on detection of virus RNA in blood by reverse transcription PCR or antigen-based ELISAs. Detection of virus in urine samples has been used for confirming infections with flaviviruses, including West Nile virus (*2*), Zika virus (*3*), dengue virus (*4*), and YFV (*5*).

Despite availability of an effective vaccine, >200,000 cases of yellow fever and >30,000 deaths occur per year (*6*). A large epidemic of yellow fever with high death rates recently occurred in Brazil. In December 2016, the first cases of yellow fever during this epidemic were reported in Minas Gerais; cases were later identified in Espírito Santo, Goiás, Mato Grosso, Pará, Rio de Janeiro, São Paulo, Tocantins, and the Federal District. There were 792 confirmed cases and 274 deaths (case-fatality rate 35%) as of July 10, 2017 (*7*). We report a case of yellow fever in a 65-year-old man who was a native of São Paulo and had not been vaccinated against yellow fever. The study protocol was approved by the Ethics Committee on Research with Human Beings at the University of São Paulo. The patient provided informed consent for use of the samples during the study.

The patient had traveled to Januária, Minas Gerais, Brazil, on December 28, 2016, and to a rural area north of São Paulo on January 3, 2017. On January 6, he had fever, chills, body pain, and nausea. During days 1–3 after symptom onset, more severe symptoms developed: persistent fever (temperature 39.5°C–40°C), headache, body pain, prostration, vomiting, dizziness, anorexia, dark stools, dark yellow urine, and bitterness in the mouth.

The patient was admitted to a public hospital in Januária on January 9. An ELISA for nonstructural protein 1 (NS1) of dengue virus showed a negative result. The patient also had severe thrombocytopenia (platelet count 77,000/mm^3 [reference range 140,000–450,000/mm^3]).

On January 13, the patient returned to São Paulo and was admitted to a public hospital. Another ELISA for dengue virus NS1 was performed and showed a negative result. His platelet count decreased to 57,000/mm^3. On January 16, the patient was admitted to a reference hospital for infectious diseases in São Paulo. He showed a moderate clinical presentation: anicteric form and mild spontaneous hemorrhage (ecchymosis in the right eye). High fever, gastrointestinal symptoms (vomiting and diarrhea), weakness, adynamia, and generalized myalgia were also observed. The patient had a weight loss of 4 kg over 8 days. Serum and urine samples were obtained (Figure, panel A; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-1310-Techapp1.pdf).

We extracted virus RNA by using the NucliSENS EasyMag Kit (bioMérieux, Marcy l'Étoile, France). We tested samples for YFV by using a real-time quantitiative reverse transcription PCR (qRT-PCR) and primers specific for YFV (*8*) and a conventional PCR and pan flavivirus primers (*9*). Serum samples showed negative results for both PCRs. However, a urine sample obtained 10 days after initial symptoms was positive for YFV RNA (cycle threshold [C_t] 17.42, 9.3×10^6 RNA copies/mL) by qRT-PCR. We also performed a qualitative IgM-capture ELISA with a specific virus antigen and obtained positive results (optical density 1.19) (*10*).

On January 27, we obtained serum, urine, and semen samples and tested them by using qRT-PCR. Urine (C_t 28.57, 3.3×10^3 RNA copies/mL) and semen (C_t 31.00, 5×10^2 RNA copies/mL) samples were positive for YFV RNA. To evaluate infectivity, we tested a urine sample obtained on this date (online Technical Appendix). We isolated YFV in cell culture, which confirmed virus integrity. We also confirmed infectivity after a second virus passage (C_t 24.35, 6.7×10^4 RNA copies/mL).

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For the urine sample that was positive for YFV RNA by qRT-PCR, we directly characterized viral diversity by using next-generation sequencing (online Technical Appendix). In South America, phylogenetic studies have inferred 2 circulating YFV genotypes. The isolate from our patient (BRMG-2017) clustered with South America I isolates, including 2 viruses isolated in 2017 in Espírito Santo, a state bordering Minas Gerais, and other viruses isolated previously in Brazil (Figure, panel B).

We did not observe any insertions or deletions in BRMG-2017 nucleotide sequences when compared with sequences of other South America I strains. However, several synapomorphic changes were detected (V108I [capsid], E1572D [NS3], R1605K [NS3], K2608R [NS5], V2645I [NS5], G2680S [NS5], N2804S [NS5], V3150A [NS5], and N3216S [NS5]). Most of these changes were located in the NS5 (RNA-dependent RNA polymerase) gene, which plays a major role in virus replication. Changes in the NS5 gene have been associated with differences in viral replication, immune response, and protein–protein interactions during virus replication.

Our results suggest that semen can be a useful clinical material for diagnosis of yellow fever and indicate the need for testing urine and semen samples from patients

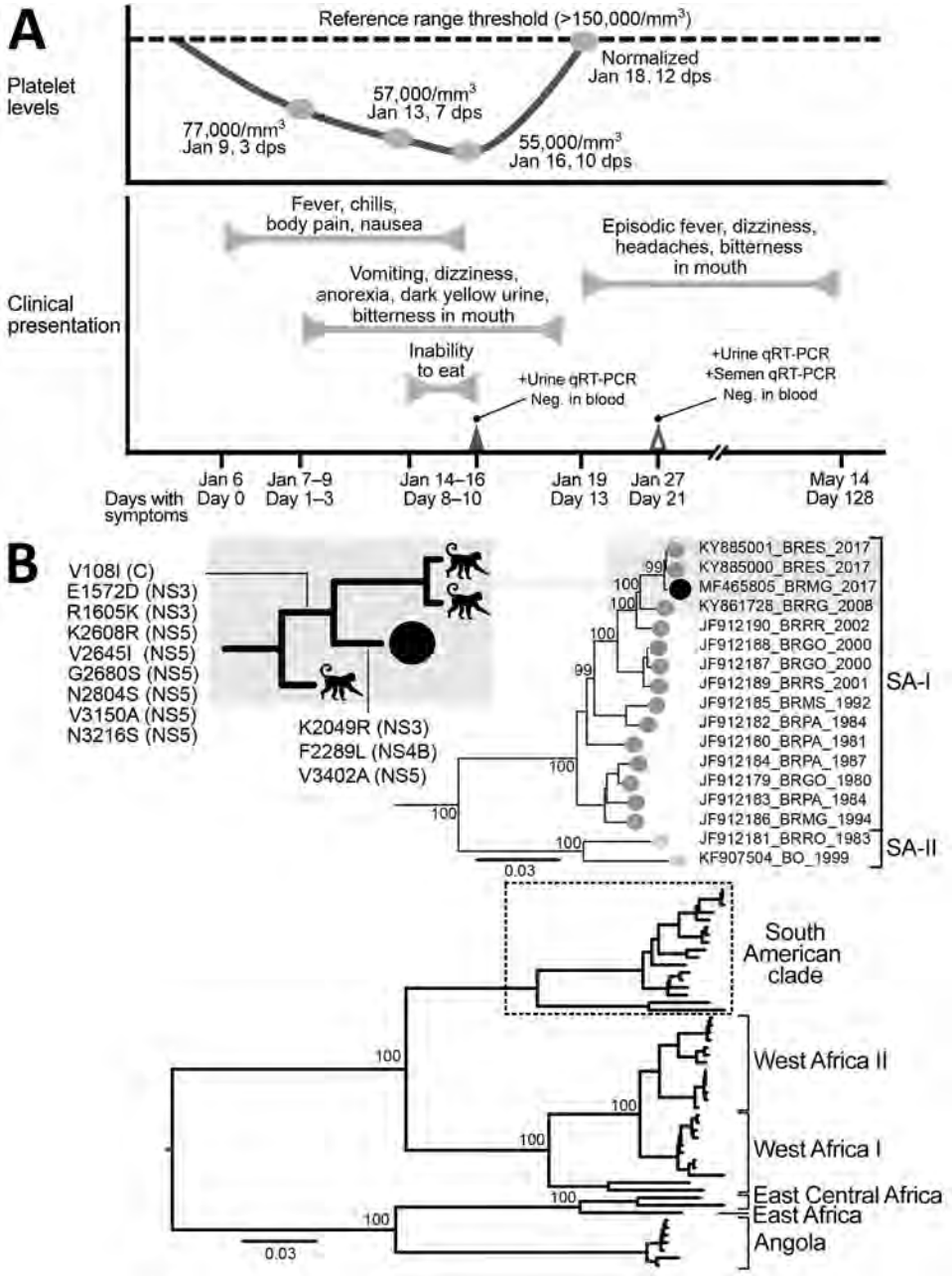

Figure. Clinical progression and detection of YFV RNA in urine and semen of convalescent patient, Brazil. A) Platelet levels, clinical parameters and symptoms, and test results over a 128-day period after initial symptoms were observed. B) Maximum-likelihood tree (midpoint-rooted) inferred by using complete genomes of YFV to distinguish major virus genotypes; dashed box indicates South American clade strains, enlarged at top. Numbers near nodes indicate percent bootstrap values after 10,000 replicates for major branches. Black circles indicate virus isolated in this study. Shaded boxes indicate monkey-derived virus sister taxa sampled during the same outbreak; inset at top left shows most parsimonious reconstructions of synapomorphic changes detected NS3, NS4B, and NS5 genes. GenBank accession number, geographic location code, and year of isolation are shown for virus isolates. Scale bars indicate nucleotide substitutions per site. C, capsid; dps, days postsymptom onset; Neg., negative; NS, nonstructural; qRT-PCR, quantitative reverse transcription PCR; SA, South America clade; YFV, yellow fever virus; +, positive.

with advanced disease. Such testing could improve diagnostics, reduce false-negative results, and strengthen the reliability of epidemiologic data during ongoing and future outbreaks.

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Molecular Characterization of Autochthonous Chikungunya Cluster in Latium Region, Italy

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We report partial molecular characterization of isolates from an autochthonous chikungunya virus cluster in the Latium Region of Italy. E1 sequences from 3 patients differ substantially from sequences from the 2007 outbreak in Italy and lack the A226V substitution associated with increased viral fitness in the *Aedes albopictus* mosquito vector.

Local transmission of chikungunya virus (CHIKV) has been confirmed in the Lazio region of Italy, with 2 related autochthonous clusters in the cities of Anzio and Rome (*1*). This event is the second known autochthonous outbreak of CHIKV in Italy; the previous one occurred in 2007 in the Emilia Romagna region and included >205 cases of CHIKV infection during July 4–Sept 27, 2007 (*2*). During the time between the 2 epidemics in Italy, autochthonous transmissions were described in France in 2010, 2014, and 2017; these events have focused attention on this infection because of the *Aedes albopictus* mosquito vector establishing itself in parts of the Mediterranean basin and beyond. *Ae. albopictus* mosquitoes are assumed to be the vector in the ongoing outbreak in Italy because *Ae. aegypti* mosquitoes are not circulating in this country. The Laboratory of Virology of Lazzaro Spallanzani National Institute for Infectious Disease (INMI) in Rome, as the regional reference laboratory for arboviral infections, is in charge of CHIKV diagnosis and surveillance for the Latium region.

CHIKV diagnosis is based on the detection of the viral genome by real-time reverse transcription PCR (RT-PCR) and of virus-specific antibodies by serologic tests. We conducted sequence analysis of endpoint PCR amplicons from selected case-patients to confirm the identity of the virus detected by real-time RT-PCR and performed virus isolation whenever possible. The first case-patient detected at INMI, a resident of Anzio (60 km from Rome) with no recent travel history abroad, was admitted to the Tropical Infectious Disease Unit on August 30, 2017, with suspected measles. Hospital staff suspected an arboviral disease on September 3, and on September 5, a serologic test was positive for chikungunya virus (E. Nicastri, unpub. data). The official notice of autochthonous cases in this region was disclosed on September 8, 2017. As of September 26, a total of 183 CHIKV infections have been reported (109 confirmed and 74 probable) (*3*), consistent with an extensive autochthonous outbreak. An intensive diagnostic campaign and backtracing of cases is under way to establish the extent of the outbreak.

Phylogenetic analyses of CHIKV strains obtained during outbreaks in different geographic regions (Réunion, Seychelles, Mauritius, Madagascar, and Mayotte) have identified the independent acquisition of a common mutation, A226V, in the E1 glycoprotein (*4*). This mutation, together with mutations M269V and D284E in E1 glycoproteins, has been described as a molecular signature of the Indian Ocean CHIKV outbreak (*5*,*6*). The A226V mutation in particular appeared in >90% of isolates after December 2005 and has been demonstrated to increase viral fitness in *Ae. albopictus* mosquitoes, expanding the potential for CHIKV to diffuse (*5*,*6*). In a previous study, we described the presence of this mutation in CHIKV in specimens from patients referred to our diagnostic facility, 5 imported to Italy and 2 linked to the 2007 outbreak in Italy (*7*). In this study, we extend the genetic analysis to samples from the ongoing outbreak.

We obtained a partial sequence of the E1 coding region directly from clinical samples of 3 patients being managed at INMI. These include the patient from Anzio and 2 additional cases belonging to a family cluster of 3 cases. On September 1, a 3-year-old child, born in Rome with neither travel history nor connection with Anzio, was brought to INMI with complaints of high-grade fever, arthralgia, rash, fatigue, headache, and retro-orbital pain. His parents reported a similar febrile syndrome 24 and 48 hours later. Samples collected on September 6 for all 3 patients tested positive for CHIKV by serology and real-time RT-PCR. We obtained E1 sequences from both parents and submitted sequences for all 3 patients to GenBank (accession nos. MF988056–8).

Further, we conducted a phylogenetic analysis that included 42 available CHIKV sequences from different parts of the world, including sequences previously analyzed in our laboratory (Figure). The analysis, based on a partial E1 sequence, showed that the virus involved in the online Latium region outbreak belongs to the broad group comprising isolates from the East/Central/South African (ECSA) clade and clusters with the Indian Ocean lineage (Figure). This finding is similar to what was observed for the 2007 Italy outbreak, but the current sequences are placed in a separate branch of the phylogenetic tree (bootstrap value 0.83). This branch also includes recent (2016) isolates from Pakistan and India, suggesting a more recent origin of the new epidemic strain, compared with the previous one affecting Italy. It is noteworthy that, unlike the isolates obtained from the 2007 outbreak in the Emilia Romagna region, the E1 sequences from the ongoing outbreak lack the A226V mutation, as do all the recent isolates placed on the same branch of the phylogenetic tree. Further study will establish the relevance of this and other genetic signatures to the fitness of the virus for the local mosquito vectors and will determine the extent of transmission cycles in humans.

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Inonotosis in Patient with Hematologic Malignancy

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We report a lung-invasive fungal disease with possible cutaneous needle tract seeding in a patient with a febrile neutropenia caused by the Basidiomycetes mold *Inonotus* spp. Although rare, *Inonotus* spp. should be added to the list of microorganisms causing invasive fungal disease in neutropenic patients with hematologic malignancies.

A 33-year-old man in Madrid, Spain, with chronic myeloid leukemia in lymphoid blastic phase underwent allogeneic stem cell transplantation (SCT) from a matched unrelated donor in 2011. Four years later, he had an extramedullary pulmonary relapse, after which he began intensive reinduction chemotherapy. After the second cycle, prolonged severe aplasia developed in the patient. Invasive fungal disease (IFD) was suspected because of the presence of persistent fever despite broad-spectrum antimicrobial drugs and the appearance of a new pulmonary nodule (Figure, panel A; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/1/17-1265-Techapp1.pdf) while the patient was receiving prophylactic micafungin (50 mg/d). Serologic fungal biomarkers were negative. A percutaneous pulmonary biopsy sample was taken, and empirical liposomal amphotericin B (3 mg/kg/d) was started (December 2015). Histology showed unspecific inflammatory tissue, and microbiology cultures were negative.

Salvage human leukocyte antigen–haploidentical SCT was performed in January 2016. Fever persisted during conditioning therapy, and a solitary cutaneous millimetric erythematous lesion appeared at the biopsy puncture site (Figure, panel B). Histopathology of the skin lesion showed dermal infiltration by periodic acid Schiff–positive elements compatible with fungal hyaline hyphae with parallel walls, regular septa, and branched hyphae with occasional bulb-like expansions; angioinvasion; and necrosis. Fungal culture of the specimen was negative, but panfungal PCR and further sequencing (*1*) revealed the presence of *Inonotus* spp. Voriconazole was added, and the lesion resolved in days. Neutrophil engraftment was achieved on day 12 post-SCT, with complete donor chimerism.

On day 34, the pulmonary lesion progressed, but we could not prove IFD as the cause of concomitant pleural effusion. Despite intensified antifungal therapy, surgical debridement was required to resolve the empyema. The patient was discharged on oral posaconazole (300 mg/d) that was eventually replaced by micafungin.

A computed tomography scan performed 6 months after the SCT showed persistence of a single mass on the left

[1]These authors contributed equally to this article.

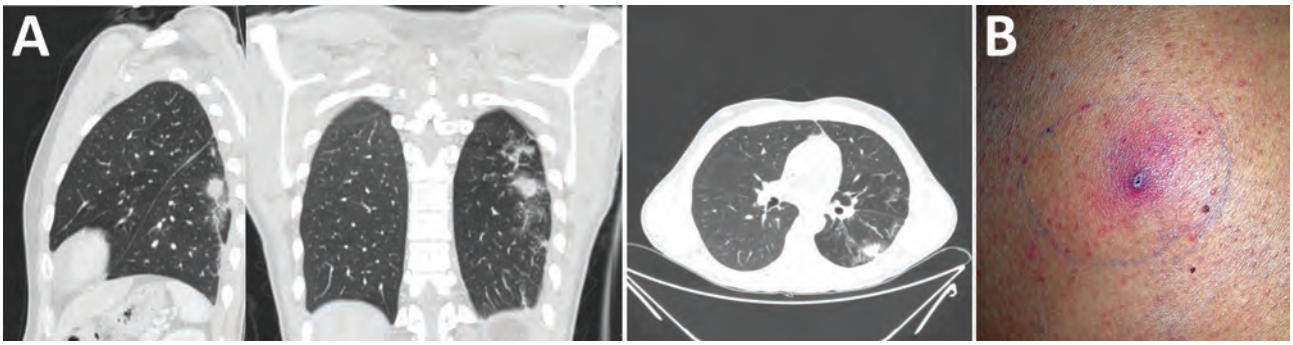

Figure. Pulmonary nodule and skin lesion in patient with invasive fungal disease caused by *Inonotus* spp., Madrid, Spain. A) Computed tomography of the lungs shows pulmonary nodule with halo sign in the left superior lobe, with peripheral distribution. B) Skin lesion at biopsy site 4 weeks after biopsy.

lung inferior lobe together with a new hepatic nodule. We performed pulmonary segmentectomy, and lung histology showed mycetoma with fungal elements similar to those observed in the previous skin biopsy. The fungal culture yielded a fluffy, white, slow-growing mold. The lack of sporulation did not permit morphologic identification, although panfungal PCR and further sequencing again revealed the presence of *Inonotus* spp. in the lung tissue sample.

Filamentous Basidiomycetes molds are ubiquitous and able to colonize in patients with chronic pulmonary disease. They cause syndromes comparable to allergic bronchopulmonary or rhinosinusal aspergillosis. However, IFD caused by Basidiomycetes molds are extremely rare (*2*). It has been hypothesized that IFD could occur in patients with mycetoma if they become immunocompromised. Clinical presentation resembles that of other IFDs, with predominantly pulmonary involvement (online Technical Appendix Table 1). Most filamentous Basidiomycetes are susceptible to antifungal drugs except for fluconazole and echinocandins. However, because many isolates do not sporulate, morphologic identification in the clinical microbiology laboratory is difficult without molecular techniques, and antifungal susceptibility testing is impossible to perform (*3*).

Invasive disease caused by *Inonotus* spp. (*Phellinus tropicalis* and *P. undulatus*) in humans has been described in 1 patient with diabetic nephropathy (*3*) and 6 patients with chronic granulomatous disease (*4–10*). Of note, 4 were breakthrough infections in patients receiving prophylactic itraconazole or posaconazole. Local infections had a favorable outcome; however, 1 patient with more extensive involvement had multiple relapses.

The infection in the patient we report mimicked other invasive mold infections in neutropenic patients with hematologic malignancies. However, we observed fungal invasion in the skin after the percutaneous puncture for the pulmonary biopsy, which suggests fungal seeding from the lung source during sample collection.

The presence of fungal elements invading the tissues supported the diagnosis of IFD; nevertheless, we did not initially consider *Inonotus* spp. to be the causative agent of the IFD in this patient because it rarely causes disease in humans. The clinical significance of the isolation of saprophytic molds in nonsterile clinical samples is difficult to ascertain. However, detection of *Inonotus* spp. in the lung tissue sample taken months after the skin lesion biopsy led us to reassess its potential role as an etiologic agent. In addition, the patient could have acquired the lung infection after inhalation of spores, and selective pressure of previous antimicrobial drugs could have triggered the breakthrough invasive *Inonotus* spp. infection. Antifungal therapy was selected without specific recommendations and without antifungal susceptibility testing (because of the poor sporulation of the isolate). Immunosuppression was more profound and prolonged than in other cases of IFD caused by *Inonotus* spp. Both surgery and antifungal therapy were required, and immunologic recovery, along with a subacute course, were probably essential for the favorable outcome of this patient.

In conclusion, *Inonotus* spp. should be added to the list of potential causal agents of IFD in neutropenic hematological patients. Systematic use of panfungal PCR targeting the internal transcribed tracer regions coupled with sequencing in patients at a high risk for IFD may be helpful for diagnosing rare invasive fungal infections.

Acknowledgments

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The subject of the case report signed an informed consent document for publication.

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Corrections

Vol. 21, No. 8

An incorrect word in a sentence in *Escherichia coli* O157 Outbreaks in the United States, 2003–2012 (K.E. Heiman et al.) inadvertently changed the meaning. The sentence should have read, "The median annual number of outbreaks reported during 2008–2012 was higher than during 2003–2007 (45 vs. 33, p = 0.12) (Figure 1)." The article has been corrected online (https://wwwnc.cdc.gov/eid/article/21/8/14-1364_article).

Vol. 23, Supplement

The names of authors Melanie E. King and Peter Wasswa were incorrectly listed and several items in the text were unclear in Surveillance Training for Ebola Preparedness in Côte d'Ivoire, Guinea-Bissau, Senegal, and Mali (V.M. Cáceres et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/13/17-0299_article).

Investigation of Pneumonic Plague, Madagascar

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To the Editor: In an investigation of a pneumonic plague outbreak in Madagascar, Ramasindrazana et al. reported isolation of *Yersinia pestis* from 2 patients and seroconversion in 2 additional patients; these data indicated 4 (28.7%) of 14 diagnosed cases among described cases (*1*). The risk for overestimation of pneumonic plague contagion was illustrated by an outbreak in the Democratic Republic of the Congo that included cases of leptospirosis (*2*). In fact, thorough investigations in Uganda indicated that 2 index patients transmitted *Y. pestis* to only 1 caregiver each and none to 23 additional untreated close contacts (*3*). Another investigation in China showed that 3 index patients exposed 214 contacts during 3–13 days; all contacts were quarantined, and no secondary cases were reported (*4*). Transmission of *Y. pestis* by respiratory droplets requires face-to-face exposure with a coughing patient, as can occur during funerals by close contact with coughing persons who may have been exposed to the pathogen while visiting or attending the patient before he or she died. Therefore, the threat for plague epidemics fueled by pneumonic plague can be reduced by measures such as isolating patients and wearing a mask when exposure is likely (*5*).

We propose the hypothesis that only the transmission of *Y. pestis* by ectoparasites, such as lice and fleas, by close contact with infected humans can sustain outbreaks and epidemics. In plague-endemic regions, to support the appropriate management of patients and provide a rapid and accurate microbiological diagnosis, we recommend point of care laboratories, some of which are now operating in a few remote regions of Africa. In addition to direct diagnosis of disease in humans, direct detection of *Y. pestis* at the point-of-care in potential sources and vectors would facilitate understanding of how plague epidemics sustain.

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Increasing Virulence in Leprosy Indicated by Global *Mycobacterium* spp.

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To the Editor: The November 2017 issue of *Emerging Infectious Diseases* had 3 articles about leprosy, including these topics: a United States–born patient who tested positive for *Mycobacterium lepromatosis* (*1*); a lethal case of *Mycobacterium leprae* manifested as Lucio's phenomenon in Peru (*2*); and pointing out that leprosy is an emerging disease in the eastern United States, including autochthonous cases without exposure to armadillos (*3*), which were previously shown to be a zoonotic source of transmission in the United States (*4*). Not only is leprosy not disappearing in the United States and globally, but the signs are pointing to a more virulent mycobacterial infection that is likely to be a microbial adaptation to the global use of multidrug therapy, as previously reported (*5*).

Lucio's phenomenon is fortunately rare; there is no proven effective therapy for this type 3 reaction in leprosy patients. Historically, Lucio's phenomenon was confined to Mexico, mostly in cases of diffuse lepromatous leprosy, also referred to as "Leprosy bonita." In recent years, it has

been discovered elsewhere, including the first known case in India in 2001 (*6*). Two additional cases of lethal Lucio's leprosy were reported in 2 immigrants from Singapore to the United States, who were shown to have *M. lepromatosis* and *M. leprae* (*7*). The report of Levis et al. is likely confirmed by the recent discovery of *M. lepromatosis* and Lucio's outside of Mexico (*5*).

In summary, leprosy is an emerging infection in the United States, including autochthonous cases in the eastern United States. The reports in the November issue of *Emerging Infectious Diseases* of autochthonous *M. lepromatosis* and a lethal case of Lucio's phenomenon outside Mexico are ominous signs of a more virulent form of emerging leprosy.

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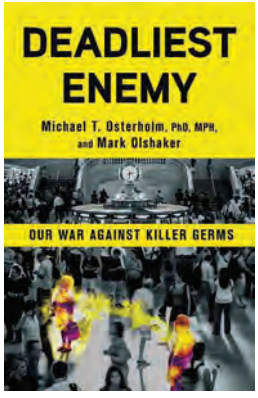
Deadliest Enemy: Our War against Killer Germs

Michael T. Osterholm and Mark Olshaker; Little, Brown and Company, Boston, Massachusetts, USA; ISBN-13: 978-0316343695; ISBN-10: 0316343692; Pages: 352; Price: US $18.30



In the world of infectious diseases, there are many microorganisms that regularly infect humans. However, certain infectious diseases have special significance to society and persons therein.

In Michael T. Osterholm and Mark Olshaker's book *Deadliest Enemy: Our War against Killer Germs*, the explicit purpose is to provide "a new paradigm for the threats posed by infectious disease outbreaks in the twenty-first century." This fast-paced and attention-grabbing book is focused on "those maladies with the potential to disrupt the social, political, economic, emotional, or existential well-being of large regions, or even the entire planet."

To anyone within the field of infectious diseases, Osterholm is a familiar, well-regarded scientist who has provided guiding insight not only to the state of Minnesota, where he was state epidemiologist, but to the entire country and world. The main value of this book is that it is an overview and analysis informed by Osterholm's unique expertise. Olshaker, his coauthor, is an extensively published writer and novelist of works such as *Mindhunter*.

The book contains 14 chapters, each addressing a major infectious disease issue. Beginning with HIV/AIDS, the book covers every major outbreak in the past 3 decades, including severe acute respiratory syndrome, Middle East respiratory syndrome, toxic shock syndrome, Zika virus disease, and Ebola. Osterholm's personal encounter with La Crosse encephalitis is harrowing. In addition, all major policy issues in the field over the past 3 decades, including bioterrorism, gain-of-function influenza research, the antivaccine movement, and antimicrobial resistance, are covered in detail.

The book is also valuable because it goes beyond a mere journalistic description of diseases and their impact as it actively offers policy- and scientific-based approaches for addressing the problems described. In the chapter titled "Taking Influenza Off the Table," the concept of "game-changing influenza vaccines" is introduced, providing a compelling rationale and strategy for developing vaccines in a realm where traditional business and technological approaches fall short. Similarly, "Fighting the Resistance" is focused on potential solutions to the public health emergency of antimicrobial drug resistance.

The most insightful aspect of the book is the authors' threat matrix. In this clarifying matrix, 4 classes of threats are identified: pathogens of pandemic potential; pathogens of critical regional importance; bioterrorism, dual-use research of concern, and gain-of-function research of concern; and endemic diseases. After subdividing infectious diseases into this matrix, the authors offer an easily understood and highly accessible agenda with several priority items to support humans against these threats.

One of the underlying themes of this book, which follows some conventions of a mystery novel in how the authors structure the chapters, is the emphasis on solving puzzles of infectious diseases through first-hand, diligent, logical thinking integrated with proper technology and appropriate laboratory studies. A good example is when one has to "play public policy Jeopardy," as Osterholm has with contentious issues involving gain-of-function research of concern.

Osterholm's mention of his childhood love of Sherlock Holmes and other mysteries is the leitmotif of this excellent book. This engaging book, which is easily accessible and requires no technical knowledge, will appeal to a broad range of practitioners engaged in public health and clinical management of infectious diseases, biomedical trainees interested in infectious diseases, and policy makers.

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Nicolas Poussin (1594–1665). *The Plague at Ashdod*, 1630. Oil on canvas. 58.3 in × 78 in/148 cm × 198 cm. Louvre Museum, Paris, France.

Of Rats and Men: Poussin's Plague at Ashdod

Victor Asensi and Joshua Fierer

Nicolas Poussin (1594–1665) was a brilliant French Baroque painter whose art was inspired by biblical and mythological scenes. Poussin depicts the *Plague at Ashdod* (1630) (Louvre Museum, Paris, France) in one of his best works, inspired by an episode from chapter 5 of the Book of Samuel. On this large canvas, rats run through buildings and among dead and dying bodies. The Book of Samuel, written during 630–540 BCE, recounts the capture of the Ark of the Covenant by the Philistines who moved it to the city of Ashdod. "Soon after receiving the Ark rats appeared in the land and death and destruction spread throughout Ashdod. The Philistines, young and old, were struck by an outbreak of tumors in the groin and died." The Philistines sent the Ark back to Israel with a guilt offering of "five gold tumors and five gold rats," models of the pestilences destroying the country.

This biblical text has been linked to bubonic plague by some, but not all, authors because black rats from the Far East did not reach the Near East until the 1st century BCE. However, fossilized remains of *Xenopsylla cheopis* fleas and the *Rattus* black rats have been found in the Egyptian Nile Valley, dating their arrival in the Middle East to 1350 BCE. The Jewish–Roman historian Flavius Josephus (37–100 ACE) attributed the epidemic to bacillary dysenter, which can lead to hemorrhoids, his translation of the Hebrew word "opalim." However, Josephus' translation of the Hebrew word has been questioned. The original Hebrew text of the Book of Samuel uses two words to describe the plague's pathology, namely *techorim* (tumor) and *ophel* (boil), both appropriate for bubonic plague.

The King James version of the Bible translates both words as "emerods" (hemorrhoids), and the New International version of the Bible translates both as "tumors." The Septuagint, a Hebrew-to-Greek translation of the Torah made in the 3rd century in Egypt by 72 Hebrew scholars, and Saint Jerome's translation of this Greek text into Latin, both expand on the original Hebrew by stating that the tumors were in the groin (*bubo* is derived from the Greek word for groin). The Septuagint translation by Hebrew scholars seems more reliable than the translation to Latin by Josephus.

It is startling that in 1630 Poussin implicated rats in the pathogenesis of the bubonic plague, a fact disregarded until the end of the 19th century. Poussin lived through the Thirty Years' War in France and Italy and might have seen cases of plague.

It was not until 1894 that Alexandre Yersin and Kitasato Shibasaburo, independently in Hong Kong isolated the bacterium responsible for the Third Bubonic Plague Pandemic. Yersin named it *Pasteurella pestis* after the Pasteur Institute, but in 1967 it was moved to a new genus and renamed *Yersinia pestis* in honor of Yersin. Yersin also noted that rats were affected by plague during human epidemics. Plague was regarded in Asia as a disease of rats. Thus, when large numbers of rats were found dead, plague outbreaks soon followed.

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Correction

Vol. 23, Supplement

Axis labels for Figure 3 were incorrect in Enhancing Workforce Capacity to Improve Vaccination Data Quality, Uganda (K. Ward et al.). The corrected figure is provided here, and the article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/13/17-0627_article).

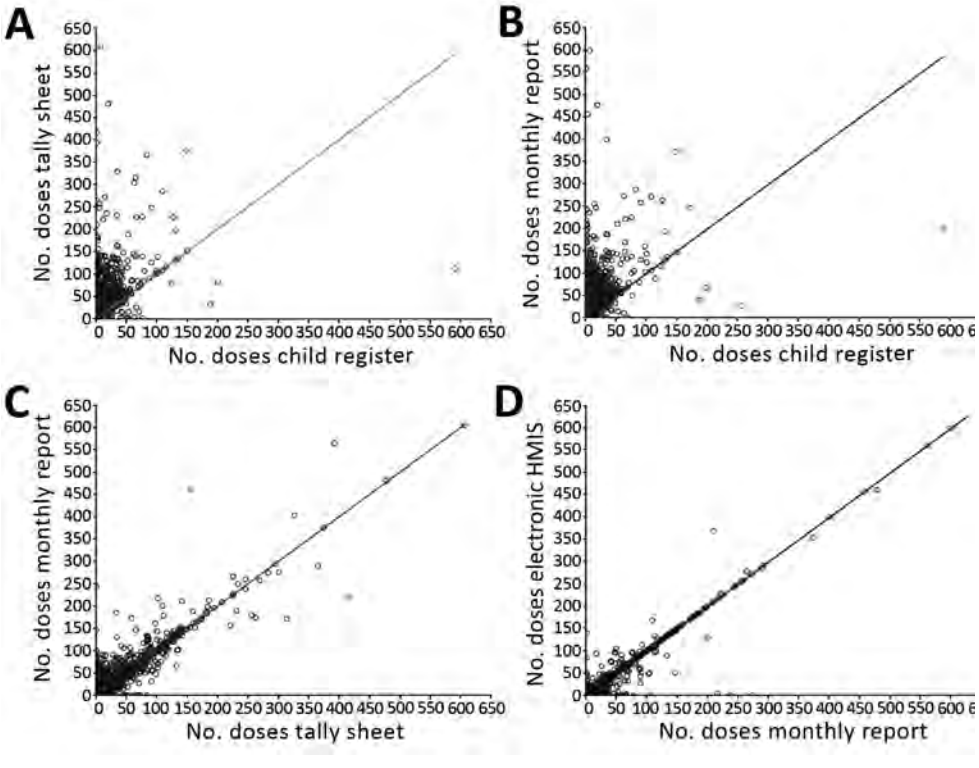

Figure 3. Comparison of the number of doses of Penta3 recorded on different vaccine dose recording and reporting tools, Uganda. A) Doses recorded on tally sheet compared with immunization register (n = 1,664 health facilities); B) doses recorded on monthly report compared with immunization register (n = 1,686 health facilities); C) doses recorded on monthly report compared with tally sheet (n = 1,713 health facilities); D) doses recorded on the HMIS compared with monthly report (n = 1,661 health facilities; 3 outliers not shown [total no. doses >650]). p<0.001 for all comparisons. Data from sample of 2015 DQI tools; 1,667 (83%) sampled from 107 districts and 343 (17%) from a census of 7 districts. Data were missing from 2 districts. HMIS, Health Management Information System; Penta3, diphtheria/tetanus/pertussis/Haemophilus influenzae type b/hepatitis B vaccine, third dose.

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- Effects of Culling on *Leptospira interrogans* Carriage by Rats
- Epidemic Varicella Zoster Virus among University Students, India
- Novel Sequence Type ST834 *Streptococcus suis* among Humans on Pig Farms, Madagascar
- In the Company of Microbes: Ten Years of Small Things Considered

Complete list of articles in the February issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 1–3, 2018
8th Advances in Aspergillosis
Lisbon, Portugal
www.AAA2018.org

February 12–14, 2018
ASM Biothreats
Baltimore, MD
https://www.asm.org/index.php/biothreats-2018

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
http://www.croiconference.org/

March 7–9, 2018
ISIRV
2nd International Meeting on Respiratory Pathogens
Singapore
https://www.isirv.org/site/

April 15–18, 2018
10th Annual Symposium on Avian Influenza (ISAI 2018)
Brighton, UK
https://science.vla.gov.uk/flu-lab-net/index.html

April 18–20 2018
ISIRV
International Society for Influenza and Other Respiratory Virus Diseases
Neglected Influenza Viruses Group
Brighton, UK
https://science.vla.gov.uk/flu-lab-net/index.html

May 6–9, 2018
ASM Clinical Virology Symposium
West Palm Beach, FL
https://www.asm.org/index.php/2018-clinical- virology-symposium

June 7–11, 2018
ASM Microbe
Atlanta GA
https://www.asm.org/index.php/ asm-microbe-2018

August 26–29, 2018
ICEID
International Conference on Emerging Infectious Diseases*
Atlanta, GA
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.

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.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 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.

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Article Title

Zika Virus Testing and Outcomes during Pregnancy, Florida, USA, 2016

CME Questions

1. Your patient is a 22-year-old pregnant woman residing in an area where transmission of the Zika virus (ZIKV) has been reported. According to the retrospective chart review by Shiu and colleagues, which of the following statements about the clinical outcomes of ZIKV infection during pregnancy is correct?

A. Both cases of probable congenital ZIKV infection in this series were from second-trimester ZIKV infections
B. Estimating the true percentage of infants affected by ZIKV may be challenging, as current testing may not provide laboratory evidence of fetal ZIKV infection after delivery
C. One patient with probable congenital ZIKV infection had intracranial calcifications detected on prenatal ultrasound examination
D. Both patients with probable congenital ZIKV infection met criteria for microcephaly

2. According to the retrospective chart review by Shiu and colleagues, which of the following statements about challenges associated with ZIKV screening and testing is correct?

A. Laboratory results were typically available within 1 week from testing
B. The longest delays in test results were at the end of the year
C. An estimated 15% of patients with false-positive ZIKV immunoglobulin M (IgM) testing results had plaque reduction neutralization testing (PRNT) results showing previous infection with dengue
D. Concern for false-positive ZIKV testing results should be weighed against concern for missing an infant with prenatal exposure to ZIKV

3. According to the retrospective chart review by Shiu and colleagues, which of the following statements about social and clinical factors associated with screening positive for ZIKV infection during pregnancy is correct?

A. Patients who primarily spoke Spanish or Haitian Creole had increased odds of positive ZIKV screening results during pregnancy
B. There did not appear to be any financial barriers to screening
C. Among women with any laboratory evidence of ZIKV infection, most were nulliparous
D. Among women with any laboratory evidence of ZIKV infection, three-quarters had symptoms suggestive of ZIKV infection

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Article Title

Recognition of Azole-Resistant Aspergillosis by Physicians Specializing in Infectious Disease, United States

CME Questions

1. You are advising a large infectious disease practice on azole-resistant *Aspergillus fumigatus* infections. According to the Emerging Infections Network (EIN) survey of US infectious disease physicians by Walker and colleagues, which of the following statements about clinician awareness of and experience with azole-resistant *A. fumigatus infections* is correct?

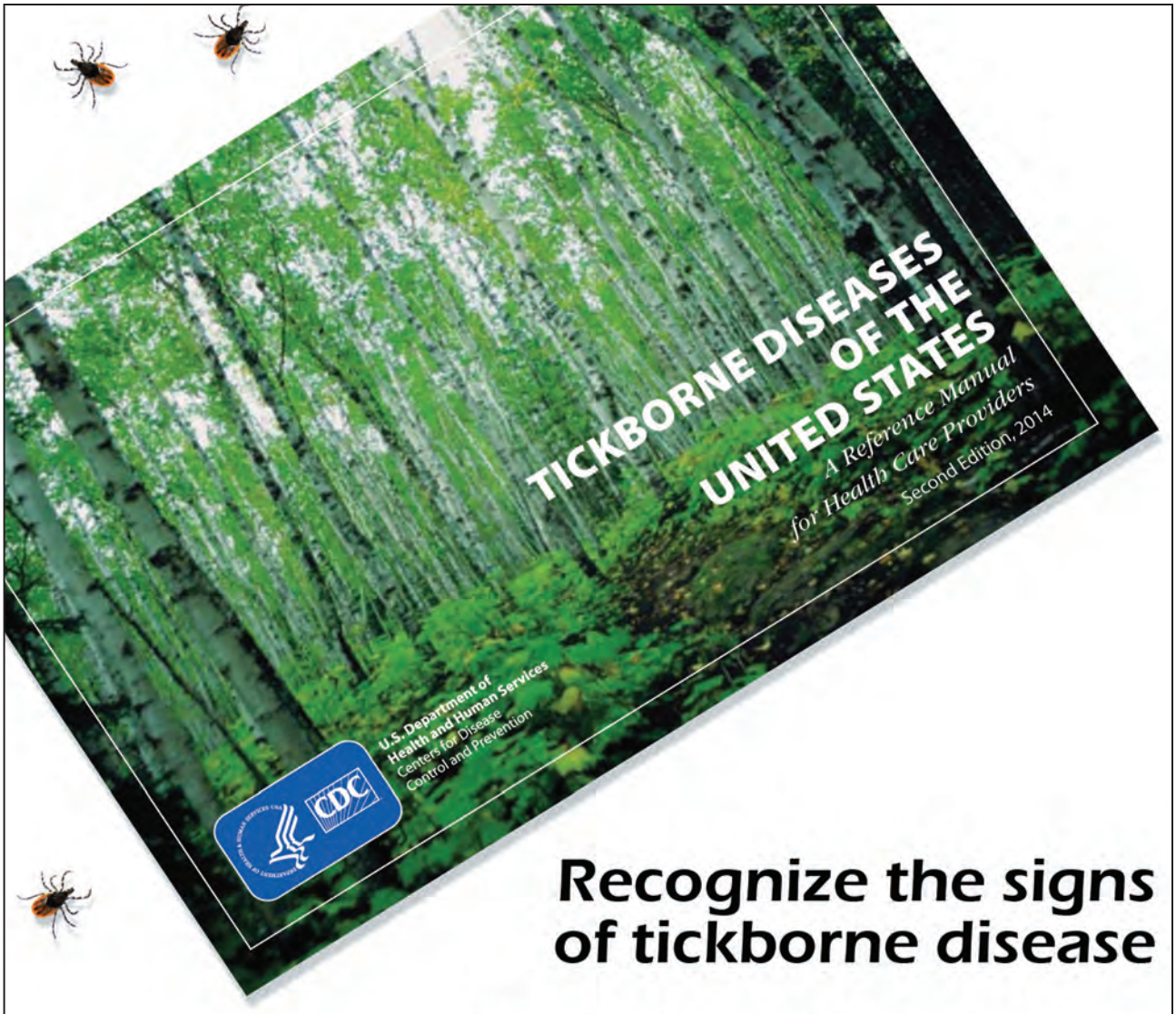
A. Approximately three-quarters of respondents were familiar with the concept of azole-resistant *A. fumigatus* infections
B. Approximately half of respondents were aware that azole resistance of *A. fumigatus* might possibly be associated with agricultural or environmental use of antifungals
C. Among 51% who reported treating at least 1 patient with aspergillosis within the past year, 38% reported at least 1 patient with clinical failure, despite use of therapeutic drug monitoring by 80%
D. No physicians reported observing azole resistance in an azole-naive patient

2. According to the EIN survey of US infectious disease physicians by Walker and colleagues, which of the following statements about availability and use of *A. fumigatus* susceptibility testing in clinical settings is correct?

A. Nearly all treating physicians had access to susceptibility testing
B. An estimated 72% who had access to testing reported that testing occurred offsite; among those, 4% typically received results within 1 week and 19% reported receiving results at 3 weeks or longer after the request
C. One-quarter of physicians who had access reported testing an isolate for at least 1 of their patients
D. No physicians reported seeing a patient with a pan-azole-resistant isolate

3. According to the EIN survey of US infectious disease physicians by Walker and colleagues, which of the following statements about regional differences in availability and use of *A. fumigatus* susceptibility testing in clinical settings is correct?

A. Proportionately more physicians from the Northeast than from other regions observed resistance to 1 or more azoles
B. Among physicians who have access to susceptibility testing, proportionately more physicians from the South reported that more than half of their patients' isolates were tested
C. Proportionately more physicians from the Midwest reported seeing more than 8 cases of aspergillosis in the past year
D. Systematic surveillance could help identify emergence of regional resistance patterns, the role of resistance in treatment failure, and locally tailored treatment options



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

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

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

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

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Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

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.

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

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

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EMERGING INFECTIOUS DISEASES

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